Faculty of Health Sciences

School of Public Health

Asthma and allergy with Eastern (China) versus Western (Australia) environment:

the role of human microbiome

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This thesis is presented for the degree of

Doctor of Philosophy

of

Curtin University

April 2020

Declaration

To the best of my knowledge and belief, this thesis titled “Asthma and allergy with Eastern (China) versus Western (Australia) environment: the role of human microbiome” contains no material previously published by any other person, except where due acknowledgement has been made.

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Human Ethics: 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 studies received human research ethics approval from the Human Research Ethics Committee at Curtin University (HRE2017-0001, HR86/2014, and HR110/2013), at Zhengzhou University and at Children's Hospital of the Capital Institute of Paediatrics in China (SHERLL2014040), the Human Research Ethics Committees of Western Australia (RGS0000002399, and RGS0000002424) and at University of Western Australia (RA/4/1/6763).

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Attribution

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

Chapter	Co-authors roles and contributions
Study 1: Chapter 3-5 **Status: Manuscript in preparation**	**Jing Guo**: concept and design of study; recruitment; laboratory work; analyses and interpretation of data; drafting and editing of manuscript **Guicheng (Brad) Zhang, Jane Scott, Gavin Pereira, Helen Brown, Chuanhe Liu**: concept and design of study; analysis and interpretation of data; critical revision of the manuscript **Guicheng (Brad) Zhang**: supervised all aspects of the study.
Study 2: Chapter 6 **Title: Western oropharyngeal and gut microbial profiles are associated with allergic conditions in Chinese immigrant children living in Australia** **Journal: World Allergy Organization Journal** **Year: 2019** **DOI: 10.1016/j.waojou.2019.100051** **Status: Published in final form**	**Jing Guo**: acquisition, analyses and interpretation of data; drafting and editing of manuscript **Quanjun Lv, Xiajie Wen, and Aarti Saiganesh**: study design, recruitment, and data acquisition. **Amir Ariff, Xiaoping Zhang, Christopher Peacock, Yong Song, Phillip Melton, Gary Dykes, Eric Moses**: verified the analytical methods, and contributed to manuscript revision **Guicheng (Brad) Zhang, Peter N. Le Souëf, Fengmin Lu:** designed and directed the study, provided critical revision of the manuscript **Guicheng (Brad) Zhang:** supervised all aspects of the study

Study 3: Chapter 7 **Title: Linking the westernised oropharyngeal microbiome to the immune response in Chinese immigrants** **Journal: Allergy Asthma and Clinical Immunology** **Year: 2020** **DOI: 10.1186/s13223-020-00465-7** **Status: Published in final form**	**Jing Guo**: study design; analyses and interpretation of data; drafting and editing of manuscript **Shu Chen, and Aarti Saiganesh**: recruitment, sample and questionnaire collection, and laboratory work **Xiaoping Zhang, Belinda Hales, Gary Dykes, Christopher Peacock**: supervised the laboratory tasks, verified the analytical methods, and contributed to manuscript revision **Guicheng (Brad) Zhang, Peter N. Le Souëf:** study design, critical revision of the manuscript **Guicheng (Brad) Zhang:** supervised all aspects of the study
Study 4: Chapter 8 **Title: Toll-like receptor signalling has inverted U-shaped response over time with the Western environment** **Journal: Allergy** **Year: 2020** **DOI: 10.1111/all.14277** **Status: Published in final form**	**Jing Guo:** experimental design and laboratory work, analyses and interpretation of data; drafting and editing of manuscript **Yong Song:** designed experiment with Jing Guo, supervised the laboratory work, and revised the manuscript. **Aarti Saiganesh:** participants recruitment, sample process, and contributed to manuscript revision. **Belinda Hales, Anthony Bosco, and Peter N. Le Souëf:** verified the analytical methods and contributed to manuscript revision. **Guicheng (Brad) Zhang:** designed and directed the study, supervised the data analysis and interpretation, and provided critical revision of the manuscript.

Abstract

Background

Asthma and allergy are distributed disproportionately across the globe and are more common in Westernised developed countries than in developing countries. Environmental factors play a vital role in the development of these conditions. Recent studies suggest that perturbations in the human microbiome is associated with an increased risk of allergic disease, while exposure to non-pathogenic microbes are important for healthy immune development and reduction of the risk for asthma and allergy. There is a lack of information about how microbiome profiles differ in asthmatic children in a developing country's environment.

Immigrants moving to Westernised countries can potentially adopt the higher prevalence of allergic conditions of their new home country, which provides a unique opportunity to investigate the role of Westernised microbiomes in the pathogenesis of allergic conditions. However, little is known about the microbiome disparities between immigrants in a Westernised environment and their ethnically comparable peers in an Eastern/developing environment.

Aims

The overarching aim of this doctoral thesis was to investigate the difference in human microbiome profiles among the homogeneous Han ethnic population in; children with and without asthma; in children living in an Eastern (developing) versus Westernised (developed) environment; and in adult immigrants living in the Westernised environment with different residence time. To achieve the research aim, four studies were undertaken.

Study one aimed to compare the differences between oropharyngeal (OP) and gut microbiome in asthmatic and non-asthmatic children in Beijing, China, and to investigate the association of microbiome profile and serum immunoglobulin E (IgE) levels among those children.

Study two aimed to compare the differences between OP and gut microbiome between Australian Chinese (AC) children in Australia and China-born Chinese (CC) children in mainland China.

Study three aimed to compare the OP microbiome between newly arrived and long-term Chinese adult immigrants living in Australia.

Study four aimed to investigate the time dependence of the Westernised environment on the expression of toll-like receptor (TLR) pathway genes, plasma cytokine levels and total and specific IgE in Chinese immigrants in Australia.

Methods

Study one was a case-control study, conducted in collaboration with the Children's Hospital of the Capital Institute of Paediatrics in Beijing, China. A total of 101 doctor-diagnosed asthmatics were recruited from the asthma outpatient clinic and 48 non-asthmatic children in the same age range were recruited from the orthopaedics inpatient clinic. OP and faecal samples were collected, and serum total IgE and allergen specific IgE were tested.

Study two included 58 AC children in Perth, Western Australia and 63 CC children from Hebi city, Henan province in China. OP and faecal samples were collected. Skin-prick tests were performed to measure the children’s atopic status.

Study three involved 44 adult Chinese immigrants: newly-arrived (n=22, living in Australia <6 months) and long-term immigrants (n=22, living in Australia >5 years), matched by age and gender. OP swabs, serum and whole blood samples were collected. Innate immune responses were determined by 23 TLR pathway cytokines using whole blood assay, while adaptive immune responses were determined by IgG-associated response to specific microbial or viral pathogens using immunoassays and immunoabsorption assays.

Study four was a cross-sectional study including 107 Chinese immigrants recruited from communities in Perth, Western Australia. The time dependence of the Westernised environment on the expression of TLR pathway genes, plasma cytokine levels, and total and specific immunoglobulin E (IgE) were investigated.

Results

In study one, the alpha diversity of OP samples was lower in asthmatic children, but no difference was evident in faecal samples. In OP microbiome comparison, the phylum Bacteroidetes and its genus *Bacteroides* were significantly higher in non-asthmatic children. In faecal microbiome abundance comparison, genus *Lachnospira* and *Ruminococcus 1* were significantly higher in asthmatic children. Nevertheless, the relative abundance of *Streptococcus* and *Escherichia-Shigella* were significantly higher in non-asthmatic children. The association patterns of microbial abundance and serum IgE levels were distinct between asthmatic and non-asthmatic children.

In study two, AC children had more allergic conditions than CC children. The alpha diversity of both OP and gut microbiome was lower in AC children compared to CC children for richness estimate (Chao1), while diversity evenness (Shannon index) was

higher in AC relative CC children. The beta diversity (community similarity) displayed a distinct separation of the OP and gut microbiota between AC and CC children. An apparent difference in microbial abundance was observed for many bacteria between the two groups. In AC children, we sought to establish consistent trends in bacterial relative abundance that were either higher or lower in AC versus CC children, and higher or lower in children with allergy versus those without allergy. The majority of OP taxa showed a consistent trend while the majority of faecal taxa did not.

In study three, the relative abundance of the genus *Leptotrichia* was higher in long-term immigrants when compared with that in newly-arrived Chinese immigrants, while the genus *Deinococcus* was significantly lower in long-term Chinese immigrants. The genera *Lachnospiraceae_uncultured*, *Erysipelotrichaceae UCG-007*, *Veillonella* and *Actinomycetales_ambiguous taxa* were negatively correlated with cytokine IL-6 in long-term Chinese immigrants (rho range: -0.46 ~ -0.73). With respect to adaptive immunity, several microbial taxa were significantly associated with IgG1 responsiveness to microbial antigens in long-term immigrants, while a significant correlation with IgG1 responsiveness to viral antigens was detected in newly-arrived immigrants.

Study four found that TLR pathway genes had an inverted U-shaped response, and total IgE and specific IgE had a quadratic increase as a function of duration of residence in Australia.

Conclusion

The overall findings of this thesis provide supporting evidence for the role of the microbiome in asthma and allergy in the context of contrasting environments,

Eastern/developing versus Westernised/developed environment. The Westernised environment has reshaped the microbial composition profile of human microbiomes, and these altered microbiomes are more likely to be a cause of high rates of asthma and allergy in Westernised countries such as Australia over the past 70 or 80 years and the cause of the continuous increase in allergic conditions in developing countries. The thesis provides a significant contribution to our understanding of underlying mechanisms for asthma and allergy, and its findings may assist in the intervention, management, treatment and possible prevention of these conditions.

Acknowledgements

First and foremost, I would like to acknowledge the support and mentorship of my great supervisors: A/Prof Guicheng (Brad) Zhang, Prof Jane Scott, A/Prof Gavin Pereira, Dr Helen Brown, and my Chairpersons: A/Prof Ben Mullins and Prof Gary Dykes. To Brad, thank you so much for letting me be your student. Thank you for the patient guidance, encouragement and advice you have provided. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. Many thanks to all my co-supervisors and Chairpersons for the thoughtful comments and suggestions during my PhD journey.

I would like to thank my research team, Dr Yong Song, Ms Khui Hung Lee, Ms Michelle Schwager, Miss Ashlyn Poole, Miss Yifan Shan, and Mr Kan Yu. To Yong, thank you for your patient guidance in the laboratory work. You helped me build the confidence in the complex experiments. To all my other team members, I am very lucky to share the highs and lows with you and thank you very much for your friendship and support.

I would also like to thank our collaborators in Children's Hospital of the Capital Institute of Paediatrics in Beijing, China. Thank you to Dr Chuanhe Liu, Dr Yuzhi Chen, and all the clinical doctors in asthma outpatient clinic. The time I spent with all of you was very enjoyable and unforgettable. I am particularly grateful to all the participants for their support for the study.

There are also many researchers to thank who have been involved in my studies both from Australia and China – Prof Peter N Le Souëf, Prof Fengmin Lu, Prof Quanjun Lv, A/Prof Christopher Peacock, A/Prof Belinda Hales, A/Prof Ben Mullins, Prof Gary Dykes, Dr Anthony Bosco, Prof Eric Moses, Dr Aarti Saiganesh, Dr Xiaoping Zhang, Dr Phillip Melton, Dr Amir Ariff, and Ms Xiajie Wen. A special acknowledge to Ms Annemieke Mulders and Dr Renée Otmar for your helping in professional editing and proofreading of my thesis.

To all the staff and students at the Centre for Genetic Origins of Health and Disease (GOHaD), thank you everyone for always being so friendly and supportive.

This research was supported by Curtin International Postgraduate Research Scholarship (CIPRS)/ Health Sciences Faculty International Research Scholarship (HSFIRS). I am very grateful for this opportunity and hope that I have met the expectation of this scholarship. I would also like to extend my utmost gratitude my school Director of Graduate Research: A/Prof Jonine Jancey, administrator officer: Ms Angela Martin, and all the GRS officers for your kind help throughout my PhD.

To my other friends in Perth - Dr Dnyanada Gokhale, Dr Rachel Jones, Ms Jacqueline Ryan, and Ms Clara Mok, I am so grateful for your encouragement when I was stressed. A special thanks to two of my best friends, Dr Yuanting Chen and Ms Zedi Niu. Thank you both for being such great friends since we were young girls. The geographic distance has never been an issue for our friendship.

Finally, to my ever-loving family, Mama and Papa. Thank you for always believing in me and being so supportive. I know it is very hard to let your only daughter to go thousands of miles away to peruse her dream. I love and miss you both every day. A big thank you to my wonderful husband Yang. You came into my life when I came to Australia for my study, made me your Mrs and now we are growing our own family with our little Sophia coming. Thank you for all your love and support over the past four years to help me through, encourage me when I was down. This thesis would not have been possible without you.

Dedication

I would like to dedicate my thesis:

In ever loving memory of my grandfather—Fangting Guo. I miss you every day and wish you could have been here with me. I hope I have made you proud.

To my loving dad and mom— Hongdong Guo and Liyun Wang, and to my beloved husband, Yang Liu. Without your support and love, this would not be possible.

Publications and Presentations

Publications during candidature

1. **Guo J**, Song Y, Saiganesh A, Hales BJ, Bosco A, Le Souef PN & Zhang G. Toll-like receptor signalling has inverted U-shaped response over time with the Western environment. Allergy. 2020.

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Presentations during candidature

1. **Poster Presentation**: Distinct Microbiome Profiles in Australian Born Chinese children in Australia and Chinese children in China. (September **2017**, Microbiota, Metagenomics & Health, Cold Spring Harbor Asia, Suzhou, China)
2. **Oral Presentation**: Risk Factors and Prognosis of Recurrent Wheezing in Chinese Young Children: A Prospective Cohort Study. (September **2018**, Mark Liveris Symposium, Curtin University)
3. **Poster presentation**: Risk Factors and Prognosis of Recurrent Wheezing in Chinese Young Children: A Prospective Cohort Study. (December **2018**, 4th Asian Pediatric Pulmonology Society (APPS) Annual Scientific Congress, Beijing.) The E-poster was awarded **outstanding E-poster presentation**.
4. **Oral presentation**: Linking the westernised oropharyngeal microbiome to the immune response in Chinese immigrants. (November **2019**, System Biology Symposium, Telethon Kids Institute, Perth, Australia)
5. **Poster presentation**: Linking the westernised oropharyngeal microbiome to the immune response in Chinese immigrants. (November **2019**, Institute for Respiratory Health: The Next 20 Years. 20th Anniversary Research Symposium, Harry Perkins Institute of Medical Research, Perth, Australia)
6. **Oral presentation**: Toll-like receptor signalling has inverted U-shaped response over time with the Western environment. (December **2019**, World Allergy Congress, Lyon, France)
7. **Poster presentation**: Linking the westernised oropharyngeal microbiome to the immune response in Chinese immigrants. (December **2019**, World Allergy Congress, Lyon, France)
8. **Poster presentation**: Increased nasal *Streptococcus pneumoniae* presence in Western environment associated with atopic eczema in Chinese immigrants. (December **2019**, World Allergy Congress, Lyon, France)

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List of Abbreviations

AC	Australian Chinese
BMI	body mass index
CC	China-Born Chinese
Df	*Dermatophagoides farina*
Dp	*Dermatophagoides pteronyssinus*
FDR	false discovery rate
Fx5	common food allergens (egg white, cow's milk. codfish, wheat, peanut, and soybean)
GF	Germ free
IBD	inflammatory bowel disease
ICS	inhaled corticosteroids
IgE	immunoglobulin E
ISAAC	The International Study of Asthma and Allergies in Childhood
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDA	The linear discriminant analysis
LEfSe	The linear discriminant analysis effect size
LPS	lipopolysaccharide

Mx1	mould mix
OP	Oropharyngeal
OTU	Operational Taxonomic Units
PBMC	peripheral blood mononuclear cell
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	ribosomal RNA
SD	standard deviation
SE	standard error
sIgE	specific immunoglobulin E
SPT	skin prick test
TLR	toll-like receptor

1 Introduction

This chapter describes the current state of knowledge about the prevalence and aetiology of asthma, its origin and its environmental risk factors. It is believed that complex interactions of multiple environmental, socioeconomic, and cultural factors contribute to the large disparity of asthma prevalence that is observed between “developed” and “developing” countries. Studies looking into risk factors for allergic diseases, including asthma, will significantly improve the understanding of the pathogenesis of these conditions. In particular, the microbiome has been noteworthy in recent research as it correlates with asthma development.

The main aim of this thesis is to investigate the role of the microbiome in asthma and allergy in the context of its environment, namely the Eastern (China) and Western (Australia) environments.

1.1 Childhood asthma prevalence

1.1.1 The prevalence of childhood asthma worldwide

Asthma is a common chronic non-communicable disease characterized by airflow limitation, bronchial hyperresponsiveness, and inflammation of the airways[1]. Additionally, the symptoms always involve wheezing, breathlessness, chest tightness or coughing [2]. It is a major global public health issue for people of all ages and affects approximately 334 million people worldwide [3]. In terms of disability-adjusted life years (DALYs), asthma is globally in the top 10 in the mid-childhood ages (5-14 years) and in the top 20 for all ages [4]. Asthma can lead to an extensive human burden because the symptoms reduce the quality of life in terms of physical, psychological and social effects [5-7].

Since the second half of the twentieth century there is increasing awareness of changes in the prevalence of asthma worldwide [8-10]. Since the 1990s, numerous epidemiological studies have been conducted around the world to estimate the prevalence of asthma and identify risk factors associated with this condition. The International Study of Asthma and Allergies in Childhood (ISAAC) initiated large multinational studies in children[11-13]. Phase One of ISAAC reported a wide variation in the prevalence of asthma between countries, with the highest prevalence as high as 15 times greater than the lowest prevalence [11]. The prevalence of wheezing in the past 12 months ranged from 4.1 to 32.1% in 6-7-year-old school children and from 2.1 to 32.2% in 13-14-year-old school children. A particularly high prevalence was observed in English-speaking countries and in Latin America [11]. After a period of 5-10 years, Phase Three of ISAAC repeated the studies of Phase One to determine if the prevalence of asthma has changed with time [12, 13]. The majority of centres reported a rise in the prevalence of asthma symptoms while

other centres with existing high prevalence in 13-14-year age group showed a decline (Figure 1-1)[13].

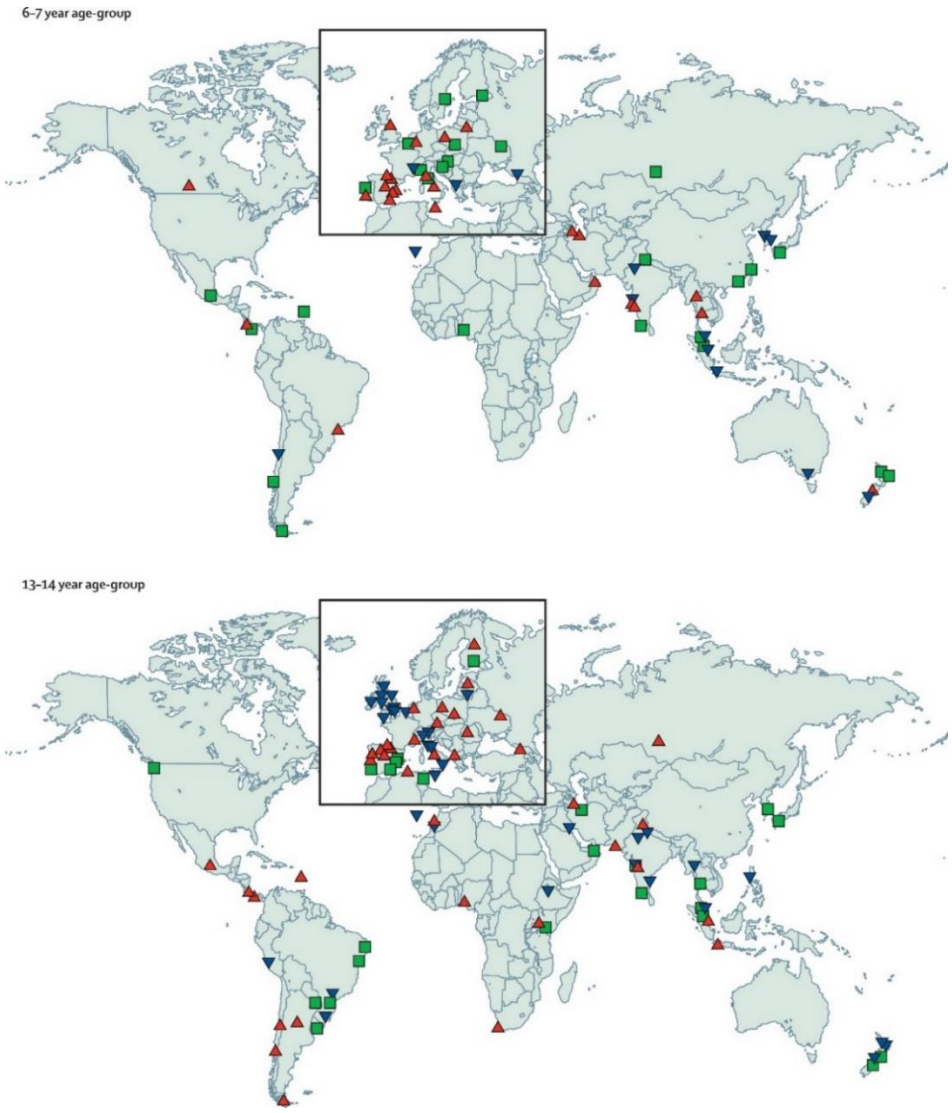


Figure 1-1 The direction of change in prevalence of asthma symptoms for children in 6–7 year age-group and adolescent in 13–14 year age-group.

Each symbol represents a centre. Blue triangles indicate prevalence reduced by at least 1 standard error (SE) per year. Green squares indicate little change (less than 1 SE per year) was observed. Red triangles indicate prevalence increased by at least 1 SE per year. Reproduced from *The Lancet*, Vol. 368, Asher MI, Montefort S, Bjorksten B, et al.; ISAAC Phase Three Study Group. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. page 733–43, copyright 2006, with permission from Elsevier (Appendix C) [13].

Generally, asthma prevalence is high in western developed countries (e.g. United Kingdom, Canada, Australia, New Zealand) and low in developing countries (e.g. China and India). Recent studies suggest that the increase in asthma prevalence seen between the 1950s and 1990s might have plateaued or even decreased in the Western world[14]. Further, the world wide disparity of asthma prevalence is becoming smaller due to the rise in prevalence among low- and middle-income countries.

Local and national studies also provide understanding of the epidemiology of asthma. Those studies suggest that the reported increase in asthma prevalence is real and cannot be completely explained by increased public awareness or greater readiness to diagnose asthma. A questionnaire study among school children in Aberdeen that took place over 35 years showed a steady increase in prevalence of asthma from 4.1% in 1964, 10.2% in 1989, 19.6% in 1994, to 24.3% in 1999 [15-17]. In a specific area of South Wales in the UK, Burr *et al.*[18] conducted questionnaire survey and exercise provocation tests in 1973 and in 1988 among 12-year-old children. They found the percentage of children with a history of wheeze rose from 17% to 22%, and with a history of asthma from 6% to 12%. In addition, the exercise provocation tests indicated both mild and severe asthma were more common in 1988. The prevalence of asthma in Australia is among the highest in the world with a quite substantial social and economic burden. In 1997, it was recorded that 27% of Australian children had current wheeze and the prevalence of children with persistent asthma had increased from 5% to 9% during the past 20 years [19].

In contrast, the prevalence of asthma in developing countries is relatively low, although it is consistently increasing. In ISAAC Phase Three [20], the lowest prevalence of wheeze in the past 12 months (current wheeze) was 0.8% in Tibet (China) in the 13-14 year olds, and 2.4% in Jodhpur (India) in the 6-7 year olds. The lowest prevalence of symptoms of severe asthma was in Pune (India) for both age groups.

African countries with mainly rural living environments have a low prevalence of childhood asthma, however, urbanized African children experienced a higher prevalence [21]. This study concluded that urban-rural disparity in asthma prevalence parallels an increasing tendency to adopt the Western lifestyle.

1.1.2 The prevalence of childhood asthma in China

China, which has the world's largest population and the third largest land area, is currently undertaking a rapid urbanisation and industrialisation progress. With an average 9% annual growth, China was the fastest growing economy in recent years. As an inevitable consequence of this economic development, the emerging environmental issues and change of people's lifestyles are severely affecting public health in China. Asthma prevalence among children has been increasing in the past two decades. The third nationwide survey of childhood asthma in urban areas of China was conducted from 2009-2010 and showed the total asthma incidence of children aged 0-14 years was 3.02%, compared to 1.50% in 2000 and 0.91% in 1990[22]. A systematic review showed that the prevalence of asthma among children aged 0-14 years ranged from 1.1% in Lhasa (Tibet) to 11.0% in Hong Kong using studies that followed the protocol of ISAAC [23]. In addition, a survey conducted between 2008 and 2009 in three major cities of China revealed high prevalence of self-reported asthma, allergic rhinitis, and atopic eczema [24]. In this study, the prevalence rates of asthma in Beijing, Chongqing, and Guangzhou were 3.15%, 7.45%, and 2.09% respectively [24]. Another study conducted in urban and rural Beijing confirmed the urban-rural difference of asthma prevalence: prevalence rates of wheeze and asthma were 1.0% and 1.1% in rural areas compared to 7.2% and 6.3% in urban areas [25]. Although the asthma prevalence is increasing in China it remains lower compared to Western countries.

1.1.3 Trends in the prevalence of asthma among immigrants

Studies among immigrants can elucidate the role of environmental factors in the pathogenesis of allergic conditions. Large cross-sectional population studies have observed disparities in the prevalence of asthma and allergy between immigrants and native inhabitants. Immigrants moving to Westernized/affluent countries gradually adopt the high prevalence of allergic conditions that native people have. However, the first generation of immigrants still appear to have a lower prevalence than the local people.

Kabesch et al. investigated the prevalence of asthma and atopy in Turkish children living in Germany in 1999 and found that Turkish children had a significant lower prevalence of those conditions than their German peers. Nevertheless, the authors proposed that the difference might be attributed to a selection bias as healthy individuals migrate [26]. However, this is too simple. A cross-sectional survey conducted in Australia investigated 9794 people aged 13-19 years [27]. They found that among non-Australian born subjects there was a 2.1-fold increased risk of having self-reported wheeze after residence for 5-9 years relative to those with a residence of 1-4 years; and the risk increased to 3.4-fold after 10-14 years residency. It indicates that there is a time dose effect on the prevalence of wheeze in immigrants born outside Australia. In agreement with this, Wang et al surveyed the prevalence of asthma and wheeze among 7794 Chinese adolescents from Vancouver, Canada, and from Guangzhou, Beijing and Hong Kong, China [28]. The prevalence of asthma symptoms was the lowest for residents of mainland China, the highest for Canadian-born Chinese, and average for Hong Kong Chinese or Chinese who migrated to Canada. The residence time-dose effect is also seen in Chinese adolescents living in Vancouver. Further, the ISAAC Phase Three Study Group found that immigrants who recently migrated to high prevalence/affluent countries have a lower prevalence of asthma and allergic disease and that the protective effect of the pre-migration

environment quickly declines with increasing residence time in the host country [29]. Several studies investigated the prevalence of asthma and allergy in second-generation immigrants. A systematic review summarized that the prevalence of allergic disorders was generally higher in second-generation immigrants compared to the first-generation [30].

The complex interaction of multiple environmental, socioeconomic, and cultural factors in developed and developing countries is attributed to the prevalence disparities of asthma worldwide. Further studies looking into the role of various risk factors in influencing prevalence of allergic diseases including asthma in immigrants would significantly improve the understanding of the pathogenesis of these conditions.

1.2 The aetiology, risk factors, and hypothesis for asthma and allergy

1.2.1 Asthma aetiology and phenotypes

Asthma is a common chronic non-communicable disease among both children and adults, of which the clinical symptoms are wheezing, breathlessness, chest tightness or coughing [1, 2]. It is suggested that the aetiology involves a range of factors that interact with each other including genetic susceptibility, host features, and environmental exposures.

It has long been recognized that asthma has a strong genetic component. As early as the 1920s, it has been suggested that asthma has familial aggregation [31, 32]. Evidence also showed that the concordance rates for asthma are significantly higher in monozygotic twins than in dizygotic twins and the heritability of asthma is approximately 36%- 75% [33]. However, the increase of asthma prevalence in the second half of the 20 century is too rapid to be explained by genetic changes in population. Therefore, there has been intensive research into the modifiable environmental risk factors that are causally related

to the development of asthma. As the rising prevalence of asthma was first observed in rich, industrialized countries, many factors associated with the Western lifestyle have been investigated. Those factors include dietary patterns, rapid urbanisation, air pollution, tobacco smoke exposure, maternal stress, mode of delivery, breastfeeding, antibiotics usage, and the microbiome [9]. Further, it seems that multiple risk factors have the same outcome resulting in a multiplicative effect on asthma and allergy.

Asthma is heterogeneous and a consequence of a multitude of gene-environment interactions. It involves different types of clinical features, ranging from mild to severe. Asthma phenotypes are defined based on clinical characteristics, lung function and the patterns of inflammation such as allergic asthma, late-onset asthma and eosinophilic and non-eosinophilic asthma.

Many children experience recurrent wheeze early in life which varies widely in clinical presentation and disease course. Although many of pre-schoolers outgrow wheezing symptoms, there are 10-15% persistent wheezers diagnosed with asthma when they reach school age [34, 35]. The commonly described wheezing phenotypes in preschool children using age 3 and 6 as cut-offs are categorised as transient wheezing, late-onset wheezing, and persistent wheezing [34]. However, these phenotypes are determined retrospectively and lack clinical relevance. Alternative preschool wheezing phenotypes are proposed by a panel of seven experienced clinicians from 4 European countries, namely “allergic wheeze”, “non-allergic wheeze due to structural airway narrowing”, and “non-allergic wheeze due to increased immune response to viral infections” [36]. Children with transient wheezing/ non-allergic wheezing often experience episodic wheezing which is usually caused by viral respiratory infection and not associated with atopy or allergic sensitization. In contrast chronic, persistent asthma is more associated with atopy, early

allergic sensitization, and severe loss of lung function. Asthma in adulthood includes some phenotypes which are rarely seen in childhood, such as occupational asthma and aspirin-induced asthma [37, 38]. Although it is infrequent, there are the cases of aspirin-induced asthma in children [39].

Identifying the phenotypes of asthma aids classifying the heterogeneity in asthma and facilitates the development of more personalized approaches for asthma management.

1.2.2 The risk factors for asthma and allergy

The risk factors for asthma and allergy include genetic, host, and environmental factors. Although a family history of asthma is quite common, it is not sufficient for asthma and allergy development [40]. This section discusses the main environmental exposures, and lifestyle factors such as breastfeeding, obesity and diet.

Environmental factors

The association of environmental factors with asthma and allergy has been studied in many large-scale cross-sectional studies. Comparison between areas of high and low asthma prevalence has shown that the Western environment and lifestyle are correlated with the development of asthma and allergy. A good example of such study followed the German reunification in 1990 as changes of asthma prevalence in a genetically homogenous population could be studied [41]. The results showed a higher prevalence of current asthma and hay fever in West Germany compared to East Germany and concluded that aeroallergens sensitization may explain the difference in the prevalence of allergic diseases. Likewise, the Finnish and Russian Karelia populations have a very divergent prevalence of asthma and allergic diseases [42, 43]. The two populations share the same ancestry and live in similar geo-climatic conditions in adjacent areas, but with a distinct

socio-economic status. Finland's fast economic growth and rapid urbanization has created a large socio-economic disparity between the two areas. Allergic symptoms were extraordinarily more common in Finnish people than in their Russian counterparts, and this allergy gap had increased further in 2011 [44]. Studies performed in Estonia and Sweden, as well as in urban and rural Mongolia and Korea have offered comparable research results [45-47]. Thus, it is noticeable that environmental factors greatly contribute to the development of asthma and allergy.

Antibiotic use and viral infections

Several studies have reported an correlation of antibiotic use in early life and the following development of asthma and allergic disease[48] . The risk of antibiotic use during pregnancy for subsequent development of atopic disease has been studied in several studies. These studies found a greater risk of persistent wheeze and asthma in early childhood and a dose-response relationship with antibiotics use [49-51].

Viral infections have a profound effect on asthma/wheeze exacerbation for patients at risk of, or with existing asthma [52]. Studies have shown that respiratory syncytial virus (RSV) and human rhinovirus (HRV) induced wheezing episodes early in life are a major risk factor for subsequent development of asthma at school age [53, 54].

Exposure of tobacco smoke and pollution

Exposure to pre- or postnatal passive smoking is associated with increased wheeze and asthma in children and young people [55]. Especially prenatal maternal smoking has been linked to wheezing at early childhood [56, 57], and a dose-response relationship was observed between exposure and impaired airway function [58]. After the childbirth, exposure to smoking also worsens asthma symptoms and can lead to severe asthma [59].

Urban air pollution mainly arises from sources such as vehicular traffic and power generation. It has been reported for several decades that air pollution can cause exacerbation of pre-existing asthma [60, 61], with accumulating evidence for causing new-onset asthma as well [62, 63]. This might be due to oxidative injury to the airways, possibly leading to inflammation, remodelling, and increased risk of sensitisation [64, 65].

Exposure to allergens

Exposure to allergens is a crucial trigger for childhood asthma exacerbations. Ambient grass pollen can trigger childhood asthma exacerbations requiring emergency department attendance [66]. Additionally, early life exposure to pollen is associated with development of childhood asthma [67]. Outdoor fungal spores exposure has also been shown to cause asthma exacerbation in children [68]. Furthermore, high concentrations of fungi or mold on peak days have been associated with asthma exacerbations and mortality in adults [69, 70]. Studies also suggest that indoor allergens, including house dust mites, molds, pets, and cockroaches, are triggers for asthma, especially in those who are sensitized [71-73].

Mode of delivery and breastfeeding

Various pre- and perinatal factors are of great importance in the development of asthma and allergic conditions. The mode of delivery has been of interest, but the results are inconsistent. Two meta-analyses reported a 20% increase risk of asthma development among children delivered by caesarean section [74, 75]. One recent study has combined 9 European cohorts (total 67,613 participants) to investigate the modes of delivery and asthma in children [76]. It concluded that, compared with vaginal delivery, caesarean

delivery was associated with an increased risk of asthma, especially elective caesarean delivery among children born at term. On the other hand, other studies found no convincing evidence for such associations [77-79]. Another consideration is that caesarean section affects the establishment of the infant's microbiome, which might lead to the development of asthma and allergy later in life [80, 81].

The influence of breastfeeding on the risk of childhood asthma and allergy remains controversial as well. A recent systematic review concluded that breastfeeding is protective for asthma, but the evidence for eczema and allergic rhinitis protection is weaker [82]. Although some studies have shown a protective effect, other studies reported higher rates of allergy and asthma among breastfed children [83, 84]. Breastfeeding is also an important influence factor for infant gut microbiome composition [80, 85]. Breastfeeding remains recognized as the "gold" standard for infant feeding.

Obesity and Dietary pattern:

Studies have indicated that being overweight or obese can worsen the asthma symptoms and influence the response to inhaled corticosteroids (ICS). A retrospective study investigated 32,321 asthmatic children aged 5-17 years old by measuring the number of dispensed β-agonist canisters and oral corticosteroid courses. It showed that overweight and obese children were more likely to have poor asthma control and more exacerbations [86]. Overweight/obese asthmatic children also showed a decreased response to ICS on measures of lung function and an increased number of emergency department visits or hospitalisations in a longitudinal analysis of an over 4-year trial [87]. Among preschool children, being overweight is also associated with greater asthma exacerbation, however, different from older asthmatic children, overweight/obese preschool children do not demonstrate reduced responsiveness to ICS [88].

Change of dietary habits, particularly deficiency in dietary antioxidants, has been addressed to be associated with increased asthma and allergy. Those changes include the Western diet which is low in fruit and vegetables and high in convenience foods which are low in fibre and antioxidants, but rich in saturated fats [89, 90]. A systematic review has summarized the protective effect of fruit and vegetable consumption against asthma [91].

Microbiome

Due to the development of sophisticated multi-omics techniques, the studies of microbiome and its correlation with asthma and allergy have attracted more attention over recent years. Observational studies demonstrated that exposure to various environmental microbes especially early in life, for example, children taken to day care, raised in homes with dogs or cats, or living on rural farms, may help protect against atopic diseases [92-94]. The commensal microbiota, which colonise heavily in the gastrointestinal tract, skin and oral mucosa, are believed to have a strong influence on the host immune system [95, 96]. Risk factors associated with the microbiome will be further discussed in the Section 1.3.

1.2.3 Innate immune responses in asthma and allergy

Innate immune responses are the first line of defence in the immune responses that keep pathogens from invading the host. They rely on germline-encoded pattern-recognition receptors (PRRs) that target conserved components of pathogens [97]. Furthermore, innate immunity also helps in activating the subsequent adaptive immune response by antigen presentation.

Toll-like receptors (TLRs) are one class of PRRs that play a vital role in recognising microbial specific components [98]. These recognitions by TLRs initiate signal transduction pathways, which activates expression of genes to control innate immune response and further activate adaptive immunity. TLR signalling pathways are regulated by Toll/IL-1 receptor (TIR) domain-containing receptors, such as MyD88, TIRAP/Mal, TRIF and TRAM. The signalling of TLRs stimulates the release of proinflammatory cytokines, including TNF-α, interferon, IL-1, IL-6, and IL-12. Those cytokines are associated with Th1-type cells maturation, as opposed to Th2-type cells which are linked to allergic responses. Thus, alternations in the innate immune response could be critical for the Th1 and Th2 response balance, and the following development of asthma and allergy [99].

1.2.4 The hygiene hypothesis and its evolution

Various hypotheses have been proposed to explain the epidemic of asthma and allergy and they are currently debated in the field. The strong inverse association between infections in early life and the development of allergic conditions led to the postulation of the “hygiene hypothesis”, proposed by Strachan [100]. The idea was that stimulation of T helper type 1 (Th1)-lymphocyte through early infection mediates immune responses by suppressing of Th2-mediated allergic responses. In developed countries, the decreased burden of microbe exposure in early childhood has redirected the immune response towards a Th2 phenotype which leads to the development of allergic conditions. However, this explanation is inconsistent with the concomitant rising prevalence of Th1-mediated autoimmune diseases that accompanied the asthma and allergy epidemic [101]. The biological mechanisms to explain the hygiene hypothesis have been expanded with

antigen competition and immunoregulation, including various regulatory T cell subsets and Toll-like receptor simulation [101].

An alternative explanation has been proposed by Rook *et al*. stating that reduced exposure to certain environmental microbes and commensal organisms is the cause for the recent epidemic asthma and allergic diseases [102]. An “old friends” hypothesis has then been proposed where environmental species rich in vast microbial diversity and the non-harmful commensals from the skin, gut, and respiratory tract play a crucial role in priming and regulating the immune system [103, 104].

Further to this, Haahtela suggested a “biodiversity hypothesis”, which expands and binds together the hypotheses of hygiene, and the “old friends” theory. It states that contact with nature environments could enrich human microbiome, elevate immune balance and prevent allergy and inflammatory diseases [105, 106]. It is warned that biodiversity continues to decline, and the loss of biodiversity could be the most dangerous global megatrend [107]. Consequently, lack of contact with natural environmental microorganisms and overall reduced biodiversity in the environment may adversely impact the human microbiota and its immunomodulatory function.

1.3 The impact of microbiome on asthma

1.3.1 Microbial community profiling methods for human microbiome studies

The development of high-throughput sequencing permits the identification of millions of microbes within or on human bodies in recent years. So far, there are two major methods for characterising the microbiome: 16S or 18S ribosomal RNA (rRNA) gene sequencing, in which the variable regions are highly conserved DNA suitable for lineages detection, and metagenomic studies, in which shotgun sequencing is used [108]. Shotgun

sequencing sequences all fragmented DNA, including that from other microorganisms and viruses.

Most microbiome studies were based on rRNA gene sequencing as it is much cheaper to obtain community profiling and determining the abundance of the microbes, but metagenomic profiles are crucial for providing more information, including encoded functions of the microbiota [109].

1.3.2 The current understanding of microbiome in asthma

There is increasing recognition that human microbiota plays a vital role in the development of asthma and allergy, as they appear to have a significant influence on the maintenance of human basal immune homoeostasis. The recent development of culture-independent molecular detection methods permits the identification of millions of microbes within or on human bodies. Studies have shown that the microbiota of humans is far greater in extent than formerly recognised [110, 111].

These bacteria that colonise in the human body are suggested to play an important role in health and disease. It is a nascent research field and early evidence shows the association between airway and gut microbiota and the development of asthma during the early infancy period. Modification of the airway and/or gut microbiome might aid the prevention and management of common inflammation diseases and conditions, including allergic asthma [112]. A broad bacterial diversity has been recognized as a critical component in in keep of the immune stability which could be measured using alpha-diversity (within samples) and beta-diversity (between samples) [113].

Germ-free (GF) animal studies have shown that the microbiota influences the maturation of the immune system. The absence of commensals in GF animals is associated with

profound effects on the development of the immune system and includes, but is not limited to, developmental defects in the lymphoid tissue within the spleen, thymus, and lymph nodes [114]. Those abnormalities are most prominent near the mucosal interface. For instance, isolated lymphoid follicles, a type of lymphoid tissue, hardly exist in the absence of microbiota in the small intestine [115, 116]. Additionally, GF animals were shown to contain smaller Peyer's patches, and mesenteric lymph nodes [116]. Thus, it is concluded that the process of microbial colonisation is important in the development of a normal immune system.

1.3.3 The role of airway microbiome in the development of asthma

Hilty et al. have documented airway microbiome colonies in asthmatic/COPD adult patients and asthmatic children and compared those with matched healthy controls using nose, oropharynx and bronchial epithelial brushings in adults and broncho-alveolar lavage samples in children. They found that *Proteobacteria*, particularly *Haemophilus* spp., were more common in asthmatic patients while, *Bacteroidetes*, particularly *Prevotella* spp, were less frequent [117]. A further study documents that the dominant microbial phyla of the healthy human lung also include Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria [118]. A pilot cross-sectional study found that asthmatics had a significantly higher bacterial burden and larger diversity relative to the healthy controls by comparing the 16S rRNA bacterial profile from bronchial epithelial brushings [119].

The microbiome of the upper airway is accessible in very early life and has been investigated for asthma development in children. A study conducted among infants through their first five years of life showed that monitoring nasopharyngeal microbiome composition might help with the early detection and intervention of recurrent wheezing

in allergic-sensitized children [120]. The study Copenhagen Prospective Studies on Asthma in Childhood 2000 (COPSAC2000) and COPSAC2010 reported that neonatal colonization of pathogenic bacteria (*Moraxella catharralis*, *Haemophilus influenzae*, and/or *Streptococcus pneumoniae*) in the hypopharynx was positively correlated with development of systemic low-grade inflammation [121]. Another study in Korea compared the composition and function difference of the upper airway microbiome between children with asthma, remission, and healthy control groups [122]. It suggested that the changes in the composition and function of the upper airway microbiome could be linked to the natural course of childhood asthma.

1.3.4 The role of gut microbiome in the development of asthma

The gut microbiome has also been studied for the association with asthma. Although the mechanism is still unclear, there is accumulating research evidence that the gastrointestinal tract is the most important route for environmental exposure of the microbiome and is crucial for the development of immune responses to allergens and pathogens [123]. The gastrointestinal tract has the greatest number and diversity of microbes and is dominated by facultative and strictly anaerobic bacteria of the phyla Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria [124].

Recent studies highlighted the importance of the gut microbiome in shaping host immune mechanisms [125]. Children at increased risk for asthma appeared to have lower relative abundance of *Bacteroides*, *Bifidobacterium*, *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* species at 3 months of age [126], and a higher relative abundance of *Streptococcus* and *Ruminococcus* species [127]. In addition, asthmatic children were reported to have lower abundance of the genus *Lachnospira* at 3 months of age [128], and lower abundance of *Akkermansia* and *Bifidobacterium* at 1 and 6 months of age [129].

Children who developed asthma at school age displayed a lower total diversity of the gut microbiota during the first month of life, compared to non-asthmatic children [130]. Colonization by species *Clostridium difficile* (phylum Firmicutes) at 1 month of age was associated with asthma at the age of 6 to 7 years [131].

Accumulating evidence shows a cross-talk between the gut and the lungs, which is called the gut-lung axis, whose effect on the maintenance of immune homeostasis has been highlighted [132]. As such dysbiosis of the gut microbiota can intervene with lung inflammation, and might be linked to the increased airway diseases, such as asthma [133].

1.3.5 The correlation of microbiome and IgE

The critical role of microbiota on immune development has been well documented in GF mice studies. A recent study shows that microbial diversity of colonization after birth is important for stimulating an immunoregulatory network that inhibits IgE induction [134]. Another recent study in human subjects indicates that there is an interaction between certain subsets of gut microbiome and IgE-mediated response to allergens in children with asthma and allergic rhinitis [135]. The interaction of human microbiome and immune response is very complex, and more studies are needed to elucidate the correlation of microbiome and IgE.

1.3.6 The environmental influence on the human microbiome and its effect on asthma

Emerging evidence suggests that some environmental microbiota may protect against the development of childhood allergy and asthma. A good example is the traditional farming environment which is rich in a wide range of microbial diversity and has a protective effect from childhood asthma and allergy. One epidemiologic study used data from two large-scale observational studies of school children living in rural areas of central Europe,

and characterized the prevalence of asthma and atopy, and the diversity of microbial exposure. They found in both studies that children who lived on farms had a lower prevalence of asthma and atopy and had a greater exposure to a variety range of environmental microorganisms [92]. A comparative study of immune response and home dust extracts from Amish and Hutterite farm children revealed that the distinct dust microbiota composition from the Amish environment (traditional farming) could protect against asthma by shaping the innate immune response [136]. Similarly, another study investigating environmental factors associated with recurrent wheezing in urban children indicated that high-level exposure to certain allergens and bacteria in early-life might be helpful for preventing recurrent wheeze [137]. The protective effect of the farming environment might come from molecules derived from bacteria, yeast, or fungi, such as endotoxin, a lipopolysaccharide (LPS) from gram-negative bacteria. Endotoxin was shown to have the function of reducing allergen activation in a mouse model and also *in vitro* in human bronchial epithelial cells [138, 139].

Microbiome and asthma and allergy studies of immigrants who migrated from areas of low prevalence to high prevalence of asthma and allergy are of interest. Particularly the shifts of microbiota diversity and abundance and change in the immunoregulatory capacity due the Western environment. However, there are sparse studies that have been conducted to investigate changes of microbiota in immigrants and their association with asthma and allergy.

1.4 Thesis Aims

The overall aim of this doctoral thesis is to investigate the role of microbiome in immigrant populations or in asthmatic children in the developing countries' environment and between children living in contrasting environments: Eastern (developing) versus

Western (developed). The microbiome has also been tested for the association with immunoglobulin E (IgE), and immune response. To meet the research aim, there are four studies in this doctoral thesis, and each study has its specific aims.

Study One: Chapter 3-5

To compare the oropharyngeal and gut microbiome differences between asthmatic and non-asthmatic children in Beijing, China; and to investigate the association of microbiome profile and serum IgE levels between those children;

Study Two: Chapter 6

To compare the oropharyngeal and gut microbiome between Australian Chinese (AC) children in Australia and China-Born Chinese (CC) children in mainland China; and to investigate the consistency of differences in microbiome abundance between the contrasting living environments (Eastern vs Western) and allergic status (with or without allergy);

Study Three: Chapter 7

To compare the oropharyngeal microbiome between newly-arrived and long-term Chinese immigrants living in Australia, and to investigate the correlation of Westernised oropharyngeal microbiome and the immune response in those immigrants.

Study Four: Chapter 8

To investigate the time dependence of the Western environment on the expression of toll-like receptor (TLRs) pathway genes, plasma cytokine levels, and total and specific IgE in Chinese immigrants.

2 Methodology

This chapter describes the methodologies that were used for the four studies presented in this thesis. The first study was a case-control study that compared the human microbiome between asthmatic and non-asthmatic children. The second study was a cross-sectional study that investigated the disparities of microbiome between Australian-born Chinese children and Chinese-born Chinese children. The third and fourth studies cross-sectionally investigated the microbiome difference, the link with the immune response, and the TLR pathway gene changes among Chinese immigrants living in Australia.

2.1 Methodology for Study One

2.1.1 Study population

Study one was a case-control study conducted at the Children's Hospital of the Capital Institute of Paediatrics in Beijing, China. Children aged 3-12 years old with doctor-diagnosed asthma were recruited as “case group” from the asthma outpatient clinic. The asthma diagnosis criteria were based on the Chinese guidelines for the diagnosis and optimal management of asthma in children.[140]

Patients were excluded if the children were diagnosed with severe pneumonia, tuberculosis, congenital pulmonary airway malformation or other chronic diseases, such as diabetes. The minimum age was 3 years old because it is problematic to make asthma diagnosis with certainty in children younger than that. Further, the composition and function of the gut microbiome steadies within the first 3 years of life [141, 142]. The “control group” was recruited from the Orthopaedics inpatient clinic using the same age range. Those patients were mainly diagnosed with fractures, but free from other diseases. The recruitment was conducted during January – July 2018. This study was approved by the human research ethics committee from the Curtin University Human Research Ethics Committee (approval number: HRE2017-0001), and the Children's Hospital of the Capital Institute of Paediatrics (SHERLL2014040).

2.1.2 Questionnaire survey

A children’s questionnaire modified from The International Study of Asthma and Allergies in Childhood Questionnaires was administered. This questionnaire was to collect the information on the child’s health status, allergic diseases history, family history of allergic diseases, and parents’ smoking.

2.1.3 Sample collection and process

The participants underwent venepuncture blood sampling for testing the levels of serum total IgE, allergen specific IgE (sIgE) using the ImmunoCap™ assays (Phadia, Sweden) on Phadia® 250 system (Thermo Fisher Scientific). The serum samples were separated at the same day of recruitment and stored in -80°C freezer. When the recruitment was finished, the samples were tested in the same batch on Phadia® 250 system. The sIgE comprised of ImmunoCAP Phadiatop (Phad), common food allergens: egg white, cow's milk. codfish, wheat, peanut, and soybean (Fx5), mould mix (Mx1), house dust mite: *Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farina* (Df), weed and flower pollen mix (Wx5: Common ragweed, Mugwort, Oxeye, Daisy, Dandelion, Golden rod). Atopic status was defined as serum total IgE level larger than 77.7 kU/L according to a population study based on Asian children [143]. Any sIgE results larger than 0.35 kU/L were defined as specific sensitization [144].

Oropharyngeal (OP) swabs were collected during the clinic visit with a sterile cotton swab and a collection tube and were immediately placed on dry ice and transported to a laboratory freezer (-80°C). Stool samples were collected during the clinical visit for asthmatic patients and in the ward for the control group. If this failed, parents were asked to collect faecal samples from their child with a "stool collection kit" at home. The "collection kit" included a stool collection guide for parents, a 25 ml faeces container with brown screw-cap lid (Sarstedt), a piece of underpad sheet, a pair of disposable gloves, and a sealed plastic bag. Parents were asked the following. First label the container and plastic bag with children's name and date of collection. The child should urinate into the toilet before bowel movement to avoid the contact of urine and stool. Then the underpad sheet is placed on the floor for the children's bowel movement. The stool specimen is collected using gloves and the spoon attached to the

lid and the specimen is placed into the container, sealed in the plastic bag and immediately frozen at −20°C in a home freezer. The samples were then collected by one of the research team members in three days and transported on dry ice to the laboratory freezers (-80°C) for storage until DNA extraction.

2.1.4 16s rRNA sequencing and Taxonomic Assignment

DNA was extracted from pharyngeal swab samples using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific), and from stool samples using QIAamp DNA Microbiome Kit (QIAGEN, cat#51704) following the manufacturer's guidelines. PCR amplification and sequencing were performed by the Beijing Novogene Bioinformatics Technology Co., Ltd. The V3–V4 region of the bacterial 16S rRNA gene of each sample were amplified with universal 16S ribosomal RNA (rRNA) gene primers using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The cycling conditions were: 98℃ for 1 min, 35 cycles of 98℃ for 10 sec, 50℃ for 30 sec, 72℃ for 30 sec; and then final extension at 72℃ for 5 min.

The resulting amplicons were mixed with an equal volume of 1X loading buffer. After operating electrophoresis on 2% agarose gel, samples with a bright main strip between 400-450bp were chosen for further experiments. PCR products were mixed with equidensity ratios and purified using QIAGEN Gel Extraction Kit (QIAGEN, Germany).

TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to generate sequencing libraries. The library quality was measured on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system before the library was sequenced on an Illumina HiSeq 2500 platform.

2.1.5 Bioinformatics analysis

FLASH V1.2.7 was used to merge the output paired-end reads [145]. Raw sequencing reads were filtrated by using Quantitative Insights Into Microbial Ecology (QIIME 1.9.1)[146] (http://qiime.org/scripts/split_libraries_fastq.html) according to the following (1) read-quality score more than 19, (2) setting length larger than 3bp, and (3) consecutive high quality base over 75%. Chimera sequences were removed with the usearch61 algorithm (http://qiime.org/scripts/identify_chimeric_seqs.html). Operational Taxonomic Units (OTUs) were picked by using the pick_open_reference_otus.py workflow against the SILVA reference database (128 release)[147] at ≥ 97% sequence identity threshold. The total number of sequences were discarded by filtering the observation counts below 0.005% [148]. OTUs were single rarefied to get even depths of 43907 for OP and 49024 for faecal samples, respectively. The α- diversity describes the number of taxa in sites or habitats at a more local scale and was determined by using the statistical methods: chao1 richness estimate, observed OTUs, Simpson, and Shannon index (http://qiime.org/scripts/compare_alpha_diversity.html). The β-diversity analysis shows the similarity between microbial communities [149]. The distances/similarities between microbial communities were calculated using weighted and unweighted UniFrac matrix. To predict the functional pathways of the microbiome, we clustered the sequences into OTUs against the Greengenes reference database (13_8 release) and performed Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis [150]. The estimated abundances from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups [151] were compared between non-asthmatic and asthmatic children using the linear discriminant analysis effect size (LEfSe) [152].

2.1.6 Statistical analysis

Statistical analysis was carried out using IBM SPSS (version 25) or within the QIIME pipeline. For the demographics and general results (Chapter 3) continuous variables were described as mean ± standard deviations (SD), namely age, body mass index (BMI), born weight and height, and the time to introduce solid food. The total and specific IgE were described as median and quantiles and compared using Mann-Whitney U test between asthmatic and non-asthmatic children. Categorical variables were described as number and percentage and compared using χ^2 test. Fisher's exact test was used when the expected value in any cell of the contingency table was below five. For the comparison of microbiome composition and diversity (Chapter 4), Mann-Whitney U tests were used to find different OTU relative abundance between asthmatic and non-asthmatic children by using "group_significance.py" within the QIIME pipeline. Alpha-diversity metrics were performed by "compare_alpha_diversity.py", and Adonis permutation ANOVA methods ("compare_categories.py") were used to analyse beta-diversity difference in QIIME. Data were visualized by bar plots or box plots using the "ggplot2" package in RStudio (Version 1.0.153). Two-dimensional Unifrac PCoA plots were created using the make_2d_plots.py in QIIME. All the p-values were corrected for False Discovery Rate (FDR) to control for multiple testing. In Chapter 5, the participants were regrouped as IgE positive/negative according to their serum total IgE levels. The microbial diversity and abundance were compared between IgE positive and negative groups. The association of serum IgE level and the relative abundance of microbiome was tested by using the Spearman correlation.

2.2 Methodology for Study Two

2.2.1 Study population

This cross-sectional study was conducted among Australian Chinese (AC) children in Australia, and China-born Chinese (CC) children in mainland China. The study is described in detail in Chapter 6 and was published in the World Allergy Organization Journal (Volume 12, Issue 8, August 2019). In summary, first 58 AC children (aged 3-18) were recruited from the local Chinese community living in Perth, Australia. Then 63 CC children were recruited by random selection from kindergartens, primary and high schools in Hebi city, Henan province so that their gender frequency and age range matched. Parents/guardians were asked to fill out a questionnaire for their child. The questionnaire collected demographic information, delivery method (Vaginal delivery/ Caesarean section), breastfeeding history, self-reported food allergic history and current wheezing (within recent 12 months that the child has had wheezing).

2.2.2 Skin prick tests (SPT)

The skin prick tests (SPT) were performed on every child by experienced nurses in order to measure atopic status with commercial allergen extracts kits. The SPT results were evaluated after 15–20 min exposure, and positive atopy was defined as a wheal size >3mm in diameter in reaction to at least at one allergen. In Australia, the common allergens include cow's milk, egg white, rye grass and mixed grass pollen, *D. farinae*, *D. pteronyssinus*, cat dander, dog dander, cockroach and moulds. In China, the common allergens were largely the same as those in Australia besides ragweed and shrimp instead of rye grass and mixed grass pollen.

2.2.3 Sample collection and process

OP swabs were collected with a sterile cotton swab into a collection tube and were immediately placed on dry ice and transported to the laboratory freezer (-80°C). Parents were asked to collect faecal samples from their child in a 25 ml faeces container (Sarstedt) provided by our laboratory. They collected approximately 10 grams of stool that was immediately frozen at −20°C in a freezer at home. The samples were then collected by research members within 3 days and transported on dry ice to the laboratory freezers (-80°C) for storage until analysis.

In total, 45 OP samples and 46 faecal samples were collected from participants in Australia. Sixty two OP samples and 53 faecal samples were collected from participants in China. Thirteen OP samples and one faecal sample from China did not pass the DNA quality control. The rest of the samples were selected for further analysis. DNA from OP and faecal samples was extracted using the QIAamp DNA Microbiome Kit (cat#51704, QIAGEN) following the instructions from the manufacturer. The DNA concentration was monitored by Nanodrop (ND-1000, Thermo scientific). Amplicons of the 16S rRNA gene V3-V4 region were sequenced on an Illumina HiSeq 2500 platform. The paired-end reads were merged, then filtered, and the sequences were assigned into Operational Taxonomic Units (OTUs) against the SILVA reference database (128 release). Bioinformatics and statistical analysis were carried out within the QIIME 1.9.1 pipeline or using RStudio (Version 1.0.153). The analysis protocol of this study was similar as described in sections 2.1.5 and 2.1.6. A detailed description of the statistical methods can be found in Chapter 6.

2.3 Methodology for Study Three

2.3.1 Study population and design

This cross-sectional study was based on "Asthma and allergy in Chinese immigrants in Australia" that was initiated in 2012 [153]. The recruiting method has been described in a previous study from our research group [154]. Briefly, twenty-two newly-arrived Chinese immigrants (living in Australia <6 months) and 22 long-term adult Chinese immigrants (living in Australia >5 years) with matched age and gender were recruited during a three-month period (Nov 2014 to Feb 2015). All participants gave informed written consent. This study has been described in detail in chapter 7 and the manuscript is under review.

2.3.2 Sample collection, processing and immune response

Oropharyngeal swab samples, serum, and whole blood samples were collected on the recruitment day. Oropharyngeal swabs were obtained using a sterile cotton swab which was immediately placed into a sterile collection tube. DNA was extracted from oropharyngeal swab samples using the QIAamp DNA Microbiome Kit (QIAGEN, cat#51704) following the manufacturer's guidelines. PCR amplification and sequencing were performed by the Australian Genome Research Facility (AGRF).

The TLR innate immune response was characterized by twenty-three cytokines that were measured ex vivo using whole blood assay under stimulation of six TLR ligands. The adaptive immune response was characterized by IgG1 responses to bacterial and viral antigens which was tested using immunoassays for total IgG1 and immunoabsorption assays for species-specific IgG1 antibody binding. The Pharmacia CAP system (Pharmacia Diagnostics AB, Uppsala, Sweden) was used to assess total

and specific IgE from serum samples at the PathWest Immunology Department (QEII Medical Centre, Perth, Australia).

2.3.3 Bioinformatics and statistical analysis

The bioinformatics analysis protocol within the QIIME 1.9.1 pipeline or using RStudio (Version 1.0.153) was similar as described in section. 2.1.5. The relative abundance and diversity of microbiome was compared between newly-arrived and long-term Chinese immigrants. The 23 cytokines, IgG1 antibody responses to pathogens, and serum IgE levels were correlated with microbial taxa (relative abundance over 1%) by using Spearman correlation test for newly-arrived and long-term immigrants, respectively. To further illustrate differences in correlation of innate response and oropharyngeal microbiome between newly-arrived and long-term immigrants, we calculated the Spearman correlation of each microbial taxa (over 1.0%) with 138 cytokine measurements (23 cytokines * six TLR ligands), and then used paired sample t test to show the strength of these correlations. Detailed descriptions are given in Chapter 7.

2.4 Methodology for Study Four

This section provides a brief summary of the methods we used. The detailed methods are described in Chapter 8.

2.4.1 Study population

As with Study Three, the cohort in Study Four was based on the “Asthma and allergy in Chinese immigrants in Australia” project. One hundred and seven adult Chinese immigrants were recruited in Perth, Western Australia by advertisements through

Chinese media. Blood samples were collected for serum, plasma and isolation of peripheral blood mononuclear cells (PBMCs).

2.4.2 TLR pathway gene expression, cytokine, and IgE measurements

We measured PBMCs gene expression levels of 10 TLR pathway genes (*TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, *TLR10* and nucleotide-binding oligomerization domain-containing protein 1 [*NOD1*]) and three cytokine genes (interleukin [*IL*]*2*, *IL13* and interferon gamma[*IFNγ*]). The gene expression levels were normalized against 18S ribosomal RNA and beta-2-microglobulin. Then, the relative expression levels were obtained using $2^{-\Delta\Delta CT}$. We also measured plasma IL-5, IL-6, IL-13, IL-17 and IFN-γ using a solid-phase sandwich ELISA assay and serum total and allergen-specific IgE using the Pharmacia CAP system.

2.4.3 Statistical analysis

The continuous variables were compared between different time groups using One-way ANOVA. A line of best fit with a coefficient of determination (r^2) was calculated using linear regression to determine the relative impacts of residence time in Australia (grouped 1: <1 year, 2: 1 to 3 years, 3: 3 to 6 years, and 4: >6 years) on the gene expression, cytokine and IgE levels.

3 Study One —Population Demographics and general results

Chapters 3-5 describe our findings of study one about the relation between the microbiome and childhood asthma in a case-control study conducted between asthmatic and non-asthmatic children in Beijing, China. Chapter 3 describes the demographics and general results followed by the microbiome profiles in Chapter 4 and the correlation with serum IgE in Chapter 5. This study is in collaboration with the Children's Hospital of the Capital Institute of Paediatrics in Beijing. This collaborative study was supported by the Curtin International Postgraduate Research Scholarship (CIPRS) and Health Sciences Faculty International Research Scholarship (HSFIRS) and has successfully built up the international research links for the Faculty of Health Sciences at Curtin.

3.1 Population demographics

One hundred and one asthmatic children and 48 non-asthmatic children participated in this case-control study. We have 44 and 100 valid questionnaires to collect the demographics and allergy disease information. 94 (93.1%) cases and 46 (95.8%) controls provided oropharyngeal specimens, while 53 (52.5%) cases and 31 (64.6%) controls provided stool samples. In total, we have collected 140 oropharyngeal swab samples and 84 faecal samples for further sequencing analysis. One hundred and forty-four (96.6%) participants have completed the questionnaire survey and 138 (92.6%) participants were tested for serum IgE level.

The demographic details are shown in Table 3-1. The gender distribution was even with 18 (37.5%) and 37 (36.6%) non-asthmatic and asthmatic female children, respectively. The mean age of the non-asthmatic children was one year older than the asthmatic children (p= 0.034). 61 (60.4%) of the asthmatic children had an acute exacerbation status while 30 (38.6%) had a persistent status.

Solid food was introduced at 5.4±1.4 months for asthmatic children, which is one month earlier than for the non-asthmatic children (6.1±1.8 months, p= 0.010). No significant differences were found in BMI, delivery method, breastfeeding history, born weight and height, antibiotic usage, daily fruit and vegetables intake between the non-asthmatic versus asthmatic children.

Table 3-1. The demographics of the study participants

General Information	Non-asthmatic children (n=48)	Asthmatic children (n=101)	Statistics	*p*
Females: n (%)	18 (37.5%)	37 (36.6%)	χ^2= 2.68	0.918
Age (y): mean (SD)	7.1 (2.8)	6.1 (2.6)	*t*= 2.15	**0.034**
BMI (kg/m^2): mean (SD)	15.9 (3.1)	16.6 (3.3)	*t*= -1.28	0.202
Delivery method				
Vaginal delivery n (%)	23 (52.3%)	53 (53.0%)	χ^2= 0.01	0.936
Caesarean section n (%)	21 (47.7%)	47 (47.0%)		
Breastfed: n (%)	42 (95.5%)	85 (85.0%)	χ^2=3.01	0.073
Birth weight (kg): mean (SD)	3.5 (0.79)	3.4 (0.50)	*t*= 1.54	0.126
Birth length (cm): mean (SD)	50.9 (2.4)	49.2 (8.1)	*t*= -1.28	0.207
Time to introduce solid food (months): mean (SD)	6.1 (1.8)	5.4 (1.4)	*t*= 2.62	**0.010**
Antibiotic used (past 2 weeks): n (%)	5 (11.6%)	22 (22.0%)	χ^2=2.11	0.146
Fruit daily intake: n (%)				
0~2 kinds	36 (81.8%)	8 (18.2%)	χ^2=0.84	0.370
>= 3 kinds	75 (75.0%)	25 (25.0%)		
Vegetable daily intake: n (%)				
0~2 kinds	25 (56.8%)	53 (53.0%)	χ^2=0.18	0.672
>= 3 kinds	19 (43.2%)	47 (47.0%)		

3.2 The comparison of allergic diseases and risk factors

Table 3-2 shows the comparison of allergic diseases between the two groups of children. Children with asthma have significant higher rates of rhinitis, eczema ever, current eczema, and allergic conjunctivitis compared to non-asthmatic children. Asthmatic children also had a higher rate of immediate-family history of allergic conditions (65.0%) compared to non-asthmatic children (34.1%, $p< 0.001$). However, there was no significant difference in the allergic history of second-degree relatives.

Table 3-2. The comparison of the allergic diseases between asthmatic and non-asthmatic children

	Non-asthmatic children (n=44)	Asthmatic children (n=100)	Statistics	*p*
Rhinitis n(%)	13 (29.5%)	70 (70.0%)	χ^2=20.4	**<0.001**
Food allergy n(%)	5 (11.4%)	10 (10.0%)	χ^2=0.06	0.805
Eczema ever n(%)	21 (47.7%)	71 (71.0%)	χ^2=7.17	**0.007**
Current eczema n(%)*****	1 (2.3%)	14 (14.1%)	χ^2=4.57	**0.038**
Urticaria n(%)	7 (15.9%)	20 (20.0%)	χ^2=0.34	0.562
Allergic conjunctivitis	1 (2.3%)	21 (21.0%)	χ^2=8.28	**0.004**
Immediate-Family history of allergic conditions n(%)	15 (34.1%)	66 (66.0%)	χ^2=12.64	**<0.001**
Second-degree relatives' history of allergic conditions n(%)	9 (20.5%)	29 (29.0%)	χ^2=1.15	0.284

* Fisher's exact test was used for groups' comparison.

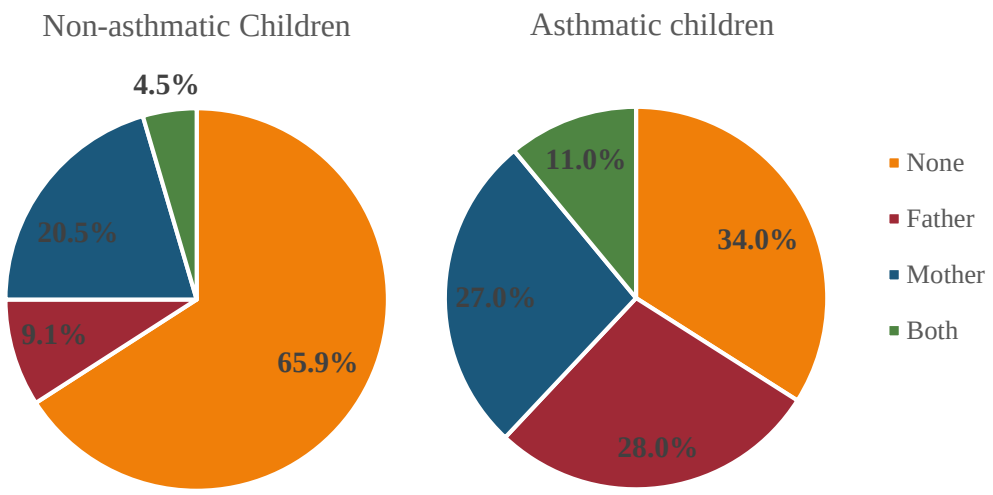


Figure 3-1. The distribution of immediate family history of allergic conditions in non-asthmatic and asthmatic children

The pie chart in Figure 3-1 shows the immediate family history of allergic conditions for non-asthmatic and asthmatic children, respectively. Asthmatic children have more often a mother or a father, or both parents, with allergies.

We have investigated other known risk factors for asthma (Table 3-3). There was no difference in pet keeping between the investigated families with non-asthmatic (13.6%) and asthmatic children (17.0%). From the six non-asthmatic children families keeping pets, one had a cat (16.7%), three had dogs (50.0%), and two had other kinds of pets (33.3%). Among the 17 asthmatic children families, three had cats (17.6%), six had dogs (35.3%), and eight had other pets (47.1%) in their homes. There is no significant difference in the distribution of the variety of pets. The passive smoking rate was slightly higher in asthmatic children, while the probiotic intake rate was lower compared to non-asthmatic children, although those differences were not significant.

Table 3-3. The comparison of the other known risk factors for asthma between asthmatic and non-asthmatic children

	Non-asthmatic children (n=44)	Asthmatic children (n=100)	Statistics	*p*
Pet keeping n(%)	6 (13.6%)	17 (17.0%)	χ^2=0.26	0.612
Passive smoking exposure n(%)	6 (13.6%)	18 (18.0%)	χ^2=0.42	0.517
Probiotic intake n(%)	8 (18.2%)	9 (9.0%)	χ^2=2.48	0.116

3.3 The comparison of serum total and specific IgE

Total and specific IgE levels were measured in 99 asthmatic and 39 non-asthmatic children. As shown in Table 3-4, the total and specific IgE levels were both higher in asthmatic children. The percentage of high serum total IgE for asthmatic children was 73.7% compared to 20.5% in non-asthmatic children. The sensitization rates for allergens were all above 80.0% among asthmatic children and significantly higher than among non-asthmatic children (all ≤15.0%).

Table 3-4. The total and specific IgE comparison between children with and without asthma

	Non-asthmatic children			Asthmatic children			
	n	median	(Q1,Q3)	n	median	(Q1,Q3)	p^*
Total IgE	39	29.4	(13.1,62.4)	99	147.0	(71.7,344)	**<0.001**
Food allergens (Fx5)	39	0.07	(0.02,0.21)	74	0.32	(0.15,0.83)	**<0.001**
Aeroallergens (Phad)	39	0.02	(0.01,0.09)	93	1.43	(0.07,19.9)	**<0.001**
Mould mix (Mx1)	39	0.06	(0.05,0.08)	95	0.16	(0.05,4.55)	**0.013**
Weed & Flower Mix (Wx5)	39	0.00	(0.00,0.01)	88	0.07	(0.03,0.41)	**<0.001**
D. pteronyssinus (dp)	39	0.01	(0.01,0.03)	97	0.12	(0.03,5.10)	**<0.001**
D. farina (df)	39	0.01	(0.00,0.04)	97	0.22	(0.03,8.18)	**<0.001**
Positive rate		n (%)			n (%)		$p^\dagger$
Total IgE	39	8 (20.5)		99	73 (73.7)		**<0.001**
Food allergens Positive (Fx5)	39	6 (15.0)		75	34 (85.0)		**0.001**
Aeroallergens Positive (Phad)	39	7 (11.3)		93	55 (88.7)		**<0.001**
Mould mix Positive (Mx1)	39	4 (8.5)		95	43 (91.5)		**<0.001**
Weed & Flower Mix (Wx5)	39	2 (7.1)		88	26 (92.9)		**0.002**
D. pteronyssinus (dp)	39	3 (7.9)		97	35 (92.1)		**0.001**
D. farina (df)	39	5 (11.1)		97	40 (88.9)		**0.001**

*: Mann-Whitney U Test was utilised for group comparison

†: χ^2 test was used to test the allergens positive frequency difference

Q1: 25th quantile; Q3: 75th quantile.

3.4 Discussion

This chapter details the demographic information, allergy risk factors, and serum IgE test results of the participants in Study One. To control for selection bias, we recruited both case and control groups from the same hospital and during the same recruitment period from January to July 2018. We acknowledge that asthmatic children recruited in this hospital had a high level of total and specific IgE. The findings from the population should be generalized to other asthmatic children populations with caution.

We found that rhinitis, eczema, and allergic conjunctivitis were significantly higher in asthmatic children. This is consistent with previous findings. Rhinitis and asthma are common morbidities, and the two diseases interact at various levels. Rhinitis usually precedes an asthma attack and may lead to poor asthma control [155]. In a survey conducted among 1549 asthmatic children in Brazil, the frequency of allergic conjunctivitis in those patients is high, yet underreported [156]. Another retrospective survey conducted in Greece showed that allergic conjunctivitis was very common in patients with symptoms of allergies and the coexistence of rhinitis and allergic conjunctivitis was the strongest indicator for IgE-mediated allergies [157]. A study that examined the association between childhood eczema and asthma incidence from preadolescence to middle age showed that childhood eczema was significantly associated with childhood asthma, and with asthma that persisted into middle age [158].

We found that asthmatic children had a significantly higher family history of allergies. Many studies have related family history of asthma and allergy to the increase of asthma in children [159, 160]. A cross-sectional survey from 5,046 Californian children has shown that a parental history of asthma and allergies is highly correlated

with an early onset persistent asthma [161]. As atopic diseases can show a transient or persistent status this cohort study has followed children from birth up to age 6~7 years [162]. They found that the father's disease history, especially the history of asthma, is more relevant to the paediatric prognosis than the mother's history. Our study also supports this finding as the disparity between the father's allergy of non-asthmatic and asthmatic children was much larger than the mother's.

In our study we also found that asthmatic children were introduced almost one month earlier to solid food during their infancy. The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life [163]. But studies about when to introduce solid food are quite conflicting and influenced by many factors, such as the food, dose, frequency, and the heritable background of the child [164]. Studies which showed that delayed introduction of solid food did not protect against the development of allergies and asthma [165, 166] defined delayed introduction to solid food as after 4 or 6 months, and sometimes after 3 months. In our study however, children from both groups fit into this "delayed introduction to solid food" category. It is hard to draw conclusions about the timing for solid-food introduction in our study and detailed information about the food variety and the introduction method are needed.

It was anticipated that the total and specific IgE levels were higher in asthmatic children. Allergenic sensitization plays an important role in the development of asthma and IgE is a central player in the allergic response [167, 168]. The IgE test is excellent to identify a sensitized state, find its triggers and provide help for selection of immunotherapy treatment [169].

In conclusion, this study cohort had comparable demographics of cases and controls. We found that the asthmatic children had higher rates of rhinitis, eczema, allergic conjunctivitis, and family allergy history. IgE testing showed the asthmatic children were more allergically sensitized.

4 The distinct microbiome profiles between asthmatic and non-asthmatic children in Beijing, China

4.1 Microbial diversity of oropharyngeal (OP) samples

The α-diversity of the OP microbiome of non-asthmatic children and asthmatic children is shown in Table 4.1 and Figure 4.1. Three of the α-diversity indexes, namely the chao1 richness estimate, the Shannon index, and the observed OTUs, were all higher in non-asthmatic children compared to asthmatic children. However, no difference was found for the Simpson index.

Table 4-1. The α-diversity indexes comparison of OP samples between asthmatic and non-asthmatic children

	Non-asthmatic children	Asthmatic children	*p*
Chao1 richness estimate	392.44±30.40	356.19±40.38	**0.001**
Shannon index	4.50±0.51	4.27±0.54	**0.012**
Observed OTUs	362.17±29.95	324.96±39.11	**0.001**
Simpson	0.86±0.06	0.85±0.07	0.112

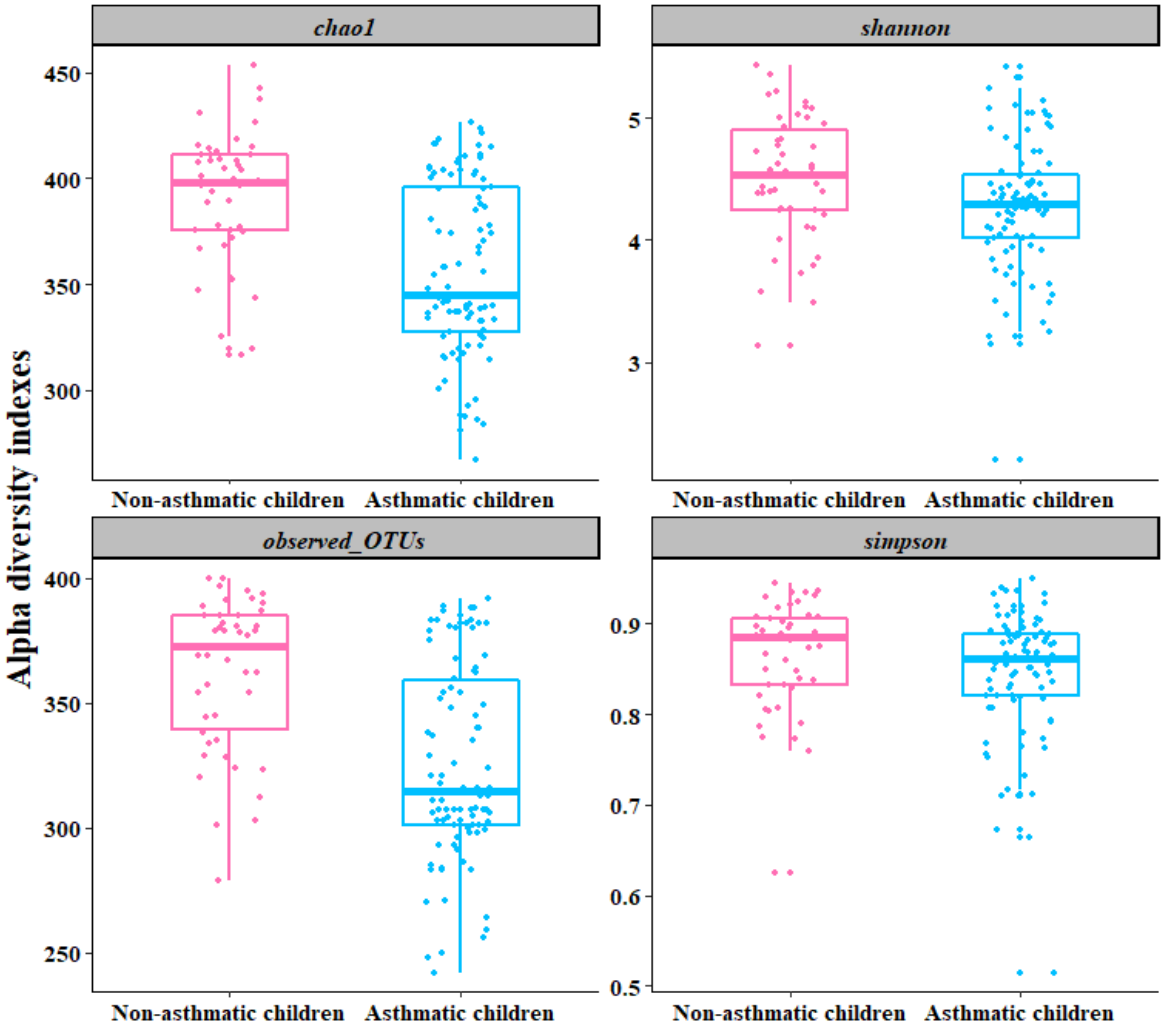


Figure 4-1. The α-diversity comparison in OP samples between non-asthmatic and asthmatic children

Figure 4-1 shows a clear difference in α-diversity between non-asthmatic (pink) and asthmatic children (blue). The top and bottom lines of the boxplots are the interquartile ranges and the thicker lines inside the boxes are the median.

Unweighted and weighted UniFrac distance matrices (β-diversity parameter) were used to compare the OP bacterial composition between children with or without asthma. Adonis permutational ANOVA test revealed a significant difference in unweighted Unifrac Principal Coordinate Analysis (PCoA) (R^2=0.09, p= 0.001), and weighted Unifrac PCoA matrix (R^2=0.03, p= 0.014) between non-asthmatic children and asthmatic children (Figure 4-2).

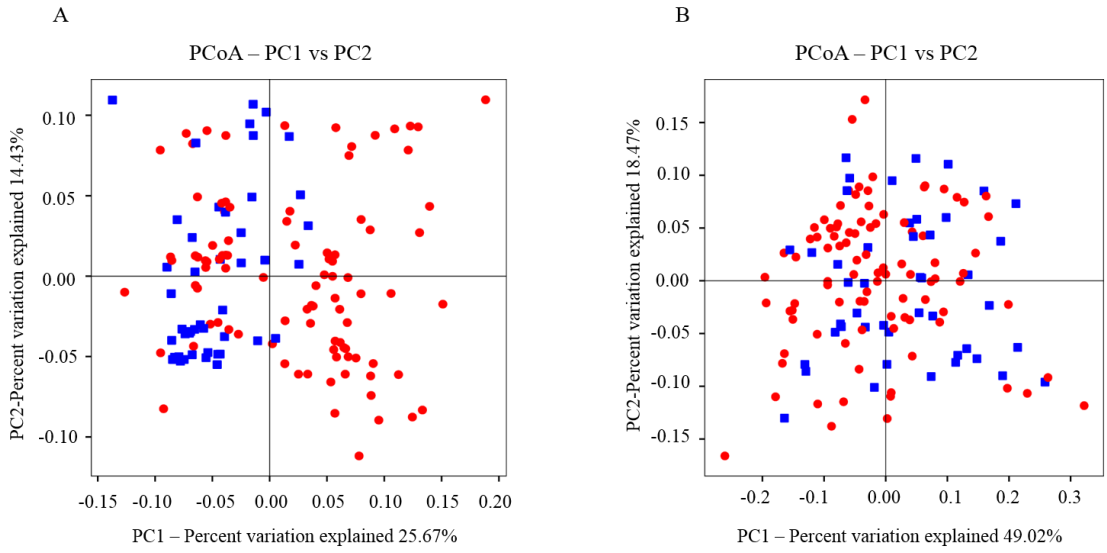


Figure 4-2. The β-diversity in oropharyngeal samples from children with or without asthma

The PCoA plots represent the β-diversity comparison between non-asthmatic children (blue) and asthmatic children (red) with two major principal components represented on the x- and y-axes (Figure 4-2). Panel A shows the unweighted Unifrac PCoA matrix, and panel B represents the weighted Unifrac PCoA matrix.

4.2 Microbial composition of OP samples

Eight phyla were assigned from OP samples and Firmicutes accounted for the largest proportion among both non-asthmatic and asthmatic children. Table 4-2 and Figure 4-3 compares the relative abundance between the two groups. For non-asthmatic children,

there were five phyla which had a relative abundance over 1.0%, namely, in descending order, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. The same five phyla were found in asthmatic children with relative abundance over 1.0%, however, Proteobacteria took the second largest proportion, followed by Bacteroidetes, Actinobacteria, and Fusobacteria. The Mann-Whiney U test showed a significant difference in the relative abundance of Bacteroidetes between children with or without asthma (FDR-corrected p= 0.043).

Table 4-2. The comparison of OP samples microbiome at phylum level between asthmatic and non-asthmatic children

Phylum	Non-asthmatic children	Asthmatic children	*p*	*FDR_p**
Firmicutes	53.85%	55.33%	0.546	0.744
Bacteroidetes	16.56%	12.35%	0.005	**0.043**
Proteobacteria	15.86%	18.23%	0.072	0.193
Actinobacteria	9.31%	10.52%	0.407	0.744
Fusobacteria	4.08%	3.32%	0.651	0.744
Saccharibacteria	0.18%	0.22%	0.585	0.744
Cyanobacteria	0.15%	0.03%	0.019	0.077
SR1 (Absconditabacteria)	0.01%	0.00%	0.982	0.982

*: The *p*-values were FDR-corrected to control for multiple testing

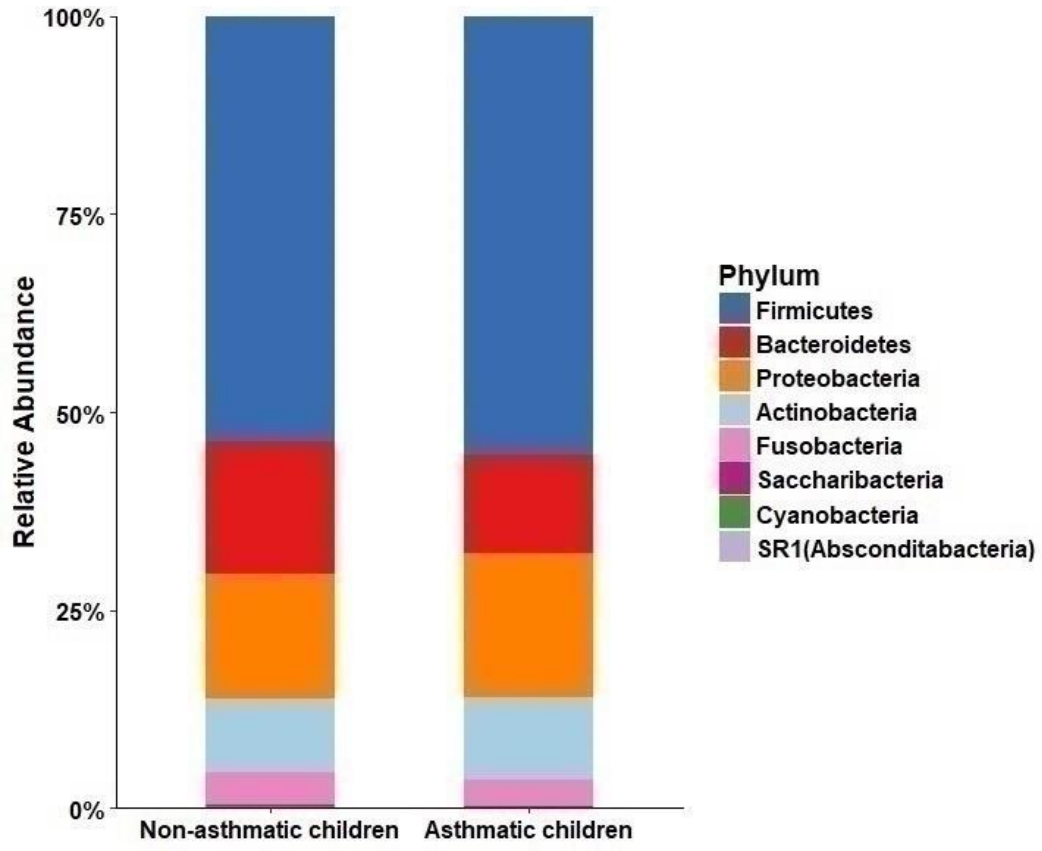


Figure 4-3. Bacteria relative abundance comparison at the phylum level for OP samples

At the genus level, a total of 118 genera were detected from OP samples of which 18 and 17 genera had a relative abundance ≥ 1.0% for non-asthmatic and asthmatic children, respectively. Genus *Streptococcus* (Firmicutes) had the highest relative abundance in both non-asthmatic children (37.48%) and asthmatic children (39.20%). These genus with relative abundance larger than 1.0% were in phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. Asthmatic children had a lower proportion of genus *Bacteroides* (Bacteroidetes) and *Faecalibacterium* (Firmicutes) with an FDR-corrected $p < 0.001$ (Table 4-3).

Table 4-3. The comparison of OP samples microbiome at genus level between asthmatic and non-asthmatic children

Genus	Non-asthmatic children	Asthmatic children	*p*	*FDR_p**
Streptococcus	37.48%	39.20%	0.356	0.467
Prevotella 7	7.52%	6.03%	0.205	0.304
Rothia	6.78%	7.46%	0.555	0.636
Gemella	6.43%	7.25%	0.143	0.225
Haemophilus	5.90%	6.88%	0.251	0.356
Neisseria	5.22%	6.07%	0.104	0.169
Granulicatella	3.20%	3.67%	0.062	0.107
Bacteroides	2.90%	1.05%	0.000	**<0.001**
Leptotrichia	2.87%	1.89%	0.492	0.574
Neisseriaceae_uncultured	2.14%	2.96%	0.080	0.134
Alloprevotella	1.99%	1.89%	0.425	0.511
Veillonella	1.57%	1.52%	0.561	0.637
Porphyromonas	1.53%	1.50%	0.373	0.473
Actinomyces	1.51%	1.63%	0.373	0.473
Prevotella	1.41%	0.94%	0.206	0.304
Actinobacillus	1.29%	1.15%	0.667	0.742
Fusobacterium	1.13%	1.35%	0.097	0.159
Faecalibacterium	0.90%	0.40%	0.000	**<0.001**

*: The *p*-values were FDR-corrected to control for multiple testing

4.3 Functional characterization of the oral microbiome

The KEGG pathway difference between the children with and without asthma was compared using the linear discriminant analysis effect size (LEfSe). Figure 4-4 shows the pathways difference with an LDA score over 2.0. We observed that the OP samples of asthmatic children showed more abundant functions of “Genetic information processing”, which included the “Translation” and the “Replication and Repair” pathways. Only two pathways under “Metabolism” were significantly increased in asthmatic children, namely “Pyruvate metabolism” (Carbohydrate Metabolism) and “Purine metabolism” (Nucleotide Metabolism).

Non-asthmatic children showed more abundant functions in “Metabolism”, “Cellular Processes”, and “Environmental Information Processing” pathways. Especially for the Metabolism pathway, five pathways were found of which the abundance was increased in non-asthmatic children. These pathways were “Starch and sucrose metabolism” (Carbohydrate Metabolism), “Methane metabolism” (Energy Metabolism), “Other glycan degradation” (Glycan Biosynthesis and Metabolism), “Peptidases” (Enzyme Families), and “Sphingolipid metabolism” (Lipid Metabolism).

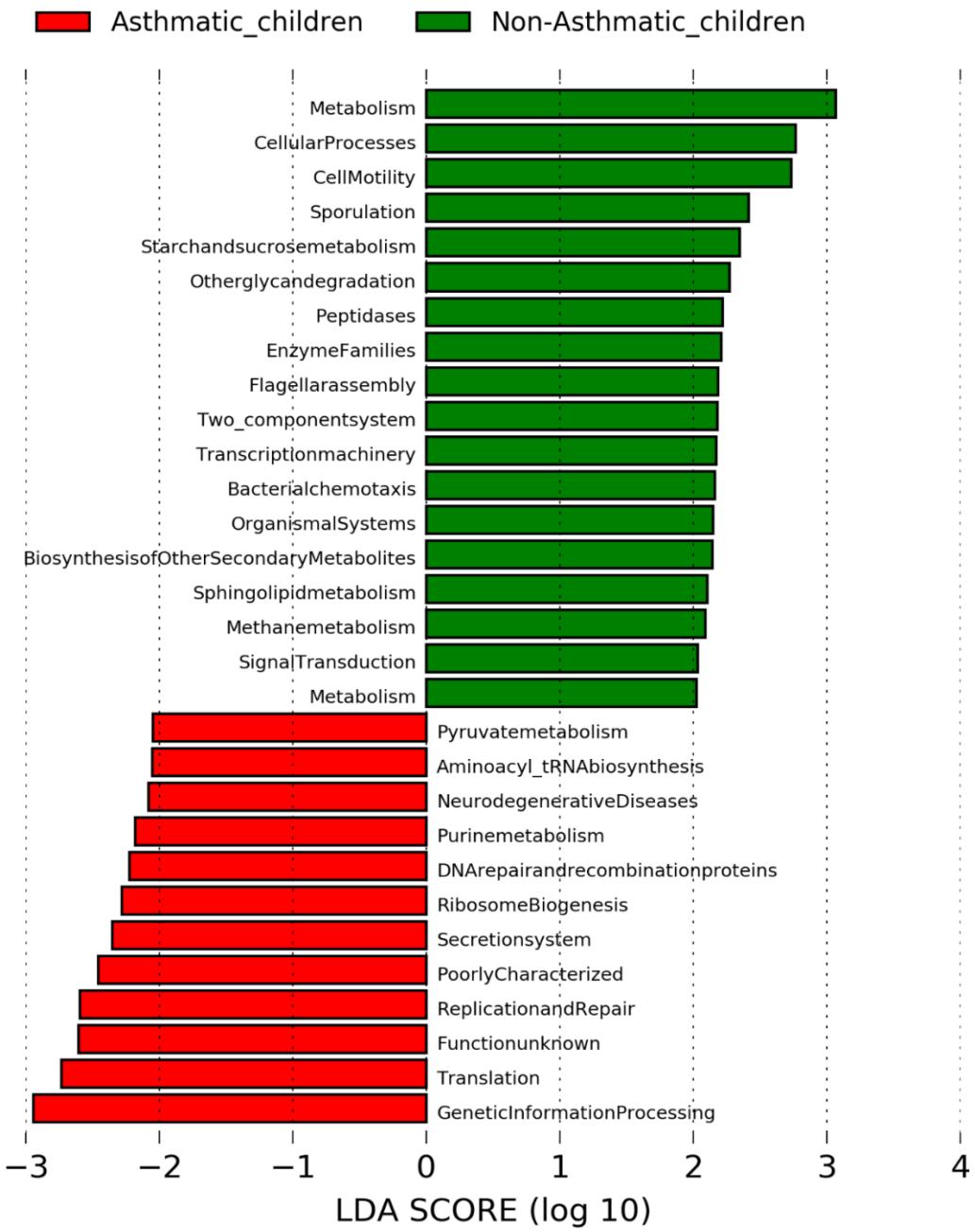


Figure 4-4. Bar graphs of linear discrimination analysis (LDA) for the mean differences of KEGG pathway for OP samples

KEGG pathways that are more present in non-asthmatic children are represented by green bars, whereas those more present in asthmatic children are represented by red bars. Note the plots have a logarithmic x-axis (of base 10) and only KEGG pathway differences with an order of 2.0 or higher are represented.

4.4 Microbial diversity of faecal samples

The α-diversity of the faecal microbiome of non-asthmatic children and asthmatic children is shown in Table 4.4 and Figure 4.5. None of the α-diversity indexes of the faecal samples showed a significant difference between non-asthmatic and asthmatic children.

Table 4-4. The α-diversity indexes comparison of faecal samples between asthmatic and non-asthmatic children

	Non-asthmatic children	Asthmatic children	*p*
Chao1 richness estimate	448.99±43.71	438.66±49.69	0.323
Shannon index	5.07±0.65	4.99±0.65	0.615
Observed OTUs	407.65±45.15	401.43±50.91	0.599
Simpson	0.90±0.07	0.90±0.06	0.658

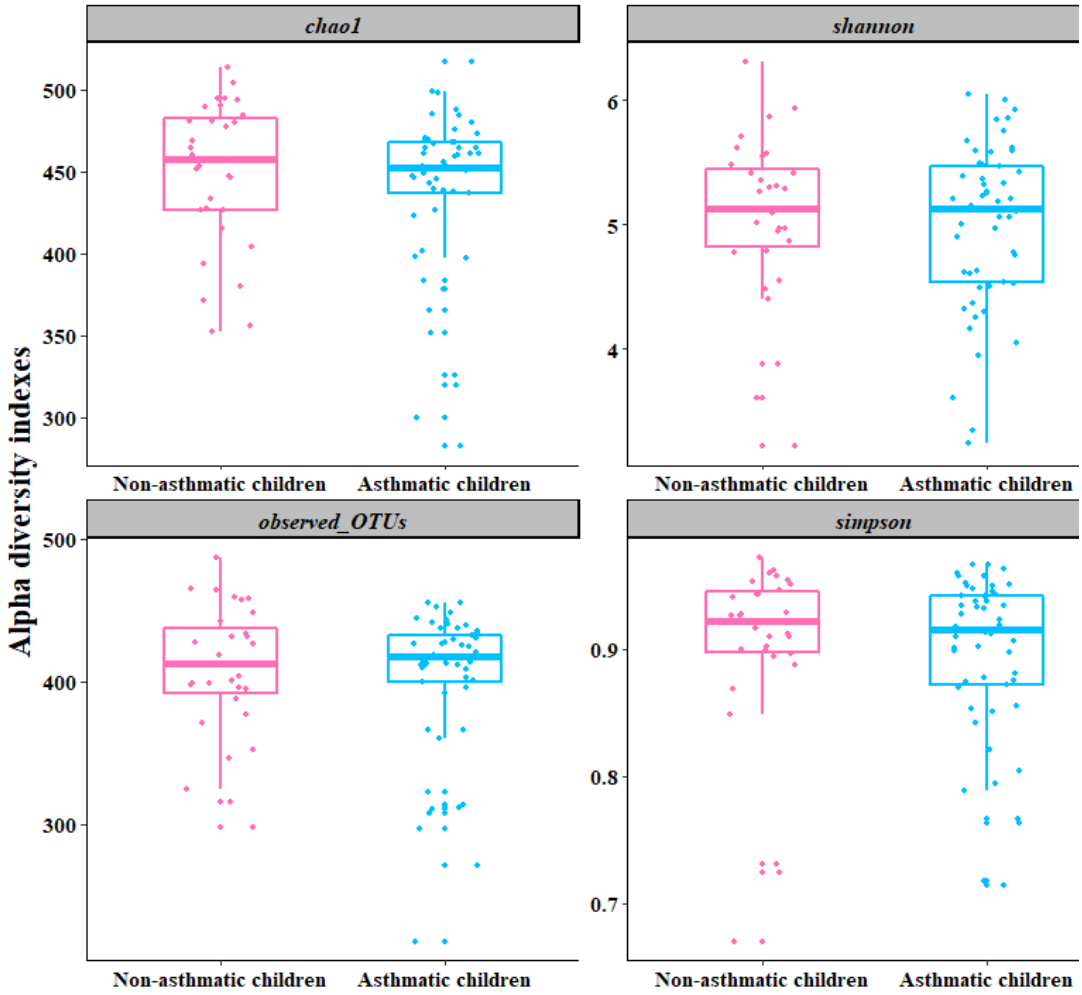


Figure 4-5 The α-diversity comparison in faecal samples between non-asthmatic and asthmatic children

Four panels of box plots show the different indexes of α-diversity comparing non-asthmatic (pink) and asthmatic children (blue). The top and bottom lines of the boxplots are the interquartile ranges and the thicker lines inside the boxes are the median.

Adonis permutational ANOVA revealed a significant difference in unweighted Unifrac PCoA (R^2=0.04, p= 0.004) and weighted Unifrac PCoA (R^2=0.04, p= 0.014) between the faecal samples of non-asthmatic children and asthmatic children (Figure 4-6).

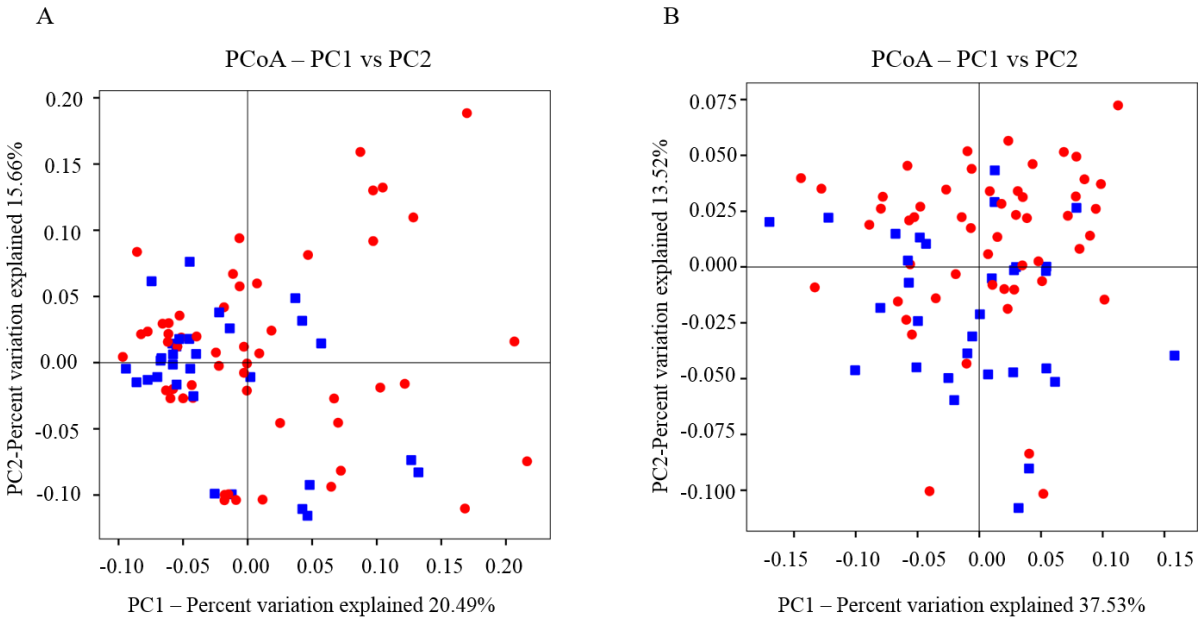


Figure 4-6. The β-diversity in faecal samples from children with or without asthma

The PCoA plots compare the β-diversity of the faecal samples from non-asthmatic children (blue) and asthmatic children (red) where two major principal components are represented on the x- and y-axes (Figure 4-6). Panel A shows the unweighted Unifrac PCoA matrix and panel B the weighted Unifrac PCoA matrix.

4.5 Microbial composition of faecal samples

The relative abundance of microbial composition was compared in faecal samples from children with and without asthma. Nine phyla were detected, of which there were four phyla with a relative abundance larger than 1.0% (Table 4-5, and Figure 4-7). Bacteroidetes and Firmicutes combined take more than 90.0% of the total bacterial load in both groups and their relative abundance shows no significant difference between them. However, Mann-Whitney test showed that Proteobacteria were significantly more present in non-asthmatic children.

Table 4-5. The comparison of faecal samples microbiome at phylum level between asthmatic and non-asthmatic children

Phylum	**Non-asthmatic children**	**Asthmatic children**	***p***	***FDR_p****
Bacteroidetes	47.75%	44.68%	0.248	0.319
Firmicutes	42.64%	49.30%	0.016	0.064
Proteobacteria	7.10%	4.48%	0.005	**0.047**
Actinobacteria	1.71%	1.15%	0.310	0.349
Verrucomicrobia	0.61%	0.14%	0.025	0.064
Fusobacteria	0.16%	0.24%	0.721	0.721
Saccharibacteria	0.01%	0.01%	0.036	0.064
Synergistetes	0.01%	0.00%	0.043	0.065
Tenericutes	0.01%	0.00%	0.029	0.064

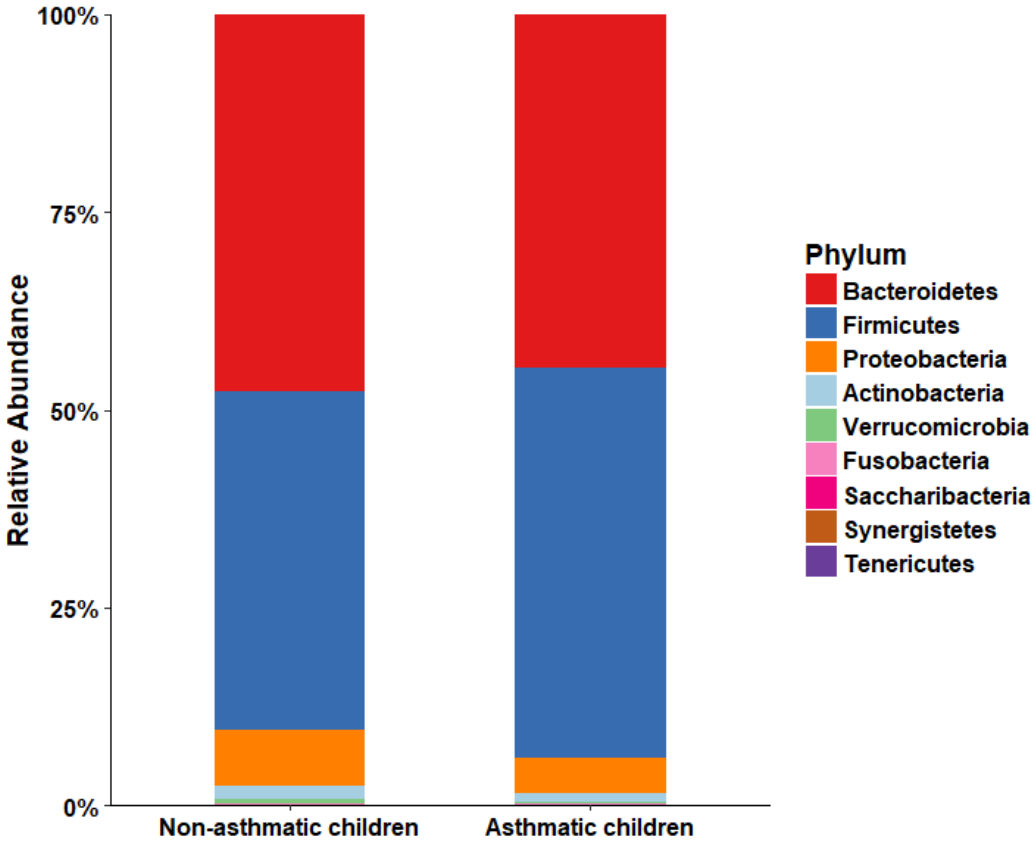


Figure 4-7. Bacteria relative abundance comparison at the phylum level for faecal samples

At genus level, 136 genera were detected of which 19 genera had a relative abundance over 1.0% (Table 4-6). Genus *Bacteroides* took the highest relative abundance in both groups. By comparing the relative abundance between children with and without asthma, we found that the percentage of *Lachnospira*, and *Ruminococcus 1* was significantly higher in asthmatic children. In contrast, the relative abundance of *Streptococcus* and *Escherichia-Shigella* are significantly higher in non-asthmatic children.

Table 4-6. The comparison of faecal samples microbiome at genus level between asthmatic and non-asthmatic children

Genus	Non-asthmatic children	Asthmatic children	*p*	*FDR_p**
Bacteroides	37.21%	35.04%	0.490	0.660
Faecalibacterium	11.73%	15.32%	0.142	0.271
Prevotella 9	3.61%	4.93%	0.287	0.451
Lachnospiraceae UCG-008	3.27%	3.90%	0.074	0.172
Parabacteroides	3.04%	2.42%	0.707	0.844
Streptococcus	2.84%	1.48%	0.000	**0.001**
Roseburia	2.76%	3.03%	0.284	0.451
Escherichia-Shigella	2.54%	0.33%	0.000	<**0.001**
Subdoligranulum	2.28%	2.80%	0.102	0.213
Dialister	1.83%	1.56%	0.937	0.944
Parasutterella	1.75%	2.08%	0.361	0.546
[Eubacterium] eligens group	1.70%	2.83%	0.017	0.058
Alistipes	1.70%	0.96%	0.112	0.231
Lachnospira	1.68%	2.66%	0.002	**0.014**
[Eubacterium] coprostanoligenes group	1.58%	1.14%	0.241	0.410
Bifidobacterium	0.97%	0.53%	0.945	0.945
Lachnoclostridium	0.80%	1.16%	0.297	0.459
Ruminococcaceae_uncultured	0.70%	1.50%	0.496	0.661
Ruminococcus 1	0.28%	1.00%	0.001	**0.009**

4.6 Functional characterization of the faecal microbiome

LEfSe was utilised to compare different features of the KEGG pathways in faecal samples between children without and with asthma (Figure 4-8). 21 pathways were found that were more abundant in non-asthmatic children, while 9 pathways were higher in asthmatic children.

More than half of the pathways which were more abundant in non-asthmatic children belonged to “Metabolism”, including Glycan Biosynthesis and Metabolism, Glycosaminoglycan degradation, Glycosyltransferases, Lipopolysaccharide biosynthesis, Metabolism of Cofactors and Vitamins: Folate biosynthesis and Ubiquinone and other terpenoid-quinone biosynthesis.

On the other hand, asthmatic children were more enriched in other Metabolism pathways such as Lysine biosynthesis (Amino Acid Metabolism), Photosynthesis proteins (Energy Metabolism), Pentosephosphate pathway, and Fructose and mannose metabolism (Carbohydrate Metabolism).

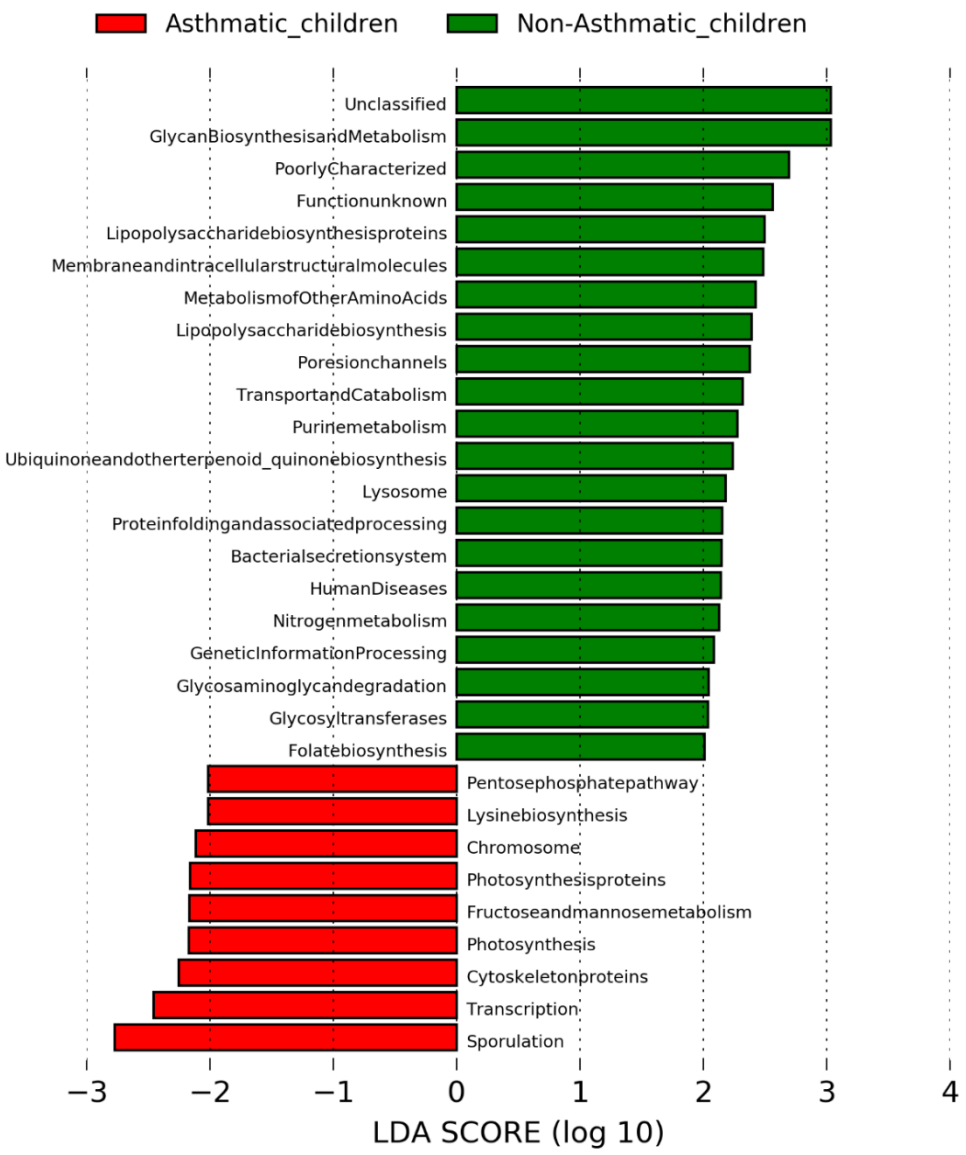


Figure 4-8. Bar graphs of linear discrimination analysis (LDA) for the mean differences of KEGG pathway for faecal samples

KEGG pathways that more present in non-asthmatic children are represented by green bars, whereas those more present in asthmatic children are represented by red bars. Note the plots have a logarithmic x-axis (of base 10) and only KEGG pathway difference for an order of 2.0 or higher are represented.

4.7 Discussion

Increasing evidence suggests that the respiratory microbiome plays a vital role in respiratory health. Many studies have identified a “critical window” for the development of microbiota in the first year of life that has its effect on developing asthma and allergy later in life [170, 171]. Studies focused on differences in the microbiome between asthmatic and healthy children have mainly been conducted in developed countries. There are few of such studies conducted in the environment of developing countries. To our knowledge, this is the first study to assess both the oropharyngeal and gut microbiome difference between asthmatic and non-asthmatic children in a developing country, namely China.

In this study we found that the alpha diversity in OP samples was significantly higher in non-asthmatic children, while no difference was found for faecal samples. The alpha diversity represents the richness of species within samples. It is suggested that the commensal microbiota interact with the environmental microbiota. Reduced exposure to a natural environmental biodiversity can adversely affect the human microbiota and its immunoregulatory capacity [172, 173]. A lower diversity of human microbiome has been associated with asthma and allergy. One study investigated mattress dust and the nasal microbiome related to farm exposure and asthma status [174]. They found that asthma was inversely associated with the alpha diversity of both mattress dust and nasal samples. Another study conducted in Northeast China compared the airway microbiome in different phenotypes of adult asthmatic patients, and they reported that asthmatics showed a significant decrease in diversity richness and evenness [175]. In this study, the alpha diversity in faecal samples showed no significant difference between asthmatic and non-asthmatic children. There is accumulating evidence that a lower gut microbial diversity in the first year of life is correlated with the development

of asthma and allergy [176-178]. But in one of these studies, Abrahamsson TR *et al*. found this association among infants at 1 week or 1 month of age and not at 1 year of life [176]. This suggests that the "critical window" for the gut microbiome to mediate immunological changes and influence the development of asthma and allergy is during the infancy period, while the airway microbiome is more dynamic and may have a direct influence on asthma development beyond early life.

In this study, the phylum Bacteroidetes from was reduced in OP samples from asthmatic children. Consistently, Hilty et al. also found Bacteroidetes were more frequent in controls than in adult or child asthmatics/COPD patients [117]. In another comparison study between severe asthma, non-severe asthma, and healthy controls, phyla Bacteroidetes and Fusobacteria were reduced in non-severe and severe asthmatic groups [179]. These two phyla were also reduced in the asthmatic groups of the previously mentioned study conducted in Northeast China [175]. Our study found genus *Lachnospira* and *Ruminococcus 1* were significantly higher in the faecal samples of asthmatic children. Genera *Lachnospira* is in the family *Lachnospiraceae*, which is a cluster of anaerobic and spore-forming bacteria. One strain of *Lachnospiraceae* was identified to influence obesity and diabetes in a mice model [180]. In addition, *Ruminococcus* is also known to be associated with atopy or inflammation [181]. The phylum Proteobacteria, mainly the genus *Escherichia-Shigella,* was significantly higher in non-asthmatic children. However, phylum Proteobacteria has been associated with inflammatory disorders, such as inflammatory bowel disease and asthma [182]. In a study comparing the gut microbiome composition in infants with or without eczema, it showed that *Escherichia-Shigella* was increased in infants with eczema [183]. Further studies are needed to determine if

this inconsistency was due to different sample sizes or sampling methods, or a feature of the microbiome in its different stages of life.

There are however some limitations in this study. Firstly, the resolution of 16s rRNA sequencing is reliable down to the genus level, and not for species and strains. Whole-genome sequencing or real-time PCR is needed to investigate the bacteria in more detail and determine differences between the different allergic condition status. Secondly, one study has shown that there are temporal dynamic changes in the nasopharyngeal microbiota [184] and future studies in airway microbiome should take this into consideration. Thirdly, different control status of asthma among asthmatic children might have an influence on the microbiome. The reason asthmatic children were not divided according to subgroups of different status was to avoid multiple comparison issues resulting from subgroup analyses.

In conclusion, this study suggests that the richness of the oropharyngeal microbiome is higher in non-asthmatic children in an eastern developing environment. This is not the case for the gut microbiome which was found the same for both non-asthmatic and asthmatic children. Differences in the relative abundance of bacteria can provide a starting point for future studies of the microbiome function in human or mice models.

5 The human microbiome and the correlation with serum IgE among asthmatic vs non-asthmatic children

5.1 Microbial comparison between serum IgE positive and negative groups

The participants were regrouped as IgE positive/negative according to their serum total IgE levels. If the serum total IgE level was larger than 77.7 kU/L the participant was grouped as IgE positive, otherwise the participant was grouped as IgE negative. The demographics were shown in Table 5-1. Among the 149 participants, 11 participants did not take part in the serum IgE test.

Table 5-1. The demographics of the participants according to the level of serum total IgE

General Information	IgE negative (n=57)	IgE positive (n=81)	Statistics	*p*
Females: n (%)	20 (35.1)	30 (37.0)	χ^2= 0.06	0.815
Age (y): mean (SD)	6.5 (2.9)	6.3 (2.5)	*t*= 0.52	0.607
BMI (kg/m^2): mean (SD)	16.3 (2.9)	16.6 (3.5)	*t*= -0.56	0.574
Delivery method				
Vaginal delivery n (%)	29 (51.8)	42 (52.5)	χ^2= 0.01	0.935
Caesarean section n (%)	27 (48.2)	38 (47.5)		
Breastfed: n (%)	52 (92.9)	67 (83.8)	χ^2= 2.50	0.114
Birth weight (kg): mean (SD)	3.4 (0.80)	3.3 (0.45)	*t*= 1.16	0.249
Birth length (cm): mean (SD)	49.3 (7.3)	51.2 (2.4)	*t*= -1.82	0.071
Time to introduce solid food (months): mean (SD)	5.6 (1.3)	5.4 (1.5)	*t*= 0.70	0.486
Antibiotic used (past 2 weeks): n (%)	9 (16.4)	15 (18.8)	χ^2= 0.13	0.722

Serum IgE was not tested in the oropharyngeal samples from one asthmatic child and from eight non-asthmatic children and in the faecal samples from five non-asthmatic children. Thus, there were 131 oropharyngeal samples and 79 faecal samples in the analysis presented in this chapter.

5.1.1 The oropharyngeal microbial comparison

5.1.1.1 The alpha and beta diversity comparison

There were 48 IgE negative and 83 IgE positive participants. After comparing the α-diversity indexes of their oropharyngeal (OP) samples, we found no significant difference between children with IgE negative and IgE positive (Table 5-1).

Table 5-2. Comparison of the α-diversity indexes of the OP samples from children with IgE negative/positive

	IgE negative	IgE positive	*p*
Chao1 richness estimate	367.98±38.99	363.85±41.28	0.586
Shannon's index	4.40±0.52	4.30±0.57	0.315
Observed OTUs	336.99±38.47	333.99±41.06	0.688
Simpson	0.862±0.06	0.844±0.07	0.164

Also Adonis permutational ANOVA tests did not show significant differences, neither in unweighted Unifrac PCoA (p= 0.320), nor in weighted Unifrac PCoA (p= 0.310). The PCoA figures are shown in Figure 5-1.

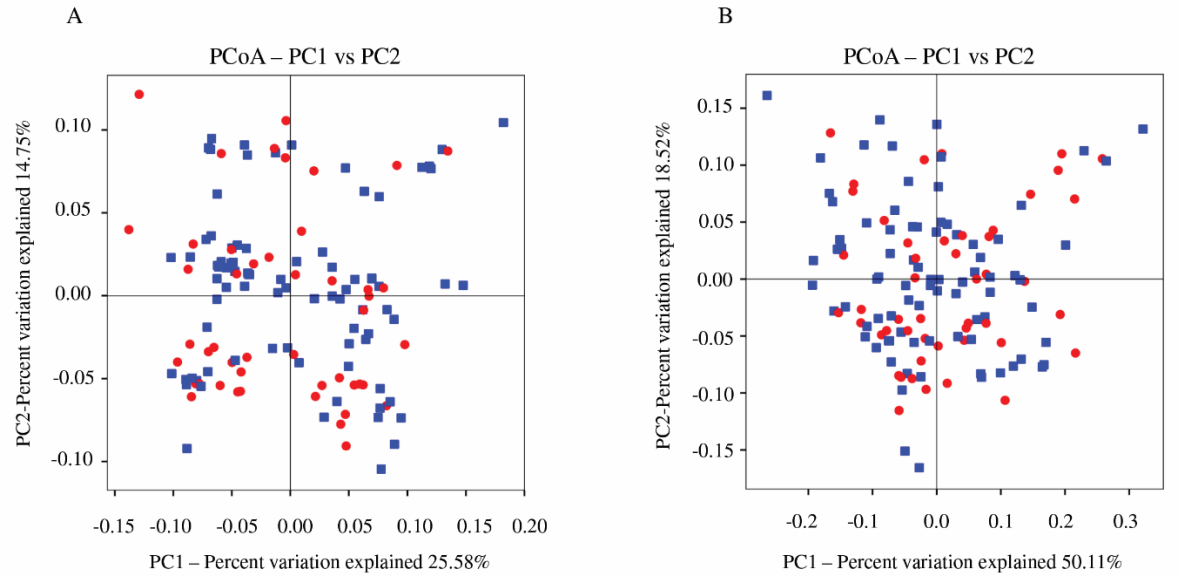


Figure 5-1. The β-diversity in OP samples from children with IgE negative/positive

The PCoA plots compare the β-diversity of the faecal samples from IgE positive children (blue) and IgE negative children (red) using two major principal components on the x- and y-axes (Figure 5-1). Panel A shows the unweighted Unifrac PCoA matrix and panel B shows the weighted Unifrac PCoA matrix.

5.1.1.2 Comparison of the microbial composition

As previously shown in Chapter 4, the same eight phyla were detected in all the children. Table 5-2 shows the comparison of the microbial composition of OP samples between children with IgE negative or positive. For both groups the largest abundance was found for Firmicutes which occupied more than 50.0%, followed by Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria. Also, here we did not find any significant difference between children who were IgE negative or positive.

Table 5-3. The comparison of OP samples microbiome at phylum level between children with IgE negative/positive

Phylum	IgE negative	IgE positive	*p*	*FDR_p**
Firmicutes	53.4%	55.7%	0.211	0.667
Proteobacteria	17.9%	17.5%	0.901	0.946
Bacteroidetes	14.7%	12.9%	0.282	0.667
Actinobacteria	9.6%	10.2%	0.860	0.946
Fusobacteria	4.1%	3.4%	0.320	0.667
Saccharibacteria	0.2%	0.2%	0.513	0.821
Cyanobacteria	0.1%	0.1%	0.334	0.667
SR1 (Absconditabacteria)	0.0%	0.0%	0.946	0.946

*: The *p*-values were FDR-corrected to control for multiple testing

At the genus level there were 17 genera with a relative abundance larger than 1.0% (Table 5-3). *Streptococcus* had the largest proportion with 37.52% in IgE negative children, and 39.41% in IgE positive children. This difference is not significant, and we did not find any other significant differences in the OP microbiome of children who were IgE negative or positive.

Table 5-4. The comparison of OP microbiome at genus level between children with IgE negative/positive

Genus	IgE negative	IgE positive	*p*	*FDR_p**
Streptococcus	37.52%	39.41%	0.221	1.000
Gemella	6.34%	7.39%	0.141	1.000
Rothia	7.28%	7.03%	0.947	1.000
Haemophilus	6.23%	6.76%	0.516	1.000
Prevotella 7	6.74%	6.16%	0.535	1.000
Neisseria	6.02%	5.81%	0.699	1.000
Granulicatella	3.84%	3.32%	0.115	1.000
Neisseriaceae_uncultured	2.98%	2.58%	0.678	1.000
Leptotrichia	2.75%	1.94%	0.448	1.000
Alloprevotella	2.25%	1.81%	0.406	1.000
Actinomyces	1.39%	1.64%	0.616	1.000
Veillonella	1.58%	1.47%	0.359	1.000
Porphyromonas	1.68%	1.46%	0.882	1.000
Bacteroides	1.85%	1.40%	0.556	1.000
Fusobacterium	1.30%	1.37%	0.830	1.000
Actinobacillus	1.33%	1.22%	0.845	1.000
Prevotella	1.18%	1.02%	0.920	1.000

*: The *p*-values were FDR-corrected to control for multiple testing

5.1.2 The faecal microbial comparison

5.1.2.1 The alpha and beta diversity comparison

There were 79 participants included in this analysis of faecal samples, of which 28 (35.4%) from IgE negative children. We did not find a significant difference in the α-diversity indexes of the OP microbiome from IgE negative and IgE positive children (Table 5-4).

Table 5-5. Comparison of the α-diversity indexes of faecal samples from children with IgE negative/positive

	IgE negative	IgE positive	*p*
Chao1 richness estimate	441.27±48.21	439.48±48.12	0.862
Shannon's index	5.01±0.70	5.02±0.65	0.951
Observed OTUs	401.71±49.51	403.59±49.56	0.860
Simpson	0.89±0.08	0.90±0.06	0.636

Adonis permutational ANOVA tests showed no significant difference in the β-diversity of the faecal samples of children with IgE negative and positive neither in unweighted Unifrac PCoA (p= 0.277), nor weighted Unifrac PCoA (p= 0.113). The PCoA figures are shown in Figure 5-2.

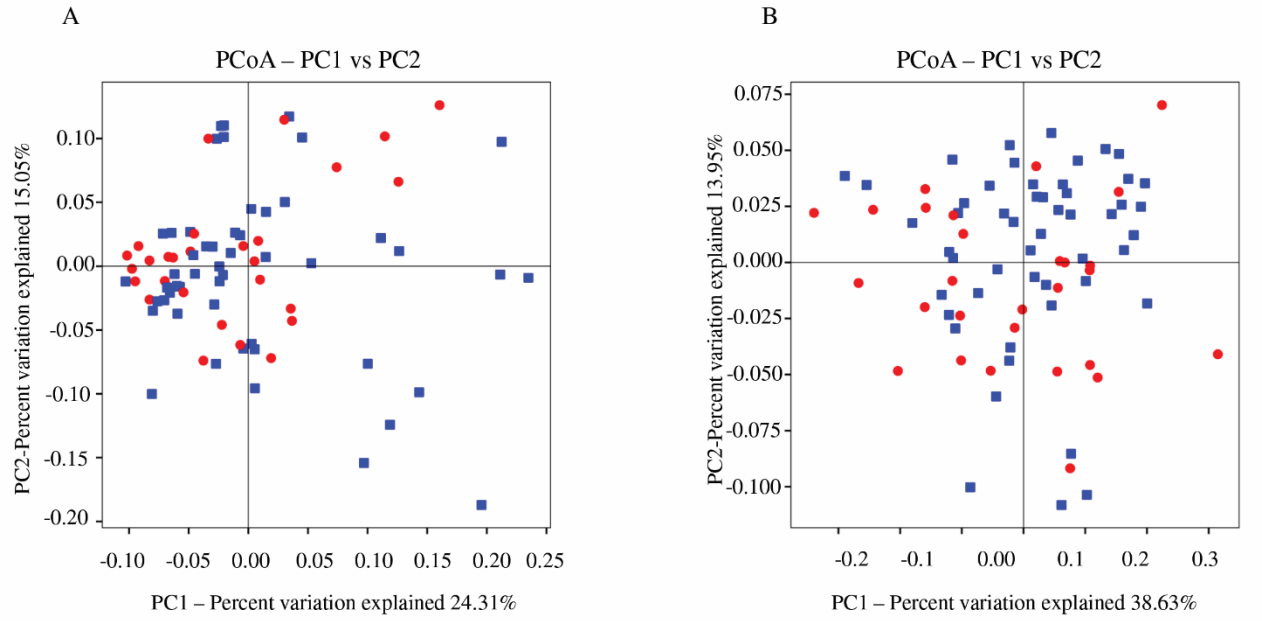


Figure 5-2. The β-diversity in faecal sampels from children with IgE negative/positive

The PCoA plots compare the β-diversity of faecal samples for IgE positive children (blue) and IgE negative children (red) using two major principal components on the x- and y-axes (Figure 5-2). Panel A shows the unweighted Unifrac PCoA matrix and panel B represents the weighted Unifrac PCoA matrix.

5.1.2.2 The microbial composition comparison

Four phyla had a relative abundance over 1.0%, namely Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria. In IgE negative children Bacteroidetes had the highest relative abundance (47.9%) while in IgE positive children it was Firmicutes which occupied the largest proportion (49.2%). However, after the comparison we found this difference was not significant (Table 5-5).

Table 5-6. The comparison of faecal microbiome at phylum level between children with IgE negative/positive

Phylum	**IgE negative**	**IgE positive**	***p***	***FDR_p****
Bacteroidetes	47.9%	44.2%	0.215	0.403
Firmicutes	43.6%	49.2%	0.067	0.300
Proteobacteria	6.3%	4.9%	0.230	0.403
Actinobacteria	1.4%	1.2%	0.135	0.403
Verrucomicrobia	0.6%	0.1%	0.048	0.300
Fusobacteria	0.1%	0.2%	0.268	0.403
Synergistetes	0.0%	0.0%	0.462	0.594
Saccharibacteria	0.0%	0.0%	0.975	0.975
Tenericutes	0.0%	0.0%	0.551	0.620

*: The *p*-values were FDR-corrected to control for multiple testing

136 genera were detected at genus level, of which 18 had a relative abundance larger than 1.0% (Table 5-6). *Bacteroides* had the highest relative abundance in both children with IgE negative (34.6%) and positive (35.9%). Comparing these 18 genera with relative abundance over 1.0% we found that *Ruminococcus 1* (from phylum Firmicutes)

was marginally higher in children with IgE positive (1.0%), compared to children with IgE negative (0.3%).

Table 5-7. The comparison of faecal microbiome at genus level between children with IgE negative/positive

Genus	IgE negative	IgE positive	*p*	*FDR_p**
Bacteroides	34.6%	35.9%	0.492	0.778
Faecalibacterium	11.9%	15.0%	0.099	0.413
Prevotella 9	6.9%	3.4%	0.621	0.871
Lachnospiraceae UCG-008	3.4%	4.0%	0.160	0.501
Parabacteroides	3.3%	2.2%	0.264	0.660
Roseburia	2.9%	3.0%	0.505	0.781
Subdoligranulum	2.5%	2.7%	0.902	0.976
Streptococcus	2.4%	1.6%	0.012	0.182
[Eubacterium] eligens group	2.0%	2.8%	0.095	0.413
Escherichia-Shigella	2.0%	0.5%	0.021	0.239
Dialister	1.9%	1.5%	0.798	0.958
Parasutterella	1.8%	2.1%	0.255	0.655
Lachnospira	1.7%	2.7%	0.080	0.413
[Eubacterium] coprostanoligenes group	1.6%	1.1%	0.454	0.751
Alistipes	1.3%	1.2%	0.362	0.683
Lachnoclostridium	0.9%	1.1%	0.806	0.958
Ruminococcaceae_uncultured	0.9%	1.5%	0.356	0.683
Ruminococcus 1	0.3%	1.0%	0.003	0.074

*: The *p*-values were FDR-corrected to control for multiple testing

5.2 The correlation of bacteria and serum IgE

5.2.1 The correlation of bacteria and IgE in all participants

Spearman correlation was used to test the association of both the oropharyngeal and faecal bacteria with serum IgE levels. The bacteria with a significant association ($p<$ 0.06, the p values have been adjusted for multiple comparison) are shown in Figure 5-3.

The associations of the bacterial taxa and serum IgE in OP samples were all negative. Genus *Candidatus Saccharimonas* (phylum Saccharibacteria), order Pseudomonadales and a downstream family Moraxellaceae, genus *Aggregatibacter* (phylum Proteobacteria) were negatively associated with sIgE for food allergens (Fx5). Phylum Cyanobacteria and the downstream class Chloroplast, and genus *Ruminiclostridium 9* (phylum Firmicutes) were negatively associated with Dp.

However, in faecal samples we only found that genus *Ruminiclostridium 9* (phylum Firmicutes) was positively associated with total IgE, aeroallergens (phad), dust mites: Dp, and Df.

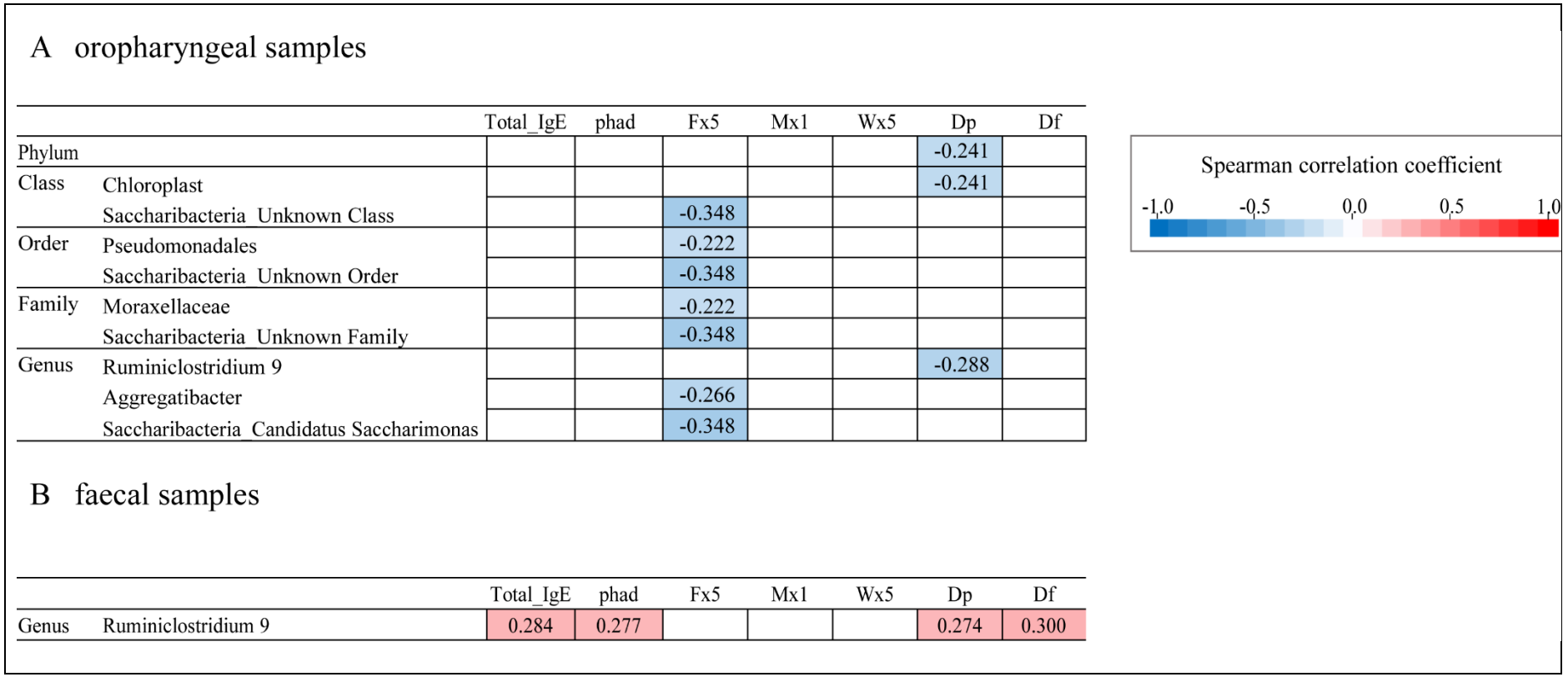


Figure 5-3. The correlation of bacteria and IgE in all children

5.2.2 The correlation of oropharyngeal bacteria and IgE in children with and without asthma

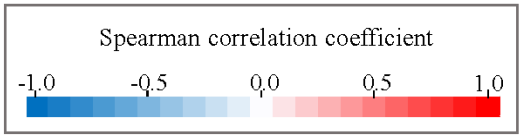
Next we investigated the correlation of OP bacteria with IgE specifically in children with or without asthma. Figure 5-4 summarises the significant associations of oropharyngeal bacterial taxa with total and specific IgE levels ($p< 0.06$, the p values have been adjusted for multiple comparison).

In non-asthmatic children, 11 taxa correlated significantly with at least one of total or specific IgE and most of them showed a negative correlation with sIgE for food allergens. Genus *Candidatus Saccharimonas* (phylum Saccharibacteria), order Pseudomonadales and a downstream family Moraxellaceae, genus *Aggregatibacter* (phylum Proteobacteria) were negatively associated with sIgE for food allergens (Fx5). Genus *[Eubacterium] nodatum group* (phylum Firmicutes) was negatively associated with both Fx5, mould mix (Mx1), and house dust mite (Df). Family Prevotellaceae and the downstream genus *Prevotella7* negatively correlated with Mx1. However, genus *Eisenbergiella* (Firmicutes) was positively associated with total IgE, aeroallergens (phad), and Dp.

In asthmatic children, we only found one genus *Eikenella* that was negatively correlated with total IgE and Mx1.

A Non-asthmatic children

		Total IgE	phad	Fx5	Mx1	Wx5	Dp	Df
Class	Saccharibacteria Unknown Class			-0.442				
Order	Pseudomonadales			-0.472				
	Saccharibacteria Unknown Order			-0.442				
Family	*Prevotellaceae*				-0.436			
	Moraxellaceae			-0.472				
	Saccharibacteria Unknown Family			-0.442				
Genus	*Prevotella7*				-0.509			
	Eubacterium Nodatum group			-0.413	-0.383			-0.369
	Eisenbergiella	0.409	0.558				0.406	
	Aggregatibacter			-0.419				
	Saccharibacteria Candidatus Saccharimonas			-0.442				



B Asthmatic children

		Total IgE	phad	Fx5	Mx1	Wx5	Dp	Df
Genus	*Eikenella*	-0.270			-0.252			

Figure 5-4. The correlation of OP bacteria and IgE in children with and without asthma

5.2.3 The correlation of faecal bacteria and IgE in children with and without asthma

The faecal bacterial taxa that have a significant association with serum IgE levels are shown in Figure 5-5 ($p < 0.06$, the p values have been adjusted for multiple comparison).

The correlation between faecal bacterial taxa and serum IgE levels was quite different for non-asthmatic children and asthmatic children. In non-asthmatic children, the bacterial taxa were mostly negatively correlated with serum IgE, especially total IgE and Mx1. Family *Rikenellaceae* and its downstream genus *Alistipes*, and family *Christensenellaceae* and its downstream genus *Christensenellaceae R7 group* were only negatively correlated with Mx1. There were seven taxa which had at least two significant correlations with serum IgE. Among those, *Clostridiales_Ambiguous taxa* had a significant negative correlation with total IgE, phad, Fx5, Mx1, and Dp. *Ruminococcaceae NK4A214 group* was negatively correlated with total IgE, phad, Mx1, Wx5, and Df. However, we also found that order Corynebacteriales and its downstream family Corynebacteriaceae, and consistently, its genus *Corynebacterium* were positively correlated with Wx5. In addition, genus Allisonella (phylum Firmicutes) was positively associated with phad, Mx1, Wx5, Dp and Df.

For asthmatic children, seven taxa were positively correlated with phad, including Order Corynebacteriales and its downstream family Corynebacteriaceae, genus *Corynebacterium*. Genus *Lachnospiraceae UCG-008* was positively correlated with phad, Mx1, and Wx5. From phylum Actinobacteria to genus *Actinomycetales ambiguous taxa* there were four taxa negatively correlated with Fx5.

A Non-asthmatic children

		Total_IgE	phad	Fx5	Mx1	Wx5	Dp	Df
Order	Corynebacteriales					0.510		
Family	*Corynebacteriaceae*					0.510		
	Rikenellaceae				-0.542			
	Clostridiales_Ambiguous taxa	-0.493	-0.408	-0.439	-0.423		-0.549	
	Christensenellaceae				-0.574			
	Family XIII	-0.435			-0.508	-0.489		
Genus	*Corynebacterium*					0.510		
	Alistipes				-0.542			
	Clostridiales_Ambiguous taxa	-0.493	-0.408	-0.439	-0.423		-0.549	
	Christensenellaceae R7 group				-0.574			
	Lachnospiraceae NC2004 group	-0.431			-0.487	-0.522		
	Peptoclostridium	-0.479			-0.490			
	Ruminococcaceae NK4A214 group	-0.518	-0.473		-0.565	-0.398		-0.524
	Eubacterium coprostanoligenes group				-0.574			
	Allisonella		0.572		0.396	0.423	0.641	0.420
	Enterobacteriaceae Ambiguous taxa	-0.532						

B Asthmatic children

		Total_IgE	phad	Fx5	Mx1	Wx5	Dp	Df
Phylum	Actinobacteria			-0.502				
Class	Actinobacteria			-0.517				
Order	Corynebacteriales		0.411					
Family	*Actinomycetales_Ambiguous taxa*			-0.460				
	Corynebacteriaceae		0.411					
Genus	*Actinomycetales_Ambiguous taxa*			-0.460				
	Corynebacterium		0.411					
	Odoribacter		0.377					
	Lachnospiraceae UCG 008		0.409		0.315	0.400		
	Faecalibacterium					-0.471		
	Ruminiclostridium 9		0.417					
	Eubacterium coprostanoligenes group		0.382					

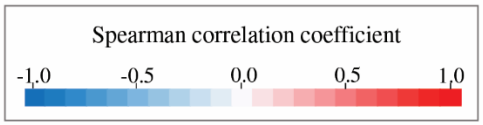


Figure 5-5. The correlation of faecal bacteria and IgE in children with and without asthma

5.3 Discussion

In this chapter we first compared the microbiome diversity and relative abundance between IgE positive and negative participants. We did not find any significant difference in alpha nor beta diversity between different IgE status. In terms of bacterial relative abundance, genus *Ruminococcus 1* was marginally higher in children with IgE positive. Second, we investigated the correlations between bacterial taxa abundance and serum IgE levels of oropharyngeal and gut microbiome for asthmatic and non-asthmatic children. We found that the pattern of correlations was quite different for asthmatic and non-asthmatic children.

The bacterial diversity has been associated with human immunological regulation. Studies have found that a reduced diversity of bacteria species is associated with the development of chronic inflammatory disorders, such as inflammatory bowel disease (IBD), obesity, and allergic conditions [105, 185, 186]. However, the diversity was not significantly different between IgE positive and IgE negative children in this study. Some studies have reported abnormally high serum IgE levels in germ-free (GF) mice, which suggest that immuneregulatory signals from microbiota are required to maintain basal IgE levels and circulating basophil populations[187, 188]. Cahenzli et al. further reported GF mice and mice with low-diversity microbiota have elevated serum IgE levels in early life. This indicates there is a critical window in early life for inducing a proper immunoregulatory network by diverse microbial stimuli to protect from induction of IgE [134]. This might indicate that the early life period, when the commensals are established, is most vital in terms of immunological regulation. Many questions remain and it is of interest to investigate the trajectory of human microbiota development and how it interacts with immune response.

The comparison of bacterial abundance in faecal samples showed *Ruminococcus 1* was marginally higher in children with IgE positive compared to children with IgE negative. In the previous chapter, we also found that *Ruminococcus 1* was significantly higher among the asthmatic children. Genus *Ruminococcus* is in the class Clostridia, phylum Firmicutes, and is an anaerobic, Gram-positive gut microbe. One study has collected faecal samples after birth and at 1 years old from 44 children in Taiwan and it showed that species *Ruminococcus gnavus* was increased in allergic infants [189]. This finding was confirmed in BALB/c mice fed with *R gnavus* who developed airway inflammation [189].

Emerging studies have investigated the association of human microbiome and IgE levels. One study reported that dysbiosis of gut microbiota can reduce faecal butyrate and was consequently associated with increased mite-specific IgE and the risk of asthma in early childhood [135]. In this study, we found *Ruminiclostridium 9* was negatively associated with sIgE for dust mite in oropharyngeal samples, but positively associated with total IgE, aeroallergens and dust mites in faecal samples. Genus *Ruminiclostridium* and *Ruminococcus* are both in the order Clostridiales, and *Ruminiclostridium* also has been found to be higher in the gut microbiota of an allergic mice model [190]. This is consistent with the positive correlation with IgE levels in the faecal samples of this study, however it is unclear why there is a different correlation in the oropharyngeal microbiome.

When the association between the microbiome and IgE levels was tested separately for asthmatic and non-asthmatic children, we found that its patterns were quite different in both oropharyngeal and gut microbiome. The oral samples of non-asthmatic children showed more significant associations between bacteria and IgE

than the oral samples of asthmatic children, and the majority of the significant associations were negative. However, some bacterial taxa in the gut microbiome were mostly negatively correlated with serum total IgE in non-asthmatic children, whereas other taxa were positively correlated with aeroallergens sIgE level. The different association patterns can be due to differences in relative abundance of bacteria in those two groups of children. It might also indicate that the function of those microbiota is mediated by the mucosa environment which is different in asthmatic children compared to non-asthmatic children.

To summarize, we found genus *Ruminococcus 1* was marginally higher in children with positive IgE. The pattern of correlations between bacteria taxa and serum IgE levels were distinctly different in asthmatic and non-asthmatic children. Additional studies are required to further investigate the function of these distinct bacteria in animal models.

6 Study Two—Western oropharyngeal and gut microbial profiles are associated with allergic conditions in Chinese immigrant children living in Australia

The work presented in this chapter was published in the World Allergy Organization Journal in August 2019. DOI: https://doi.org/10.1016/j.waojou.2019.100051. (see Appendix A).

6.1 Abstract

Background: The allergy epidemic resulting from the western environment/lifestyles is potentially due to modifications of the human microbiome. Therefore it is of interest to study immigrants living in a western environment as well as their counterparts in the country of origin to understand differences in their microbiomes and health status.

Methods: We investigated 58 Australian Chinese (AC) children from Perth, Western Australia as well as 63 Chinese-born Chinese (CC) children from a city in China. Oropharyngeal (OP) and faecal samples were collected. To assess the microbiomes, 16s ribosomal RNA (rRNA) sequencing for variable regions V3 and V4 was used. Skin prick tests (SPT) were performed to measure the children's atopic status. Information on food allergy and wheezing were acquired from a questionnaire.

Results: AC children had more allergic conditions than CC children. The alpha diversity (mean species diversity) of both OP and gut microbiome was lower in AC children compared to CC children for richness estimate (Chao1), while diversity evenness (Shannon index) was higher. The beta diversity (community similarity) displayed a distinct separation of the OP and gut microbiota between AC and CC children. An apparent difference in microbial abundance was observed for many bacteria. In AC children, we sought to establish consistent trends in bacterial relative abundance that are either higher or lower in AC versus CC children and higher or lower in children with allergy versus those without allergy. The majority of OP taxa showed a consistent trend while the majority of faecal taxa showed a contrasting trend.

Conclusion: Distinct differences in microbiome compositions were found in both oropharyngeal and faecal samples of AC and CC children. The association of the OP microbiome with allergic condition is different from that of the gut microbiome in AC

children. The microbiome profiles are changed by the western environment/lifestyle and are associated with allergies in Chinese immigrant children in Australia.

6.2 Introduction

The rising prevalence of asthma and allergies has become a global public health concern, and there are wide variations between countries [13, 191]. The prevalence of adult asthma is highest in developed countries, such as Australia (31.0%), while it is the lowest in developing countries, such as China (0.2%) [192]. The substantial difference in allergy prevalence indicates that environmental factors play a vital role in the development of these conditions [30]. Immigrant populations in industrialized countries represent a unique opportunity to examine western environmental influences [193]. Immigrants moving from less affluent countries (asthma-low risk) to more affluent countries (asthma-high risk) experience a gradually increased prevalence of allergies and asthma, correlated with the length of residence in the more affluent country [194]. For example, a cross-sectional survey of school-age children reported that compared to a residence in Australia from zero to four years, residence for five to nine years after migration was associated with a two-fold increase in reported wheezing, and this increased to a three-and-a-half-fold for 10 to 14 years after migration [27]. This time-dependent effect points to a gradual change of individual homeostasis, potentially related to ongoing modifications of the human microbiome due to western environmental risk factors.

Studies have shown that perturbations in the human microbiome are associated with an increased risk of allergic disease [176, 195]. This agrees with the well-known “hygiene hypothesis” that suggests early exposure of children to high microbial abundance and increased biodiversity protects against development of allergic diseases [101, 196]. Our recent studies showed that Chinese immigrants in Australia had a significant shift in the

innate and adaptive immune response [154, 197]. Chinese immigrants living in Australia for more than five years had reduced innate immune cytokine production and weaker adaptive antibody responses to pathogen-associated antigens relative to recently-arrived Chinese immigrants [154, 197]. We presume that the human microbiome inherent to the western environment may regulate the priming of immune response and modulate the susceptibility to allergic disorders [43, 135, 198]. However, there is a lack of knowledge about the difference in human microbiome between immigrants and their counterparts in the country of origin. Chinese immigrant children in Australia with matched Chinese children in China are a relatively homogeneous population yet living in an industrialized or non-industrialized environment. Therefore we compared the oropharyngeal (OP) and gut microbiome of Australian Chinese (AC) children in Australia and China-Born Chinese (CC) children in mainland China. The two cohorts were strictly matched for age-range, gender-frequency, and season of recruitment to control for potential confounders.

6.3 Methods

6.3.1 Study design and recruitment

This study is a cross-sectional investigation of which the participants are living in Australia and China. First we recruited AC children from the local Chinese community living in Perth, Western Australia by advertisements through Chinese media− eg. radio and newspaper (from March to May 2015). Chinese children aged 3 to 18 and residing in Australia were recruited. Second we recruited CC children from cluster randomly selected students from kindergartens, primary and high schools in Hebi City in the northern Henan Province. Gender frequency and age range were matched with the AC children (from September to October 2015). The recruitment took place during autumn considering the countries are in opposite hemispheres. Hebi city is a relatively less affluent (prefecture-level) city in China, where agriculture has traditionally been a pillar of its economy. In

total we recruited 58 AC children (aged 3-18) and 63 CC children (aged 2-17), all of whom were of Han Chinese descent. The Han is the majority ethic group in China.

OP swabs and faecal samples were collected from the participants and one of parents/guardians was asked to fill out a questionnaire for their child. The questionnaire collected demographic information, delivery method (Vaginal delivery/ Caesarean section), breastfeeding history, self-reported food allergic history and current wheezing status (within recent 12 months). At recruitment, skin prick tests (SPT) were performed to measure the child's atopic status. The SPT results were evaluated after 15–20 min exposure, and positive atopy was defined as a wheal size >3mm diameter in reaction to at least at one allergen [199].

This study was approved by the Human Research Ethics Committee of Western Australia (RGS0000002424), at Curtin University (HR86/2014), the University of Western Australia (RA/4/1/6763) and at the College of Public Health, Zhengzhou University in China. All parents provided informed consent on behalf of their children.

6.3.2 16S rRNA gene sequencing, Bioinformatics and Statistical analysis

Amplicons of the 16S rRNA gene V3-V4 region were sequenced on an Illumina HiSeq 2500 platform. The paired-end reads were merged, then filtered, and the sequences were assigned into Operational Taxonomic Units (OTUs) against the SILVA reference database (128 release). Bioinformatics and statistical analysis were carried out within the Quantitative Insights Into Microbial Ecology (QIIME 1.9.1) pipeline or using RStudio (Version 1.0.153). OTUs were single rarefied to get even depths of 19785.0 for OP and 27240.0 for faecal samples, respectively. Alpha diversity, which describes the number of taxa in sites or habitats at a more local scale, was estimated using the chao1 richness

estimate and Shannon index. Beta diversity, which indicates the extent of similarity between microbial communities, was measured using weighted and unweighted UniFrac. To infer the microbiome phenotypes and functional pathways associated with the bacterial taxa, we used Bugbase and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. The predictions of functional pathways were collapsed into Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups and they were compared between AC and CC children using the linear discriminant analysis effect size (LEfSe) with the linear discriminant analysis (LDA) cutoff of 2.5.

Mann-Whitney U tests were used to compare the group difference between AC and CC children and we selected taxa with relative abundance over 1.0% to illustrate with figures and tables. Linear regression was used to investigate the associations of bacterial relative abundance and AC/CC groups, after adjusting for age, gender, BMI, breastfeeding percentage, and antibiotic usage. Alpha-diversity metrics were performed by "compare_alpha_diversity.py", and ANOSIM and Adonis methods ("compare_categories.py") were used to analyze beta-diversity difference in QIIME. Data were visualized by bar plots or box plots using the "ggplot2" package in RStudio. All the p-values were False Discovery Rate (FDR)-corrected to control for multiple testing.

If the taxa that show a change in abundance in AC children relative to CC children play a role in the occurrence of allergic conditions, we expect that these taxa will show a similar change in children with allergic conditions compared to children without allergic conditions. To examine such trend, we selected taxa with significant differences (at two significance levels: $p<0.05$ and $p<0.01$) in relative abundance between AC and CC children at five taxonomic levels (phylum, class, order, family, and genus). Subsequently

the mean difference of taxa abundance between children with positive and negative allergic conditions (atopy, food allergy, and wheezing) was calculated in AC children, and those with mean difference over 0.01% and 0.1% were selected for further analysis respectively. If the mean relative abundance of a bacterium is higher or lower in AC children (compared to CC children) and also higher or lower in children with allergic conditions (compared to children without allergic conditions), a "1" was assigned to the bacterium, otherwise a "0" was assigned. A new variable with these binomial values (1 and 0) was created for all the selected taxa and used to test for consistency using a binomial probability test. The null hypothesis for the binomial probability tests is that the proportion of zero (inconsistent) or one (consistent) is equal to 50% which indicates that there is no consistent trend. We used this consistency test of the major distinct taxa to infer the influence of the western environment on the human microbiome and its relation with allergic conditions.

6.4 Results

6.4.1 Characteristics of the study population

As shown in Table 6-1, there were no significant differences in gender, age, body mass index (BMI), delivery method, breastfed percentage and antibiotic usage between the AC and CC children. 42 (72.4%) of the AC children were born in Australia and 16 (27.6%) were born in China and had been living in Australia with a median duration of 4.6 years. The percentages of atopy, food allergy, and current wheeze were all significantly higher among AC children than among CC children.

Table 6-1. The characteristics of participants

Characteristic	**AC(n=58)**	**CC (n=63)**	***p***
General Information			
Females: n (%)	26 (44.8%)	30 (47.6%)	0.758
Age (y): mean (SD)	8.6 (3.5)	7.7 (3.7)	0.196
BMI (kg/m^2): mean (SD)	17.1 (2.6)	17.6 (4.3)	0.391
Delivery method			
Vaginal delivery n (%)	34 (58.6%)	32 (50.8%)	0.701
Caesarean section n (%)	22 (37.9%)	24 (38.1%)	
Breastfed: n (%)	46 (79.3%)	53 (84.1%)	0.584
Clinical Information			
Antibiotic used (past 2 weeks): n (%)	2 (3.4%)	8 (12.7%)	0.179
Atopy: n (%)	36 (62.1%)	8 (12.7%)	**<0.001**
Food allergy: n (%)	15 (25.9%)	5 (7.9%)	**0.017**
Wheezing: n (%)	16 (27.6%)	2 (3.2%)	**<0.001**

6.4.2 Microbial diversity and composition

The microbiome composition between the AC children born in China or Australia was similar and therefore grouped for further analysis. Two AC and eight CC participants had used antibiotics two weeks prior to sample collection, and we performed a sensitivity test without those subjects which gave a consistent result.

6.4.2.1 Microbial Diversity

The Chao1 richness estimates are consistently lower in AC children for both OP (257.70±43.22) and faecal samples (330.24±41.65) compared to CC children (288.62±43.03, 345.00±33.39, p= 0.002, 0.046) (Figure 6-1 a and d). Conversely, the Shannon indices were significantly higher in AC children (OP: 4.46±0.59, faecal: 5.58±0.73) than in CC children (OP: 3.99±0.95, faecal: 5.06±0.67, p= 0.009, 0.002).

A distinct clustering was observed of the OP and faecal bacteria communities between the AC and CC children using both the unweighted and weighted UniFrac matrix presented by Principal Coordinate Analysis (PCoA) plots (Figure 6-1 b, c, e, f). Additionally, ANOSIM and Adonis statistical model analyses further showed a significant difference between both OP and faecal bacterial communities of AC and CC children (Supplemental Table S6-1).

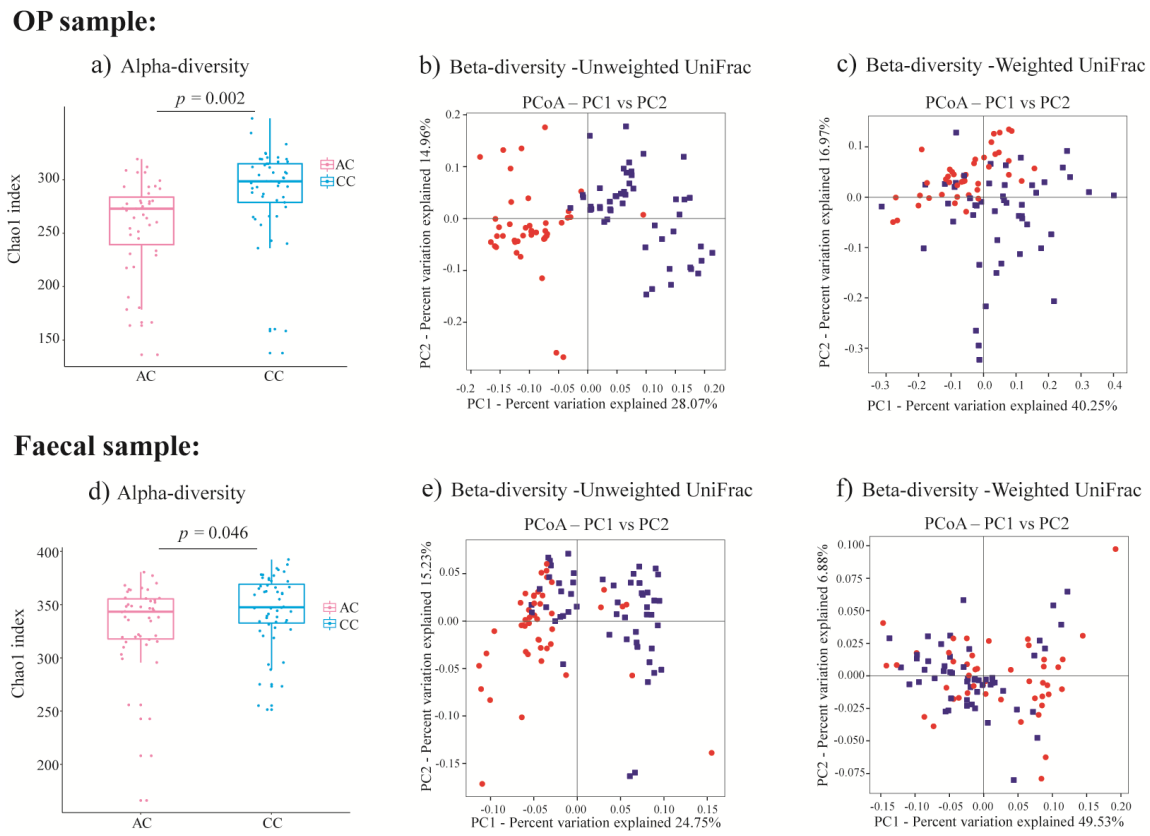


Figure 6-1. The alpha and beta diversity of OP and faecal samples from AC and CC children

Alpha diversities are exemplified by the Chao1 index for oropharyngeal (OP) samples in panel a), as well as faecal samples in panel d). The top and bottom lines of box plots showed the interquartile range, and lines inside the boxes represented medians. Beta diversities for OP samples are represented by Principal coordinate analysis (PCoA) plots of b), unweighted and c), weighted UniFrac matrix, whereas those for faecal samples are similarly represented in panels e), unweighted and f), weighted UniFrac matrix. For beta diversity analyses, data points represent either AC samples (red) or CC samples (blue), and the two major principle components are respectively represented on the x- and y-axes.

6.4.2.2 Oropharyngeal sample bacterial composition

A total of 16 bacterial phyla were detected from OP swabs (Figure 6-2 a, Supplemental Table S6-2). Phylum-level taxonomical assignment showed that *Firmicutes* and *Proteobacteria* were dominant in both AC (49.6%, 19.8%) and CC children (46.2%, 26.6%). At the genus level, 12 genera accounted for 80.8% of the abundance in AC and 15 genera accounted for 82.3% of the abundance in CC children, using a minimum relative abundance of 1.0% (Supplemental Figure S6-1 a, Supplemental Table S6-2).

Among the total of 16 bacterial phyla and 193 genera, 6 (37.5%) phyla and 113 (58.5%) genera were significantly different between AC and CC children. AC children had a lower proportion of phylum Deinococcus-Thermus ($p<0.001$), and higher proportions of Actinobacteria ($p<0.001$), Fusobacteria ($p<0.001$), and Bacteroidetes ($p= 0.013$) (Figure 6-2 b, Supplemental Table S6-2). The relative abundance of genus *Thermus* (phylum *Deinococcus-Thermus*) was lower in AC children, whereas genera *Rothia*, *Actinomyces* (phylum *Actinobacteria*), *Leptotrichia*, *Fusobacterium* (phylum *Fusobacteria*), and *Bacteroides* (phylum *Bacteroidetes*) were all present in significantly higher proportions in AC. The *Firmicutes* did not show a difference at phylum level, however the genera *Streptococcus* and *Granulicatella* were present in higher proportions in AC children, while the genera *Gemella*, *Ammoniphilus* were present in higher proportions in CC children. In addition, the genera of the phylum *Proteobacteria*, including *Actinobacillus*, *Pseudomonas*, and *Sphingomonas* were all present in higher proportions in CC children. The differences between the two groups of children are shown for the 10 most abundant genera in Supplemental Figure S6-1 b. These differences remained significant after further adjustment for confounders (age, gender, BMI, breastfed percentage, and antibiotic use) using linear regression.

6.4.2.3 Faecal sample bacterial composition

We observed 10 distinct phyla in the faecal microbiomes of AC and CC children (Figure 6-2 c, Supplemental Table S6-3). The phylum Firmicutes (49.4%) was dominant in AC children, whereas the phylum Bacteroidetes (47.5%) had the highest proportion in CC children. These two phyla made up the vast majority of OTUs, namely 89.3% in AC and 88.4% in CC children. Seven (70.0%) phyla and 91 (62.8%, 91/145) genera were significantly different between AC and CC children, with the major differences shown in Figure 6-2 d, Supplemental Figure S6-2. The phylum Firmicutes was more abundant in AC children (p= 0.008), and included the genera *Ruminococcus1*, *Lachnospira*, *Ruminococcaceae UCG-002*, *Eubacterium*, and *Peptoclostridium*. The genus *Blautia* was present in a significantly higher frequency in CC children (p= 0.019). The phylum Bacteroidetes was more abundant in CC children (p= 0.033), but the downstream genera *Alistipes* and *Barnesiella* were significantly higher in AC children (p= 0.001, and p= 0.001). Within the phylum Proteobacteria, the genus *Sutterella* was present in a significantly higher frequency in AC children, while *Parasutterella*, and *Escherichia-Shigella* were more abundant in CC children (p<0.001).

After adjusting for confounders, the relative abundance of phyla Firmicutes, and Bacteroidetes, genera *Ruminococcus1*, *Lachnospira*, *Eubacterium*, *Peptoclostridium*, *Barnesiella*, *Parasutterella*, and *Escherichia-Shigella* remained significantly different between AC and CC children.

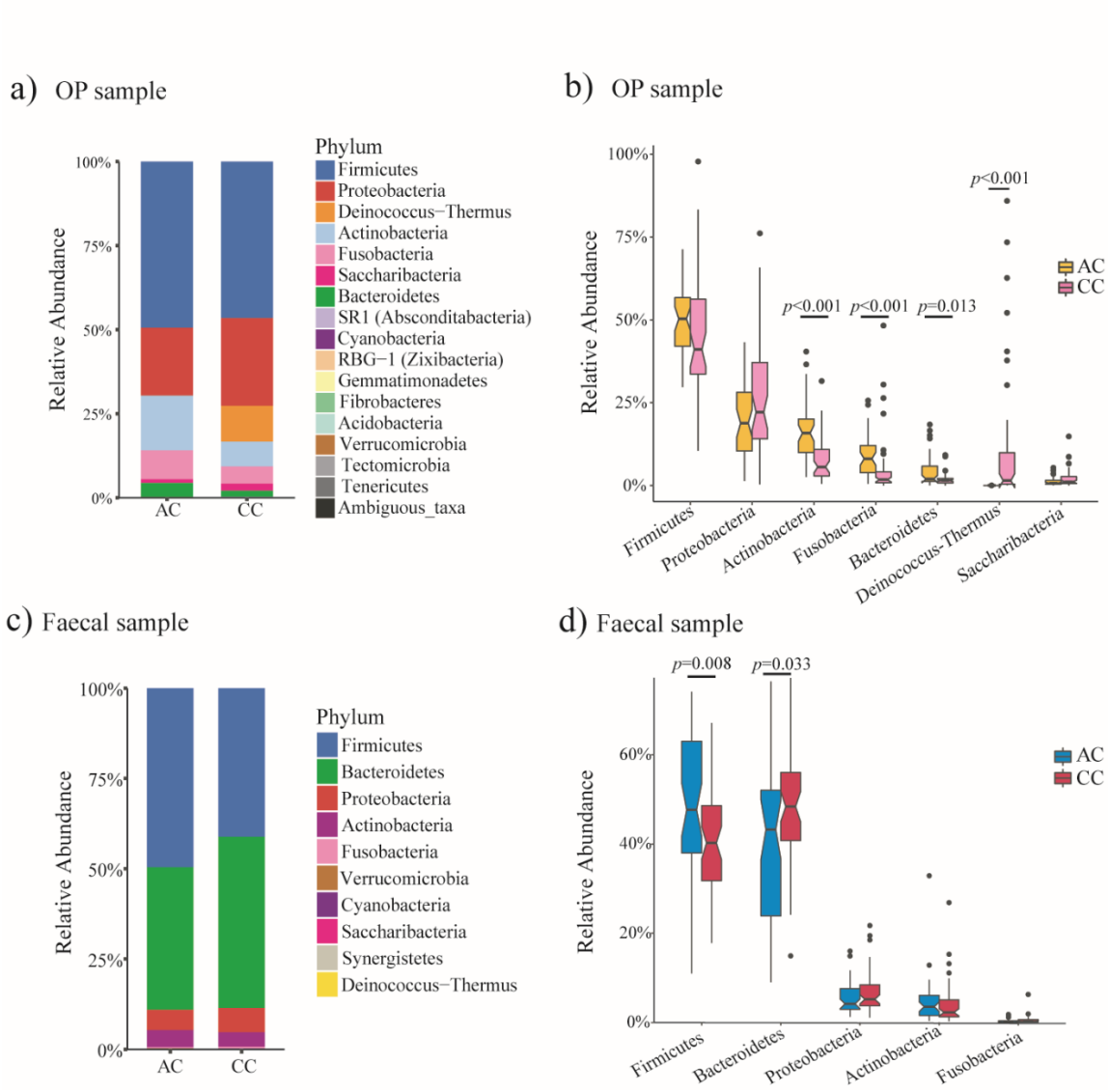


Figure 6-2. Bacteria relative abundance composition and comparison of OP and faecal samples at the phylum level

The composition of bacterial relative abundance are shown in bar plots: panel a) oropharyngeal (OP) samples and c) faecal samples. The major phyla (relative abundance >1.0%) comparisons between AC and CC children are shown on box plots in panel b) OP samples and d) faecal samples. The top and bottom lines of box plots show the interquartile range, and lines inside the boxes represent medians, and black dots represent outliers.

6.4.3 Functional characterization of the microbiome communities

The OP microbiome of AC children had a higher frequency of Gram-positive, anaerobic, facultative anaerobe and potentially pathogenic bacteria, while the OP microbiome of CC children had a higher frequency of Gram-negative bacteria. The Gram-reaction groupings as well as the facultative anaerobic bacteria findings were the same for the faecal samples of both groups (Supplemental Table S6-4). The KEGG pathways showed larger disparities in functional profiles between AC and CC children in OP samples, compared to faecal samples (Supplemental Figure S6-3 and S6-4).

For OP samples comparison, genetic information processing (DNA repair and recombination proteins), environmental information processing (transporters and phosphotransferase system PTS) and glycan biosynthesis and metabolism were increased most significantly (LDA > 3) in AC children relative to CC children, whereas xenobiotics biodegradation and metabolism, amino acid metabolism (valine leucine and isoleucine degradation) and cellular process (cell motility) were increased most significantly among CC children (LDA >3, Supplemental Figure S6-3). For faecal samples, cellular process (cell motility) and genetic information processing (transcription) were increased in AC children, while several metabolic pathways were increased in CC children (Supplemental Figure S6-4).

6.4.4 Trend consistency of taxonomic abundance with the western environment and allergy

We identified taxa that were different between the AC and CC children across the five taxonomic levels. We selected 204 OP taxa with a significance level $p< 0.05$ and 141 OP taxa with a significance level $p< 0.01$. For faecal samples this was 123 and 81 taxa, respectively. Combined with the mean difference of taxa abundance between positive and negative allergic conditions (> 0.01% and > 0.1%) we performed four sets of binomial

tests each for atopy, food allergy, and wheezing among AC children (Supplemental Table S6-5).

We discuss the binomial test results for the analysis of $p < 0.05$ and difference > 0.01% (Supplemental Table S6-5). In OP samples, 84.0% (63/75) of the taxa showed a consistent trend for food allergy that is significantly higher than 50% ($p < 0.001$). Such trend was also found for the faecal taxa and atopy (61.0% (36/59)). In contrast, only 23.6% (13/55) and 29.8% (14/47) of the faecal taxa showed a consistent trend for food allergy and wheezing, significantly lower than 50% with a p value of < 0.001 and 0.008, respectively. This indicates that the trend is inverse, an increase in abundance of the faecal taxa in AC children corresponds to a decrease in these taxa in children with food allergy or wheezing. These findings are observed for all the four analyses presented in Supplemental Table S6-5 albeit different cut-off points of significance and difference. Figure 6-3 shows the proportion of consistency in OP and faecal samples for food allergy and wheezing.

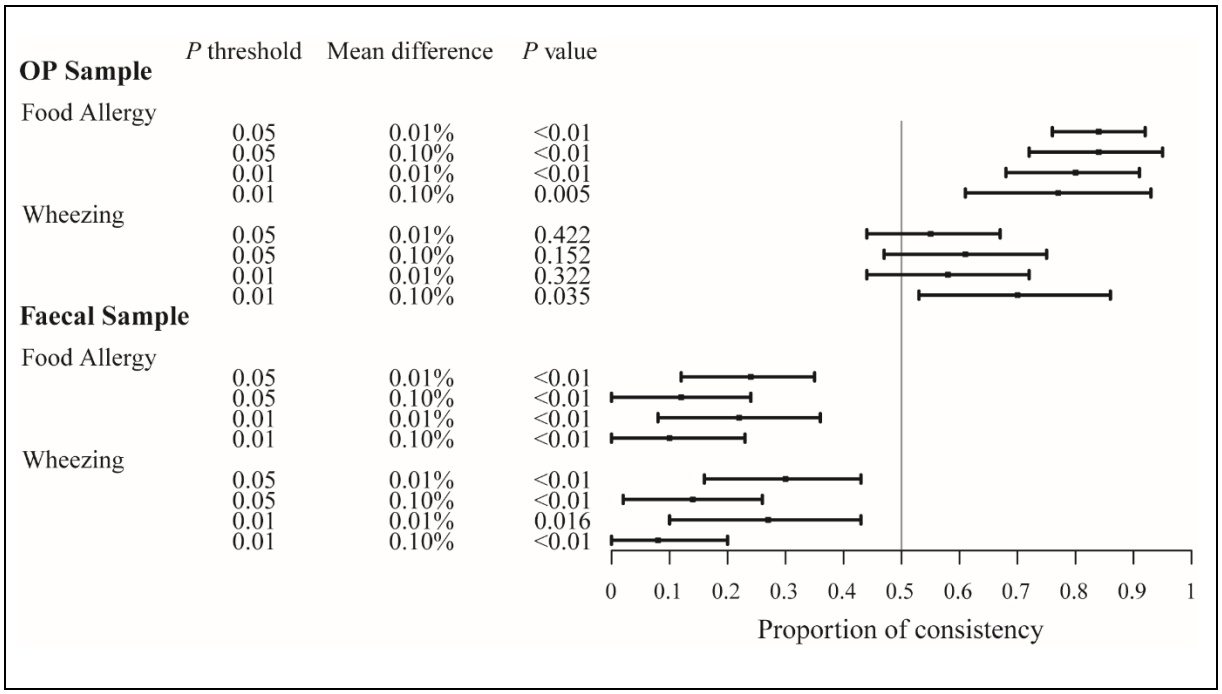


Figure 6-3. Consistency in trends of taxa abundance between the OP and faecal microbiome

Left columns indicate the taxa selection thresholds p values (0.05 and 0.01) for the abundance comparison between AC and CC children, and the mean difference between with or without allergic conditions (over 0.01% or 0.10%). The "P value" column represents the significance of each binomial probability test. The horizontal axis of the plot represents the proportion of consistency of taxa abundance that is higher or lower in AC children (compared to CC children) and in children with allergic conditions (relative to children without allergies) among AC children. At the vertical "line of null effect" there is no consistent trend. Each horizontal line on the plot represents a consistent trend under certain thresholds. The black box indicates the mean value of the proportion of consistency, and the horizontal line represents the 95% confidence intervals.

6.5 Discussion

This is the first study that compares the diversity and composition of human OP and faecal microbiomes in a single ethnic (Han) group of children living in either a Western (Australia) or Eastern (China) environment. Here, we use the term 'Western environment' as a phrase to collectively denote the socio-cultural, lifestyle, and geographical environment in industrialized countries, such as Australia, and the 'Eastern environment' as a collective term to indicate non-industrialized counties, such as China. The children were matched in age and gender and we also matched the recruitment season in Australia and China. We selected this homogenous population to control the genetic influence so that only environmental exposure is varied, which is a main advantage of this study.

As expected, AC children had higher rates of atopy, food allergy, and wheezing compared to CC children. We hypothesised that the Australian environment has modified the human microbiome in Chinese immigrant children (microbiome modification by the western environment), thereby leading to more allergy in AC children (the western microbiome causing allergy). Consistent with the first part of our hypothesis, we found significant differences in microbial diversity, composition, and functional pathway expression in both OP and faecal microbiota between Chinese children living in Australia and China. We designed the study to measure significant differences in the microbiome between AC and CC children, rather than identifying specific taxa associated with allergic conditions in the population. To examine the hypothesis of the western microbiome relating to allergy we investigate if the change in relative abundance of taxa in AC children (compared to CC children) is also apparent in children with allergic conditions (relative to children without allergies) among the AC population to examine the hypothesis of the western microbiome relating to allergy. Such a consistent trend was significant (>50%) for food allergy and wheezing in the OP microbiome. This means that OP taxa which

increase/decrease in the western environment are likely to show the same increase/decrease in children with food allergy and wheezing. In contrast, we found that such trend was significantly lower than 50% for faecal samples with these two phenotypes. Faecal taxa which increase/decrease in the western environment likely show an opposite effect, namely decrease/increase, in children with food allergy or wheezing. Conceivably, the environment of upper respiratory tract and gastrointestinal tract are very different, and the way the microbial component interacts with the immune system differs considerably. The gut has numerous immunogenic regions, where microbial elements interact rapidly with regulatory T cells [200]. The segmented filamentous bacteria have the function of promoting intestinal T helper type (Th17) responses [201]. Therefore, the mechanism of interaction could be very different between the two sites as is the diversity and taxonomic groups in those two distinct areas.

This is a cross-sectional study and these trend consistencies do not indicate a cause-effect relationship. Children with food allergy and wheezing may have a changed immune status that changes the abundance of taxa. This may partly explain the inverse trend mentioned above. We think that the observed trends are unlikely to be false findings as they are consistently significant in all four analyses. Our study shows that western oropharyngeal and gut microbial flora are associated with allergic conditions in Chinese immigrant children. More studies are required to clarify the opposite trend that is observed for OP and gut microbiomes with food allergy and wheezing in industrialized countries.

Environmental biodiversity is important for human health. Lynch *et al*. reported that healthy children were exposed to richer and more diverse bacterial communities in the first year of life, compared to those children that developed either atopy or recurrent wheeze [137]. Another recent study compares of the prevalence of asthma between Amish and Hutterites schoolchildren (similar genetic ancestries and lifestyle). It revealed that

Amish children, living on a traditional farm, have been exposed to a more enriched microbiota environment and demonstrate low rate of asthma, compared to Hutterites children whose farming practice is industrialized [136]. There is emerging evidence that the environmental influence (environmental microbiomes) on shaping human microbiomes is a key element in tuning immune system and development of allergy. Several studies have shown that a reduced diversity of the human microbiome may be a risk for asthma and allergy [105]. A low microbial diversity in early infancy can potentially predict atopic dermatitis [202]. A longitudinal study demonstrated a lower oral bacterial diversity among children who developed allergic disease, particularly asthma at an age of 7 years [203]. There are different indexes that estimate microbial diversity such as the Chao1 index as a richness estimator and the Shannon index for the bacterial evenness [204]. In our study both OP and faecal samples in AC children had a lower Chao1 index but a higher Shannon index. This indicates that the western environment has shaped the microbiome to have less richness and more evenness. In another population comparison study it was found that the alpha-diversity of the faecal microbiome (Chao1 and Shannon indexes) was higher in African children (non-industrialized) compared to those of European (industrialized environment like Australia) children [205]. The findings of these studies are largely consistent with our study. Microbial diversity variations in the human microbiome related to the western environment may provide a mechanistic explanation for the allergy epidemic in the past 60 or 70 years.

The microbiome profiles in AC children are significantly different from CC children. This indicates that western and eastern populations may be living with a different genus and species group of commensal microorganisms. This present study is not designed to ascertain which bacteria that are commonly present in western populations cause allergy as it is likely a combination effect of many. Rather we analyse the difference between

microbiome profiles in western and eastern populations to aid further studies to clarify their causal effects on asthma and allergy.

We discuss a few dominant taxa and compare our findings with recent literature. The genus *Streptococcus* (Firmicutes), a Gram-positive bacterium, has the largest abundance in both AC and CC children in OP samples but in a significantly higher proportion among AC children compared to CC children. Studies have shown that *Streptococcus* is associated with allergic symptoms. A 234 children cohort study revealed that early colonization of *Streptococcus* in the nasopharyngeal microbiota was a strong predictor for asthma during the first year of life, and its colonization was linked to atopy by the age of two years and chronic wheeze at age five [206]. Similarly, another study of neonatal oropharynx bacteria showed that a high burden of *Streptococcus* within the first month of life increased the risk for recurrent wheeze and asthma development [207]. That the gut microbiota is critical for immune development has been well documented [208]. The majority of genera that showed a significantly higher abundance in AC children compared to CC children were in the class Clostridia of phylum Firmicutes. Interestingly, a recent study found that the same class and phylum were enriched in faecal microbiome of food-allergic children compared to siblings and healthy children, but other *Clostridium* species were enriched in non-food-allergic subjects [209]. Class Clostridia has been associated with immune tolerance in mouse models of allergy and aids protection from allergic inflammation [210]. Our inverse association between the western faecal microbiome and food allergy and wheezing partly supports the association of the class Clostridia with food allergy reported in the literature.

The cell walls of Gram-negative bacteria contain lipopolysaccharide (LPS), which contribute to innate immune tolerance and help to prevent inappropriate immune stimulation through the microbiota-epithelial crosstalk [211]. Indeed, we found Gram-

negative bacteria were higher among CC children in both OP and faecal samples using BugBase. Although the KEGG pathways provide limited understanding of the actual bacterial potential functions, differences in the expression of certain pathways can indicate potential associations.

One limitation is that this study is cross-sectional and the results cannot determine causality. To Chinese migrants, the change to a Western environment is the combination of a different diet, less air pollution, exposure to new allergens and, greater hygiene, all of which can lead to different microbiota composition/diversity, and contribute to the increased allergies in AC children [212, 213]. However, in this study we could only focus on the overall influence of Western environment on the microbiome and the relation with allergy. Thirdly, recent antibiotic usages are known to have a significant impact on the human microbiome. Antibiotic use during the two-week period prior to the recruitment was three to four times more common in CC than in AC children. The disparity of antibiotic use in the two population may confound the findings in this study. Unfortunately, we did not collect a detailed history of antibiotic use in this population. In addition, the resolution of 16s rRNA sequencing is reliable down to the genus level. Studies utilising whole-genome sequencing or real-time PCR, are of interest to further investigate the species and strains of bacteria that are different between the industrialized and non-industrialized environment, as well as to understand how the western microbiome shapes the immune system, leading to the development of asthma and allergy. Moreover, a comparison of microbiota and allergies present before and after immigration is worthy of investigating for future study.

6.6 Conclusion

We found evident differences in the compositions of the OP and gut microbiome between AC and CC children. The AC children demonstrated a lower microbial diversity richness and higher diversity evenness compared to CC children. The association of the OP microbiome with food allergy and wheezing is different from the gut microbiome in Chinese immigrant children in Australia. The western environment/lifestyle promotes a different human microbiome profile that may significantly contribute to the increased prevalence of asthma and allergy in industrialized countries.

6.7 Supplemental information

6.7.1 Supplemental tables

Supplemental table S6-1. The p-values of β-diversity comparison between AC and CC children

***β*-diversity**	**OP samples**		**faecal samples**	
	ANOSIM	Adonis	ANOSIM	Adonis
Unweighted UniFrac PCoA	0.001	0.001	0.001	0.001
Weighted UniFrac PCoA	0.001	0.001	0.001	0.003

Supplemental table S6-2. The phylum and genus level of OP samples microbiome compared between AC and CC children

Phylum	**AC**	**CC**	***p***	***FDR_p****
Firmicutes	49.63%	46.22%	0.186	0.198
Proteobacteria	19.80%	26.57%	0.149	0.181
Actinobacteria	16.04%	7.61%	0.000	**<0.001**
Fusobacteria	8.92%	4.95%	0.000	**<0.001**
Bacteroidetes	4.41%	1.83%	0.005	**0.013**
Saccharibacteria	1.13%	2.12%	0.045	0.086
Cyanobacteria	0.02%	0.08%	0.000	0.001
SR1 (Absconditabacteria)	0.02%	0.11%	0.046	0.086
Tenericutes	0.01%	0.00%	0.015	0.036
Deinococcus-Thermus	0.00%	10.34%	0.000	**<0.001**
Fibrobacteres	0.00%	0.01%	0.120	0.157
RBG-1 (Zixibacteria)	0.00%	0.06%	0.101	0.143
Gemmatimonadetes	0.00%	0.04%	0.101	0.143
Tectomicrobia	0.00%	0.01%	0.101	0.143
Acidobacteria	0.00%	0.01%	0.183	0.198
Verrucomicrobia	0.00%	0.01%	0.349	0.349
Ambiguous taxa	0.01%	0.04%	0.000	0.000
Genus				
Streptococcus (Firmicutes; Bacilli; Lactobacillales; Streptococcaceae)	26.59%	13.91%	0.000	**<0.001**
Rothia (Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae)	9.47%	4.69%	0.000	**0.001**
Haemophilus (Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae)	8.18%	6.65%	0.287	0.344

Leptotrichia (Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae)	7.80%	4.68%	0.000	**<0.001**
Neisseria (Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae)	7.77%	8.01%	0.555	0.595
Veillonella (Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae)	6.31%	7.41%	0.087	0.128
Actinomyces (Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae)	4.44%	1.29%	0.000	**<0.001**
Gemella (Firmicutes; Bacilli; Bacillales; Family XI)	3.60%	13.79%	0.000	**<0.001**
Granulicatella (Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae)	2.13%	1.31%	0.001	**0.004**
Bacteroides (Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae)	1.90%	0.23%	0.002	**0.004**
Fusobacterium (Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae)	1.12%	0.26%	0.000	**<0.001**
Erysipelotrichaceae UCG-007 (Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae)	1.08%	1.85%	0.315	0.368
Actinobacillus (Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae)	0.42%	4.53%	0.000	**0.002**
Pseudomonas (Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae)	0.01%	1.23%	0.000	**<0.001**
Sphingomonas (Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae)	0.00%	1.06%	0.000	**<0.001**
Thermus (Deinococcus-Thermus; Deinococci; Thermales; Thermaceae)	0.00%	10.34%	0.000	**<0.001**
Ammoniphilus (Firmicutes; Bacilli; Bacillales; Paenibacillaceae)	0.00%	1.49%	0.000	**<0.001**

*: The *p*-values were FDR-corrected to control for multiple testing.

Supplemental table S6-3. The phylum and genus level of faecal samples microbiome compared between AC and CC children

Phylum	**AC**	**CC**	***p***	***FDR_p****
Firmicutes	49.42%	40.85%	0.004	**0.008**
Bacteroidetes	39.87%	47.52%	0.020	**0.033**
Proteobacteria	5.55%	6.83%	0.121	0.151
Actinobacteria	4.53%	4.04%	0.240	0.240
Fusobacteria	0.31%	0.48%	0.003	**0.006**
Verrucomicrobia	0.22%	0.01%	0.000	**<0.001**
Cyanobacteria	0.05%	0.17%	0.165	0.183
Saccharibacteria	0.03%	0.06%	0.000	**<0.001**
Synergistetes	0.01%	0.00%	0.034	**0.049**
Deinococcus-Thermus	0.00%	0.03%	0.000	**<0.001**
Genus				
Bacteroides (Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae)	27.37%	33.85%	0.059	0.085
Faecalibacterium (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	10.40%	9.21%	0.056	0.082
Lachnospiraceae UCG-008 (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	5.27%	5.40%	0.550	0.582
Prevotella 9 (Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae)	4.69%	7.18%	0.086	0.119
Alistipes (Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae)	4.57%	2.81%	0.000	**0.001**
Bifidobacterium (Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae)	3.64%	3.03%	0.261	0.307
Sutterella (Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae)	2.35%	1.29%	0.003	**0.005**

Ruminococcus 1 (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	2.03%	0.52%	0.000	**<0.001**
Subdoligranulum (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	2.03%	1.68%	0.045	0.069
Roseburia (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.75%	1.91%	0.825	0.843
Parabacteroides (Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae)	1.64%	2.00%	0.185	0.234
Lachnospira (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.56%	0.89%	0.004	**0.007**
Ruminococcus 2 (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	1.55%	1.21%	0.060	0.086
Blautia (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.51%	2.32%	0.011	**0.019**
Fusicatenibacter (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.33%	1.68%	0.063	0.089
Dialister (Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae)	1.29%	1.84%	0.477	0.516
Ruminococcaceae UCG-002 (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	1.26%	0.57%	0.000	**<0.001**
Parasutterella (Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae)	1.25%	2.38%	0.000	**<0.001**
[Eubacterium] eligens group (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.24%	0.36%	0.000	**<0.001**
[Eubacterium] coprostanoligenes group (Firmicutes; Clostridia; Clostridiales; Ruminococcaceae)	1.23%	0.57%	0.000	**<0.001**
Peptoclostridium (Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae)	1.21%	0.49%	0.000	**<0.001**
Haemophilus (Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae)	1.21%	0.74%	0.049	0.074

[Eubacterium] oxidoreducens group (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	1.06%	0.49%	0.001	**0.002**
Barnesiella (Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae)	1.03%	0.31%	0.000	**0.001**
Escherichia-Shigella (Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae)	0.17%	1.13%	0.000	**<0.001**

*: The *p*-values were FDR-corrected to control for multiple testing

Supplemental table S6-4. Phenotypes of bacteria function comparison between AC and CC children in OP and faecal samples

	AC	CC	*FDR_p**
OP samples			
Gram Positive	0.64±0.10	0.45±0.19	**<0.001**
Gram Negative	0.36±0.10	0.55±0.19	**<0.001**
Oxygen Utilizing			
Aerobic	0.31±0.13	0.39±0.22	0.330
Anaerobic	0.31±0.15	0.23±0.16	**0.016**
Facultatively anaerobic	0.21±0.07	0.11±0.05	**<0.001**
Biofilm Forming	0.41±0.13	0.36±0.20	0.120
Pathogenic Potential	0.92±0.07	0.80±0.23	**0.002**
Mobile Element Containing	0.50±0.09	0.51±0.22	0.907
Oxidative Stress Tolerant	0.18±0.11	0.22±0.16	0.445
Faecal samples			
Gram Positive	0.70±0.16	0.64±0.12	**0.012**
Gram Negative	0.30±0.16	0.36±0.12	**0.012**
Oxygen Utilizing			
Aerobic	0.03±0.03	0.03±0.02	0.515
Anaerobic	0.93±0.04	0.92±0.05	0.968
Facultatively anaerobic	0.02±0.01	0.02±0.02	**0.041**
Biofilm Forming	0.09±0.05	0.10±0.06	0.974
Pathogenic Potential	0.52±0.13	0.51±0.12	0.823
Mobile Element Containing	0.78±0.13	0.73±0.11	**0.011**
Oxidative Stress Tolerant	0.04±0.03	0.04±0.03	0.172

Bugbase tool was used to predict the organism level of microbiome phenotypes.

*: The *p*-values were FDR-corrected to control for multiple testing.

Supplemental table S6-5. Binomial probability test to determine consistent differences in distinct taxa between AC and CC children and different allergic conditions in AC children

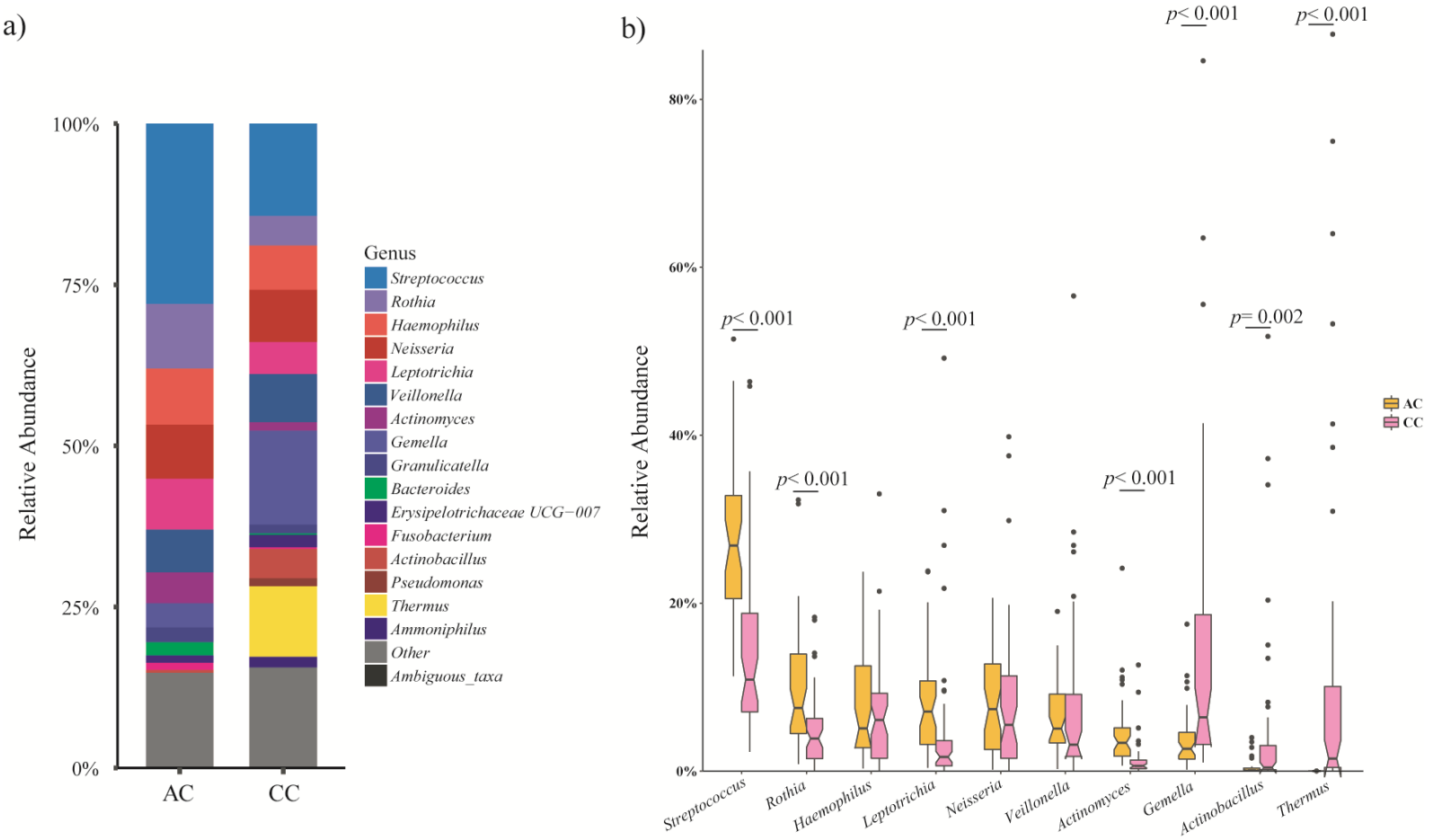
	significance level: 0.05 *						significance level: 0.01 *					
	Mean difference #: 0.01%			Mean difference #: 0.1%			Mean difference #: 0.01%			Mean difference #: 0.1%		
OP	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$
Atopy	69	42(60.8)	0.091	39	22(56.4)	0.522	42	27(64.3)	0.088	24	17(70.8)	0.064
Food allergy	75	63(84.0)	**<0.001**	43	36(83.7)	**<0.001**	49	39(79.6)	**<0.001**	30	23(76.7)	**0.005**
Wheezing	76	42(55.3)	0.422	49	30(61.2)	0.152	50	29(58.0)	0.322	33	23(69.7)	**0.035**
Faecal	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$	N	n(%)	$P^{\$}$
Atopy	59	36(61.0)	0.117	30	21(70.0)	**0.043**	39	22(56.4)	0.522	17	12(70.6)	0.143
Food allergy	55	13(23.6)	**<0.001**	33	4(12.1)	**<0.001**	37	8(21.6)	**0.001**	21	2(9.5)	**<0.001**
Wheezing	47	14(29.8)	**0.008**	36	5(13.9)	**<0.001**	30	8(26.7)	**0.016**	24	2(8.3)	**<0.001**

*: selected taxa with significant differences (at two significance levels: $p<0.05$ and $p<0.01$) in relative abundance between AC and CC children at five taxonomic levels (phylum, class, order, family, and genus)

#: mean difference of taxa abundance between allergic condition positives and negatives was calculated, and those with mean difference over 0.01% or 0.1% were selected.

$: Binomial probability test was used to evaluate the consistent trend.

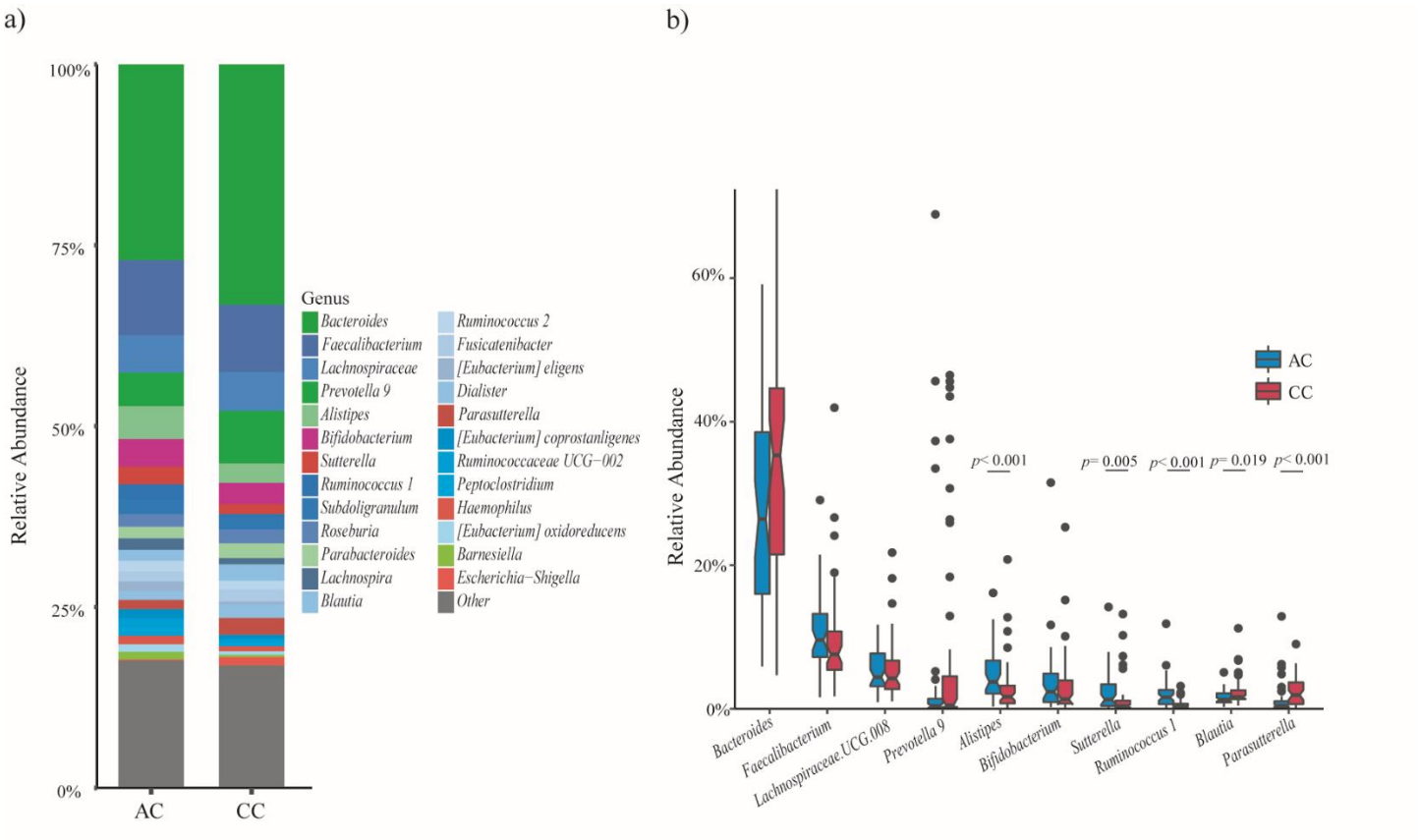
6.7.2 Supplemental figures



a) The bar plot shows the composition of bacterial relative abundance. Major genera (relative abundance >1.0%) are drawn individually and taxa with relative abundances less than 1.0% are combined into the 'Other' category.

b) The box plot shows the comparison of top ten major genera in oropharyngeal samples between AC and CC children. The notched boxes show the interquartile range, and lines inside the boxes represent medians, and black dots represent outliers.

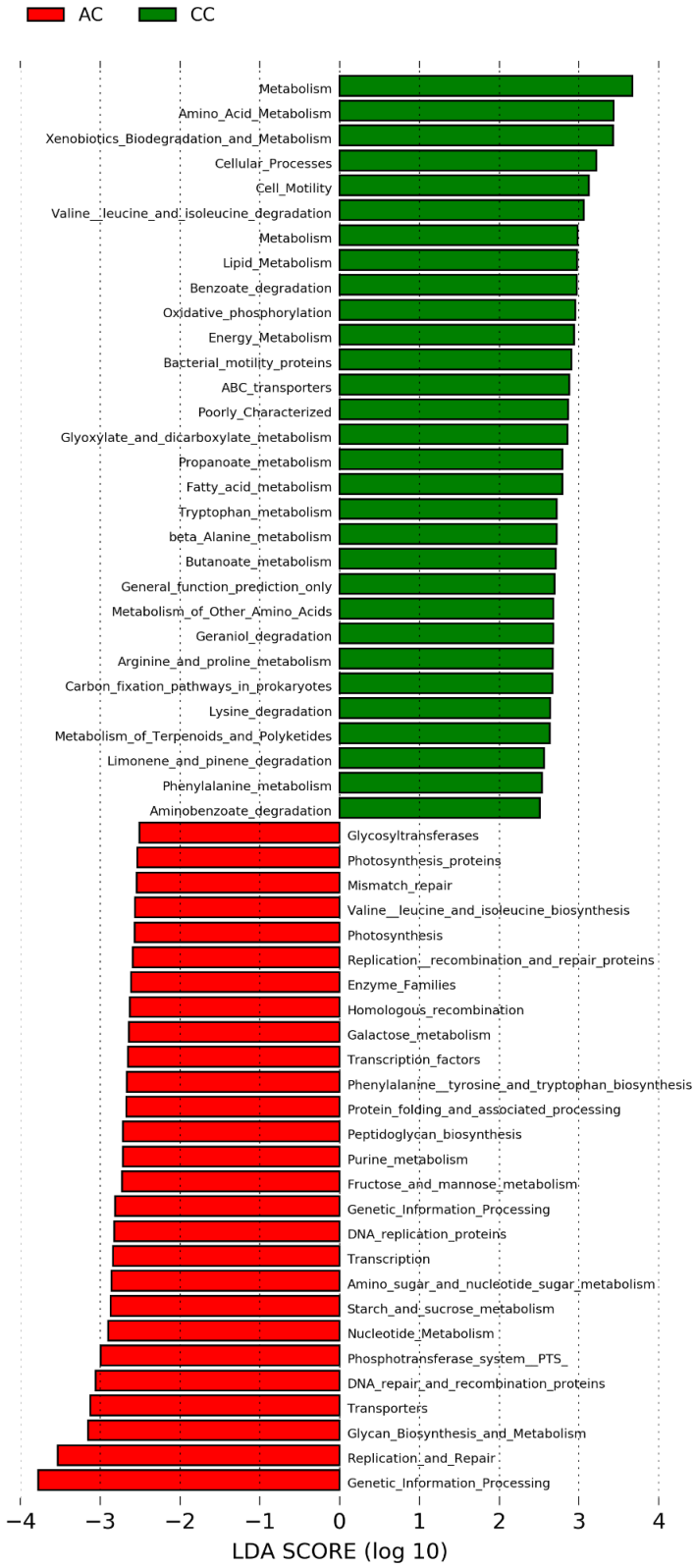
Supplemental figures S6-1. Bacterial relative abundance composition and comparison of oropharyngeal samples at the genus level



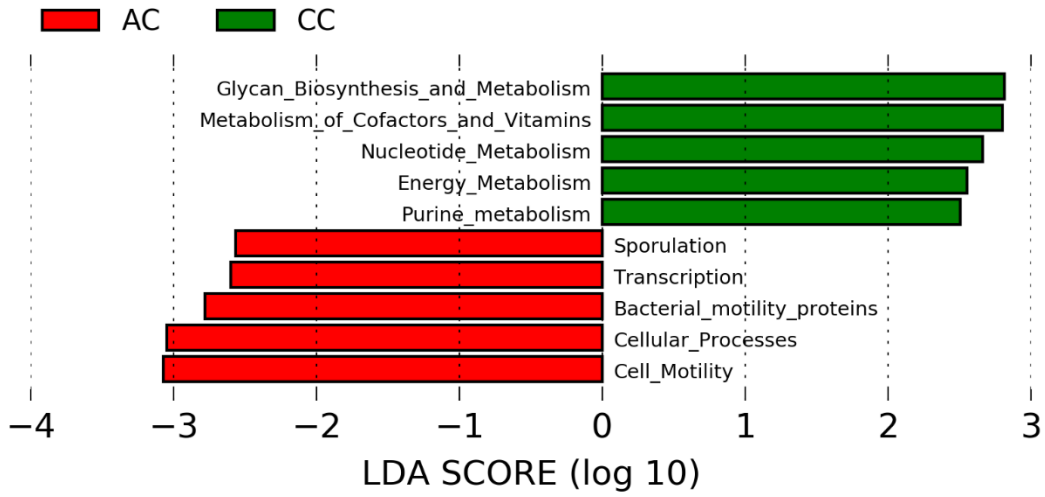
a) The bar plot shows the composition of bacterial relative abundance. Major genera (relative abundance >1.0%) are drawn individually and taxa with relative abundances less than 1.0% are combined into the 'Other' category.

b) The box plot shows the comparison of top ten major genera in faecal samples between AC and CC children. The notched boxes show the interquartile range, and lines inside the boxes represent medians, and black dots represent outliers.

Supplemental figures S6-2. Bacterial relative abundance composition and comparison of faecal samples at the genus level



Supplemental figures S6-3. Bar graphs of linear discrimination analysis (LDA) for the mean differences of KEGG pathway for oropharyngeal samples



Supplemental figures S6-4. Bar graphs of linear discrimination analysis (LDA) for the mean differences of KEGG pathway for faecal samples

KEGG pathway more present in Australian Chinese (AC) children are represented by red bars, whereas those more abundant in China-Born Chinese (CC) children are represented by green bars. Note the plots are a logarithmic x-axis (of base 10) and only KEGG pathway difference for an order of 2.0 or higher are represented.

7 Study Three—Linking the westernised oropharyngeal microbiome to the immune response in Chinese immigrants

In this chapter, we demonstrate that the composition of oropharyngeal microbiome and the patterns of microbiome-host interactions are distinctly different between newly-arrived and long-term Chinese immigrants in Australia. To the best of our knowledge, this is the first study to investigate the oropharyngeal microbiome and its correlation with the immune response and IgE sensitization in an ethnically homogeneous immigrant population in Western/developed (Australia) versus Eastern/developing (China) environments.

The work presented in this chapter was published in Allergy Asthma and Clinical Immunology. 2020 Jul 25;16:67. doi: 10.1186/s13223-020-00465-7.

7.1 Abstract

Background: Human microbiota plays a fundamental role in modulating the immune response, and these microbiota are associated with Western environment and lifestyle. Here, we investigated how differences in composition of oropharyngeal microbiome may contribute to patterns of interaction between the microbiome and immune system in Chinese immigrants living in Australia.

Methods: We recruited 44 adult Chinese immigrants: newly-arrived (n=22, living in Australia <6 months) and long-term Chinese immigrants (n=22, living in Australia >5 years), with age and gender matched. Oropharyngeal swabs, serum and whole blood were collected. The 16s ribosomal RNA gene from the swabs was sequenced on the Illumina MiSeq platform. Innate immune responses were determined by 23 Toll-like receptors (TLR) pathway cytokines, while adaptive immune responses were determined by IgG-associated response to specific microbial/viral pathogens.

Results: The relative abundance of the genus *Leptotrichia* was higher in long-term immigrants as compared to that in newly-arrived Chinese immigrants, while the genus *Deinococcus* was significantly lower in long-term Chinese immigrants. The genera *Lachnospiraceae_uncultured*, *Erysipelotrichaceae UCG-007*, *Veillonella*, and *Actinomycetales_ambiguous taxa* were negatively correlated with cytokine IL-6 in long-term Chinese immigrants (rho range: -0.46 ~ -0.73). With respect to adaptive immunity, several microbial taxa were significantly associated with IgG1 responsiveness to microbial antigens in long-term immigrants, while a significant correlation with IgG1 responsiveness to viral antigens was detected in newly-arrived immigrants.

Conclusion: The composition of the oropharyngeal microbiome varies between newly-arrived and long-term Chinese immigrants. Specific microbial taxa are significantly associated with immunological parameters but with different association patterns between newly-arrived and long-term Chinese immigrants.

7.2 Introduction

"Western-developed" vs. "Eastern-developing" gradients in many inflammatory conditions, such as asthma and allergic diseases, are significant.[44, 214] The International Study of Asthma and Allergies in Childhood confirmed these variations, showing that the self-reported asthma prevalence varied from 2-3% in developing countries to 20-40% in developed countries.[215] In addition, immigrants from a developing (Eastern) to a developed (Western) country are at an increased risk of asthma and allergy, with gradually increasing prevalence related to their years of residence in the developed country.[30] These differences in disease prevalence are thought to be related to lower levels of 'hygiene' in Eastern countries relative to Western countries[216], consistent with the 'hygiene hypothesis'.[100] Elucidation of the mechanisms underlying the inequality of asthma prevalence between Western and Eastern countries, and the increased trend of allergic conditions in immigrants from a low to a high asthma risk country, may hold the key to understanding why these conditions have increased in developed countries and are increasing in developing countries.[153]

Microbes associated with humans play a pivotal role in maintaining human health.[217] Recent studies have shown that the microbiome is associated with diverse health conditions, such as inflammatory bowel diseases, autoimmune disorders, and allergy.[112, 218] All these conditions and diseases share a common chronic

inflammatory mechanism associated with environmental and lifestyle risk factors. Recent research emphasizes on microbiota-host interactions with the discovery that commensal microbes link the innate and adaptive response by producing small molecules.[219] It has been suggested that Western environments and lifestyles may alter human microbiota resulting in a microbiome profile that fails to prime the immune system. This in turn leads to chronic inflammation and causes a range of conditions that have increased during the past several decades in Western countries. It is therefore timely to investigate the influence of the Western environment and lifestyle on the human microbiome as well as the interaction between the microbiome and immune response in a homogeneous immigrant population to elucidate mechanisms underlying the development of asthma and other chronic inflammatory diseases.

Australia and China have one of the highest and lowest worldwide prevalence of asthma and allergies, respectively.[215] Chinese immigrants living in Australia have entered a 'natural experimental environment' with a high risk of developing asthma and allergy and provide a unique population to investigate microbiota changes resulting from the Western environment. We have found a marked shift in the innate and adaptive immune response between Chinese immigrants living in Australia for less than 6 months and more than five years. This was achieved by comparing the whole blood toll-like receptors (TLRs) pathway cytokine response and sera immunoglobulin G (IgG)-associated responsiveness to specific microbial/virus pathogens.[154, 197] Recently, our team reported that Chinese immigrant children living in Australia show different microbiome diversity and composition compared to Chinese-born Chinese children.[220] The aim of this study is to compare the oropharyngeal microbial profiles between newly-arrived (< 6 months) adult Chinese immigrants and long-term (> 5 years) adult Chinese immigrants. We also investigated

the correlation between the oropharyngeal bacterial composition and innate immune response, adaptive immune response and atopic indices (serum IgE levels). An oropharyngeal swab was used as it is a non-invasive method and sampling creates little discomfort and also oropharyngeal microbiota have been successfully used to investigate the upper airway microbiome.[221, 222] This study contributes to an understanding of the Western environment-microbiome-chronic inflammation paradigm. The findings provide valuable insights into the treatment and prevention strategies for chronic inflammatory conditions such as asthma and allergy.

7.3 Methods

7.3.1 Study participants and design

We initiated a study on asthma and allergy in Chinese immigrants in Australia[153] in 2012. This current study is part of a large Chinese immigrant study and the recruiting method has been described elsewhere.[154] The participates were recruited from the local Chinese community living in Perth, Western Australia by advertisements through Chinese media− eg. radio and newspaper. Twenty-two newly-arrived Chinese immigrants (living in Australia <6 months) and 22 long-term adult Chinese immigrants (living in Australia >5 years) were recruited with age and gender matched over three month period (Nov 2014 to Feb 2015). We excluded participants that used antibiotics or had infections in the last 4 weeks. Most pairs of newly arrived and long-term Chinese immigrants were recruited on the same day and a few pairs were recruited within a two- or three-day days of each other. Oropharyngeal swab samples, serum, and whole blood samples were collected on the recruitment day. This study design followed the principles of the Declaration of Helsinki and was approved by the Human Research Ethics Committee of Western Australia (RGS0000002399), at Curtin University (HR110/2013), and University of Western Australia (RA/4/1/6763). All participants gave informed written consent prior to participation. Oropharyngeal swabs were obtained using a sterile cotton swab which were immediately placed into a sterile collection tube. The swabs were sent to our laboratory on dry ice and kept frozen at −80 °C freezer until further processing. A study overview is shown in Figure 7-1 A).

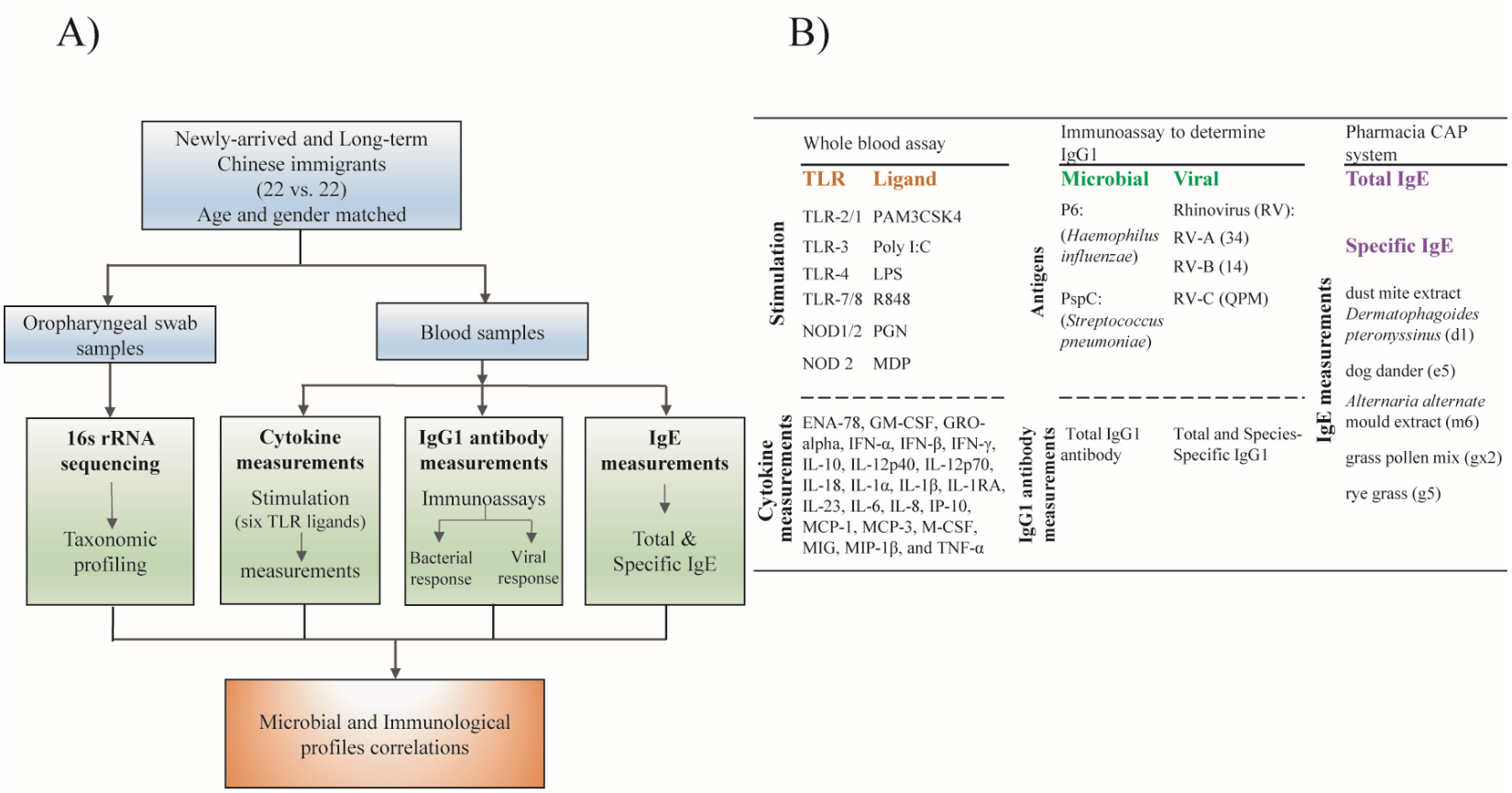


Figure 7-1. A whole study overview

A) Study workflow. **B)** The immune response measurements

7.3.2 High-throughput 16S rRNA gene paired-end sequencing

DNA was extracted from pharyngeal swab samples using the QIAamp DNA Microbiome Kit (QIAGEN, cat#51704) following the manufacturer's guidelines. PCR amplification and sequencing were performed by the Australian Genome Research Facility (AGRF). Briefly, universal 16S ribosomal RNA (rRNA) gene primers were used to amplify the V3–V4 region of the bacterial 16S rRNA gene of each sample, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia) for the primary PCR. A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The cycling conditions consisted of 95 ℃ for 7 min; 29 cycles of 94 ℃ for 30 s, 50 ℃ for 60 s, and 72 ℃ for 60 s; and then final extension at 72 ℃ for 7 min. The resulting amplicons were quantified using the fluorescent PicoGren assay and measured by fluorometry (Invitrogen Picogreen) and normalised. The eqimolar pool was then measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry. Negative (HPLC water) and positive (the BEI 16S gDNA mock community 782D) controls, as well as all samples, were sequenced on the same batch.

7.3.3 Bioinformatics analysis

The output paired-end reads were merged using PANDASeq assembler[223] with the parameters at a minimum of 400 bp length and maximum of 500 bp length, and then analysed by downstream computational pipelines of the open source software package Quantitative Insights Into Microbial Ecology (QIIME v1.9.1).[146] Chimera checking was performed with the usearch61 algorithm.[224] Sequences were clustered into Operational Taxonomic Units (OTUs) by UCLUST method[225] using the open-reference OUT-picking workflow pipelines against SILVA reference database (128

release), and a 97% similarity threshold. OTUs with abundance below 0.005% of the total number of sequences were discarded.[148] A total of 2,516,134 sequences that passed the quality check were used in further analysis. An even depth of 2,289 sequences per sample was used for alpha and beta diversity as all samples had at least this number of sequences. Alpha diversity was estimated using the chao1 richness estimate and Shannon index. Beta diversity was measured using the Bray-Curtis distance metric.

7.3.4 Cytokine, IgG1 response to stimulation/antigens, and Immunoglobulin E (IgE) measurement

Twenty-three cytokines, representing the TLR innate immune response, were measured *ex vivo* using whole blood assay under stimulation of six TLR ligands (Figure 7-1 B). IgG1 responses to bacterial and viral antigens (Figure 7-1 B) that represent the adaptive immune response were tested using immunoassays for total IgG1 and immunoabsorption assays for species-specific IgG1 antibody binding. The detailed method was described in a previous paper.[197] The Pharmacia CAP system (Pharmacia Diagnostics AB, Uppsala, Sweden) was used to assess total and specific IgE (Figure 7-1 B) from serum samples at the PathWest Immunology Department (QEII Medical Centre, Perth, Australia). The specific IgE tests included house dust mite extract *Dermatophagoides pteronyssinus* (d1), dog dander (e5), *Alternaria alternate* mould extract (m6), grass pollen mix (gx2) and rye grass (g5).

7.3.5 Statistical analysis

The relative abundance of bacterial taxa in samples from newly-arrived and long-term Chinese immigrants were compared using the Mann-Whitney U test (Statistical Package Social Science, SPSS, version 25.0). The linear discriminant analysis (LDA)

effect size (LEfSe) method[152] was used to compare the microbial composition of the two Chinese immigrant groups. The compare_alpha_diversity.py script was used to compare the alpha diversity (within-sample diversity) metrics in the QIIME pipeline, which implements a nonparametric two-sample t-test with 999 Monte Carlo permutations. Beta diversity (between-sample diversity) comparisons were completed using analysis of similarities (ANOSIM) (compare_categories.py; QIIME). A value of $p < 0.05$ was considered statistically significant.

Principal component analysis (PCA) was used to extract the first PC representing the major variation of the six cytokine measurements (stimulated by six TLR ligands) for each of the 23 cytokines. The 23 cytokine PC1 scores were correlated with microbial taxa (over 1.0%) using Spearman correlation test. The microbial taxa (over 1.0%) were also correlated with IgG1 antibody responses to microbial and virus pathogens, and total and specific IgE for newly-arrived and long-term immigrants, respectively.

We also calculated the Spearman correlation of each microbial taxa (over 1.0%) with 138 cytokine measurements (23 cytokines * six TLR ligands) in those two immigrant groups, respectively. The correlation coefficients (ρ) calculated in long-term versus newly-arrived immigrants, respectively, were treated as a pair. Among the 138 pairs (as we have 138 cytokine measurements), those pairs with a ρ coefficient that is significant ($p < 0.05$) either in long-term or newly-arrived immigrants were selected. The Spearman ρ represents the correlation strength between taxa and innate immune response. A paired sample *t* test was used to test the difference in the strength of these correlations between the two immigrant groups in different taxonomic levels. All the *p* values were adjusted with Benjamini-Hochberg false discovery rate (FDR) correction.

7.4 Results

7.4.1 Oropharyngeal microbiota composition and diversity

Eleven bacterial phyla were detected in the oropharyngeal microbiome of newly-arrived and long-term Chinese immigrants (Figure 7-2 A, Supplemental Table S7-1). Firmicutes was dominant in both groups, comprising 54.3% and 53.4% of sequences in newly-arrived and long-term Chinese immigrants, respectively, followed by Actinobacteria, and Proteobacteria. At the genus level (Supplemental Figure S7-1, Supplemental Table S7-2) there were 20 genera that account for 89.7% and 88.1% of the two groups respectively, with a minimum relative abundance of ≥1.0%. The relative abundance of Fusobacteria in newly-arrived immigrants (2.6%) was significantly lower than that of long-term immigrants (4.8%) ($p< 0.01$; FDR-corrected $p= 0.066$).

To further compare the microbial composition of long-term Chinese immigrants and newly-arrived Chinese immigrants, LefSe was used (Figure 7-2 B, threshold of LDA score: 2). The relative abundance of the genus *Leptotrichia* was higher among long-term immigrants, compared to that of newly-arrived Chinese immigrants. However, the abundance of genus *Deinococcus* was significantly lower in long-term immigrants Chinese immigrants. (Figure 7-2 B).

There was no significant difference in alpha diversity indices between the newly-arrived and long-term Chinese immigrants (Supplemental Table S7-3 and Figure 7-2 C). The Bray-Curtis distance measurer was applied to examine differences in bacterial community composition and structure between the newly-arrived and long-term Chinese immigrants (Figure 7-2 D). No significant difference was observed in beta diversity according to the Bray-Curtis distance measure (ANOSIM, $p= 0.431$).

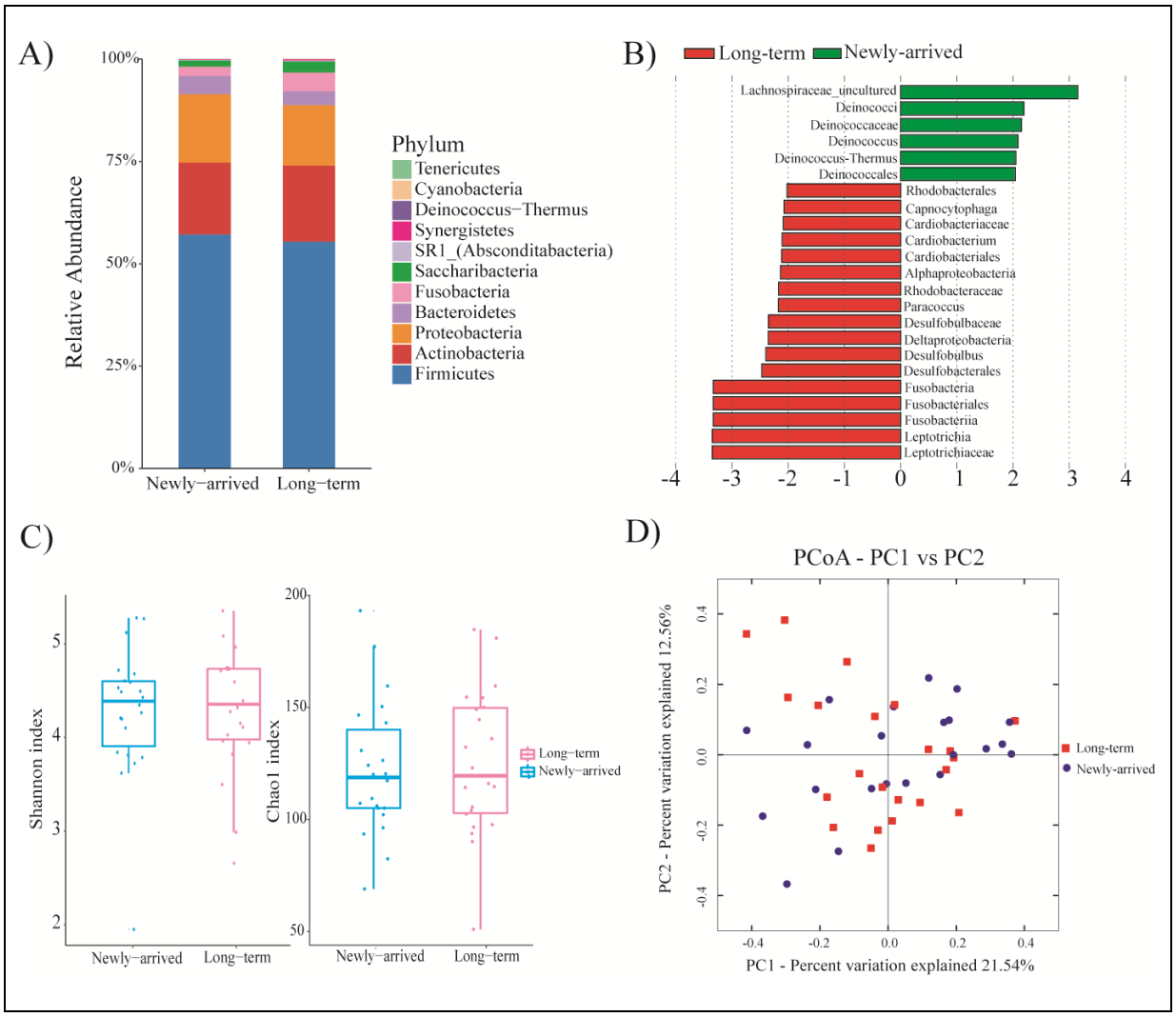


Figure 7-2. Comparison of the composition and diversity of newly-arrived and long-term Chinese immigrants

A) The relative abundance of identified phyla in newly-arrived and long-term Chinese immigrants. The Y-axes represent relative phylum abundance and the x-axes represent sampling cohort, either from newly-arrived or long-term Chinese immigrants. **B) LefSe analysis of microbiome changes.** Taxa enriched in newly-arrived Chinese immigrants are indicated with a positive LDA score (green), and taxa enriched in long-term Chinese immigrants have a negative score (red). Only taxa meeting an LDA significant threshold of >2 are shown. **C) Alpha diversity analysis among samples.** Box-and-whisker plots of the Alpha diversities are exemplified by the Shannon and Chao1 index between newly-arrived (blue) and long-term (pink) Chinese immigrants. No difference was observed in Shannon and Chao1 indices. **D) Beta diversity analysis among samples was carried out according to the Bray-Curtis distance.** Data points represent either long-term Chinese immigrant samples (red square) or new-arrived Chinese immigrants samples (blue dots), and the two major principle components are respectively represented on the x- and y- axes.

7.4.2 Oropharyngeal microbial taxa are significantly associated with immunological parameters

A Spearman correlation was used to test for the association of bacterial taxa with innate immune responses, adaptive immune responses and IgE levels. Figure 7-3 summarizes the results with a significance ($p< 0.1$, the p values have been adjusted for multiple comparison). In long-term Chinese immigrants, 25 taxa significantly correlated with at least one of these immunological parameters, namely, cytokine IL-6, IgG1 binding to bacterial antigens, and IgE, with majority of them showing negative correlations. In newly-arrived Chinese immigrant, seven taxa were significantly correlated with at least one immunological parameters, namely, IgG1 binding to virus antigens and IgE, with majority of them demonstrating positive correlations.

Interestingly, negative microbial taxa correlations with PC1s of cytokine IL-6 were found exclusively significant among 11 taxa in long-term immigrants (rho range: -0.60 ~ -0.72). Further, Spearman correlation tests were also conducted between these microbial taxa and cytokine IL-6 stimulated by six TLR ligands (rho range: -0.46 ~ -0.73, Figure 3 A). The taxa *Lachnospiraceae_uncultured* (Firmicutes), *Erysipelotrichaceae UCG-007* (Firmicutes), *Veillonella* (Firmicutes), and *Actinomycetales_ambiguous taxa* (Actinobacteria) were negatively correlated with IL-6 stimulated by TLR ligands of Poly I:C, R848 and/or LPS.

In long-term Chinese immigrants the genera *Oribacterium* (Firmicutes), *Neisseria* (Proteobacteria) and *Porphyromonas* (Bacteroidetes) had a negative correlation with IgG1 antibody responses to bacterial antigen P6 or PspC, whereas *Prevotella 7* (Bacteroidetes) was positively correlated with P6 (Figure 7-3 A). In newly-arrived Chinese immigrants the phylum Bacteroidetes was positively correlated with viral

specific antigen to RV-A, but the order Actinomycetales (Actinobacteria) was negatively correlated with viral total antigen to RV-C (Figure 7-3 B).

A total of 12 taxa were significantly negatively correlated with total IgE or specific IgE to dog dander, while only the genus *Neisseria* was positively correlated with mould extract in long-term immigrants. The genus *Saccharibacteria_Ambiguous* was negatively correlated with total IgE in long-term immigrants, but the phylum Saccharibacteria was positively associated with total IgE, specific IgE for grass pollen mix and rye grass in newly-arrived immigrants (Figure 7-3).

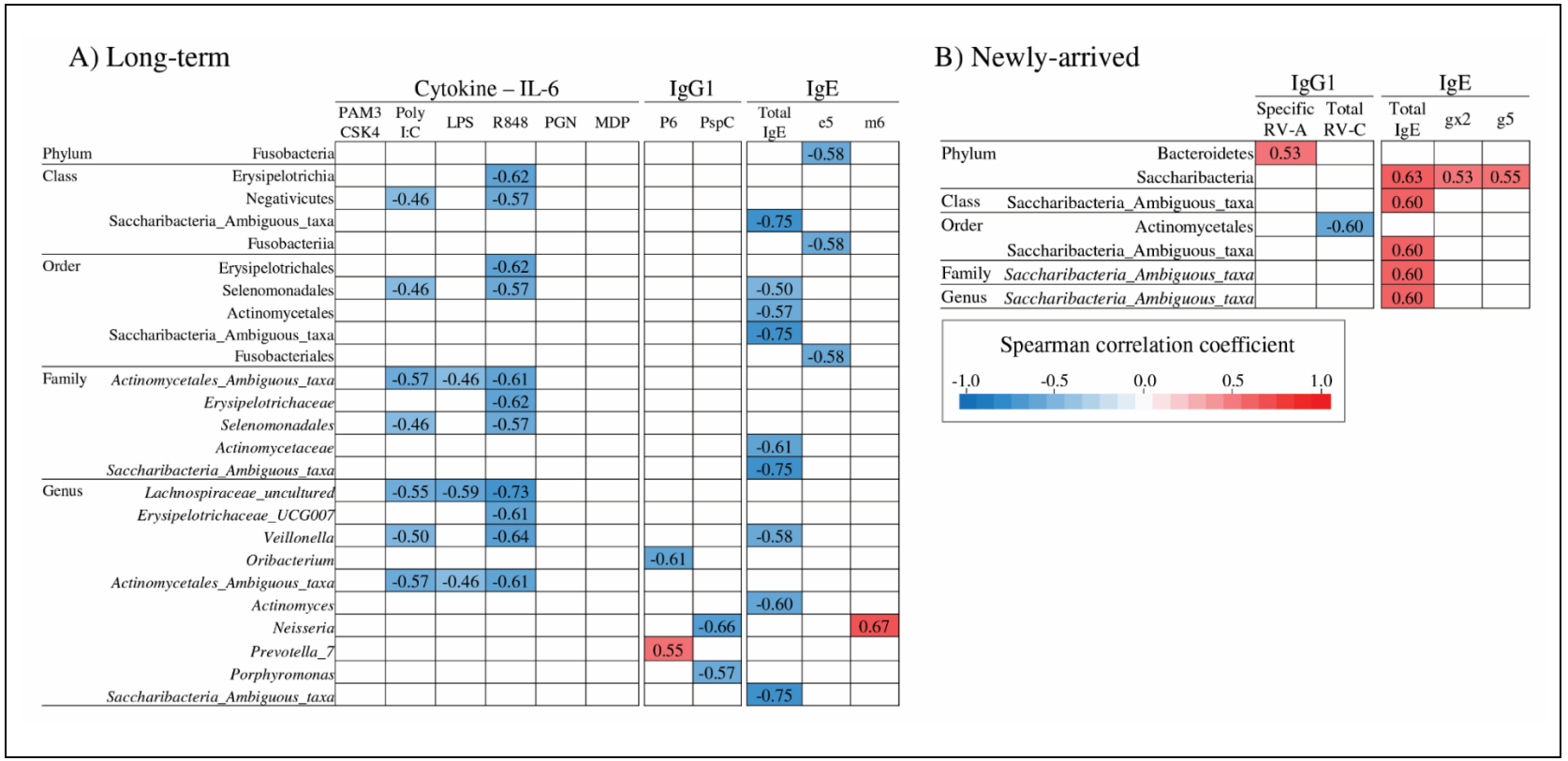


Figure 7-3. Significant correlation between oropharyngeal microbial taxonomies and immunological parameters (cytokine response, IgG1 antibody response, and IgE levels) among long-term Chinese immigrants (A) and newly-arrived Chinese immigrants (B).

Spearman correlation test was used with Benjamini-Hochberg FDR correction (FDR-corrected $p < 0.1$). All taxa had a minimum relative abundance of ≥ 1.0% and only the taxa which had a significant correlation with at least one immunological parameters are displayed.

7.4.3 Different microbial taxonomies and cytokine correlation comparison between long-term and newly-arrived Chinese immigrants

The paired-sample *t* test was used to investigate the correlation strengths between these bacterial taxa and the overall innate immune response (represented by the 138 cytokine measurements) in newly-arrived and long-term Chinese immigrants, respectively (Supplemental Table S7-4). Correlation strength of bacteria with overall innate response was significantly different between newly-arrived and long-term Chinese immigrants after adjusting for multiple comparisons. Correlation trends for the genera *Gemella* (Firmicutes), *Neisseria* (Proteobacteria) and *Kingella* (Proteobacteria) were positive in newly-arrived Chinese immigrants, but negatively associated with long-term immigrants. Conversely, *Prevotella 7* (Bacteroidetes) and *Saccharibacteria_Ambiguous* (Saccharibacteria) had negative correlation with the innate-immunity of newly-arrived Chinese immigrants, but positive correlation with that of long-term immigrants (Figure 7-4).

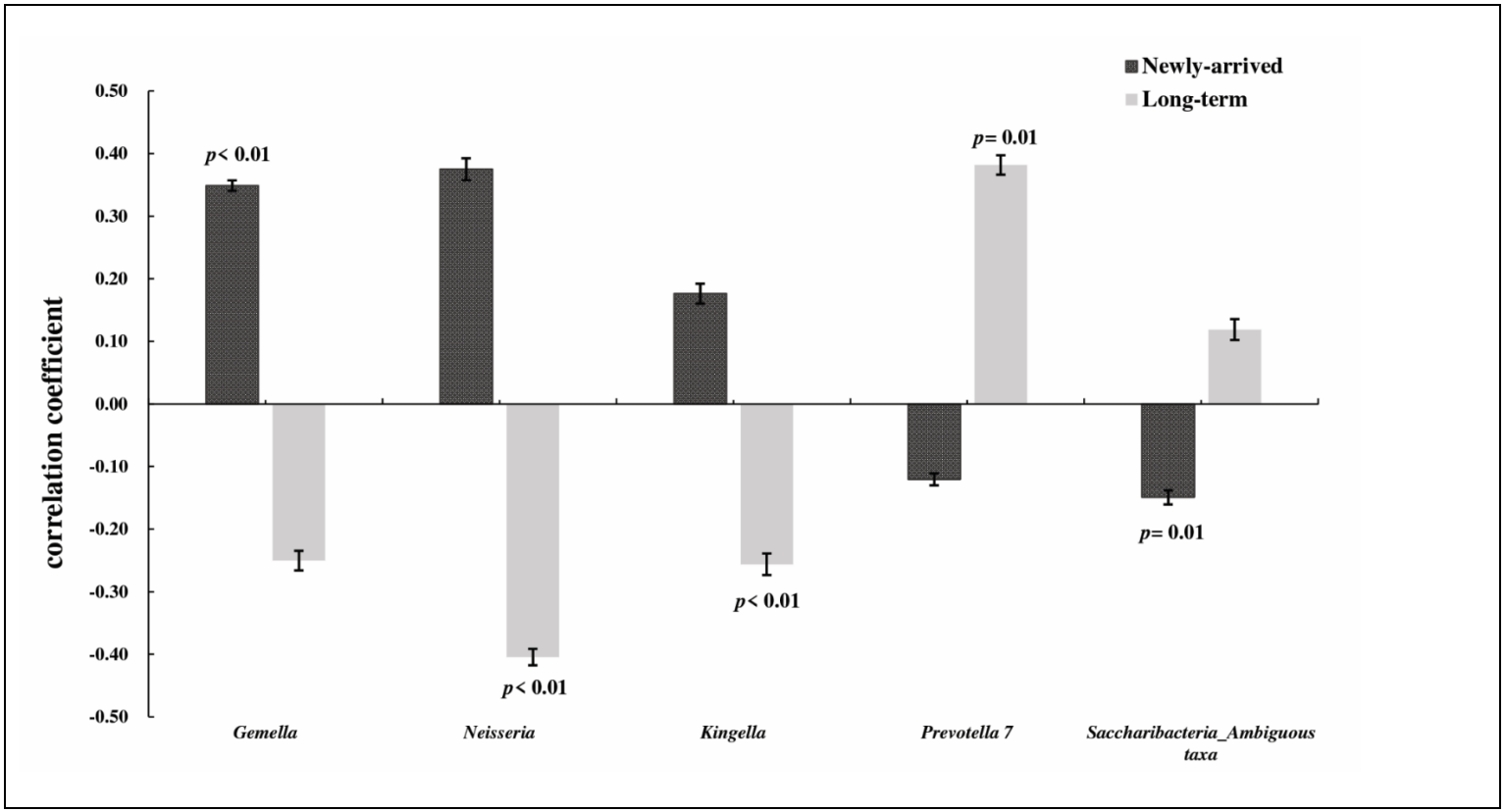


Figure 7-4. Comparison of the correlation strength of bacterial genera (over 1.0%) and the overall innate immune response (138 cytokine measurements) between newly-arrived and long-term Chinese immigrants

Y-axes represents the spearman correlation coefficients. The paired-sample t test was used to test the correlation strength difference. Only genera significantly different between the two groups were displayed.

7.5 Discussion

To our knowledge this is the first study to investigate the oropharyngeal microbiome and its correlation with the immune response and IgE sensitization in an ethnically homogeneous immigrant population in Australia. In this study we use the phrase 'Western environment' as a collective term to denote the socio-cultural, physical and geographical environment in developed countries, such as the United States and Australia, and the 'Eastern environment' as a collective term to denote developing counties, such as China. Our analysis indicates that the variation of oropharyngeal microbial composition existed between newly-arrived (exposed to an Eastern environment) and long-term (exposed to a Western environment) Chinese immigrants. It is evident that the oropharyngeal microbiome had significant correlations with immune response and atopic sensitization, with distinctive association profiles present in newly-arrived and long-term Chinese immigrants.

The increased prevalence of allergic diseases in Westernised countries suggests that environment plays a vital role.[226] In this cohort, long-term Chinese immigrants living in Australia with a Western environment had more allergic symptoms compared with age and gender matched newly-arrived Chinese immigrants.[197] The increased allergy in long-term Chinese immigrants reported in our population and the substantial West versus East gradient of allergy prevalence between developed and developing countries indicates that the Western environment/lifestyle is the cause of allergic conditions. How or through what intermediates the Western environment causes allergies is poorly understood. With a population of 100 trillion cells, the human microbiome outnumbers human cells by at least a factor of ten.[227] A rising understanding of the fundamental role of the microbiota in the immune system has taken a centre stage in the field of immunology in decades. It

is envisaged that environmental-host-microbial interaction should be critical for the development of allergic diseases. We surmise that the Western environment in Australia has altered the oropharyngeal microbiome in long-term Chinese immigrants, which leads to modifications of their innate and adaptive immunity, resulting in the development of allergies.

Our study found that individual pharynx microbiomes were dominated by phyla Firmicutes, Actinobacteria or Proteobacteria in Chinese immigrants, which is consistent with previous reports.[228, 229] Some differences in microbiome profiles between newly-arrived and long-term Chinese immigrants were found, which may partly be attributed to the Western vs Eastern environmental influence. The relative abundance of the genus *Leptotrichia* (Fusobacteria) was higher among long-term Chinese immigrants, compared to newly-arrived Chinese immigrants. The genus *Leptotrichia* is an anaerobic, Gram negative bacillus and part of the normal human oral flora. It may occasionally causes disease in immunocompromised hosts.[230] An asthma-related study reported that *Leptotrichia* was commonly detected in patients with corticosteroid-resistant asthma.[231] A recent study showed that *Leptotrichia* was strongly associated with mite sensitization as well as asthma.[232] In contrast to the Gram-negative *Leptotrichia*, *Deinococcus*, which is a Gram-positive genus, decreased in their relative abundance in long-term Chinese immigrants. *Deinococcus* is highly resistant to environmental hazards and its association with disease is rarely investigated, although there are a few studies that investigated its immunogenicity.[233, 234] Considering the difference between the environments in Australia and China and the large time difference (10 years) of residence in Australia, changes in the microbiome are expected. However, limited sample size and statistical power have precluded the identification of more taxa with a difference that is statistically significant after adjusting for the multiple comparisons. It is expected that a

comparison study with a large sample size will identify more bacteria that have significantly different relative abundances between newly arrived and long-term immigrants.

Microbiome diversity has been associated with asthma and allergy.[235, 236] De Filippo et al [205] found that the Chao1and Shannon's index in gut microbiome from Burkina Faso were higher ($p < 0.01$) than in EU samples at the Operational Taxonomic Unit (OTU) cutoff 0.10. Lin et al [237] found Bangladeshi children exhibited greater gut microbiome diversity than the U.S. children. These two studies may support the assumption that the Western environment (such as Europe and USA) has a decreased microbiome diversity, which may affect the development of asthma and allergy in Western countries. We initially hypothesized Chinese immigrants would have decreased airway microbiome diversity after living in the Western environment for a sufficient period of time. However, our result show no difference between newly-arrived and long-term Chinese immigrants in alpha and beta diversity of oropharyngeal microbiota. Therefore, this initial hypothesis was not confirmed. However, considering that the 16S sequencing method is measuring the relative abundance rather than absolute taxa, our findings do not conflict with the assumption that there may be less microbiome taxa in terms of copy numbers of bacteria existing in the human pharynx, in the long-term relative to newly-arrived Chinese immigrants. The assumption was based on the premise that hygienic environments are more prevalent in Western countries relative to Eastern countries. The copy numbers of bacterial taxa in the human pharynx need to be further investigated with a focus on comparing Western versus Eastern environments.

The immune system is made up of a complex network of innate and adaptive responses capable of adapting to highly-diverse challenges.[238] The innate immune system is the first line of defence against pathogens and acts by detecting conserved microbial

structures through TLRs. Cumulative studies revealed, in Westernised countries, changes in diets/lifestyle, overuse of antibiotics, removal of parasitic infections, may have resulted in a lack of microbiome resilience and diversity to establish balanced immune responses.[218] Our previous study indicated that long-term Chinese immigrants had attenuated innate cytokine responses.[154] Present study found a significant correlation between the relative abundance of several bacterial and immune response measurements, such as TLR pathway cytokines, specific IgE and bacterial or virial antibody response. This indicates that the human microbiome has an influence on host immune response. In long-term Chinese immigrant IL6 was negatively correlated with several bacterial genera from the phyla Firmicutes and Actinobacteria. The correlations were more apparent for IL-6 stimulated by viral (TLR3, TLR7/8) and/or Gram-negative bacterial (TLR4) ligands. In a cross-sectional investigation it is not possible to determine a cause-effect relationship. A further investigation is warranted to determine if these bacteria have inhibited the whole blood IL-6 response in the TLR pathway or if the IL-6 response has prohibited the proliferation of these taxa in Chinese immigrants living in a Western environment for a significant period. Importantly, the present study has identified a convincing link between the oropharyngeal microbiome and innate and adaptive immunity in the Chinese immigrant population. It should be highlighted that these interactions of the microbiome and the overall innate immune responses are distinctly different in newly-arrived and long-term Chinese immigrants. Considering our previous findings that a stronger positive correlation between innate and adaptive responses were apparent among newly-arrived Chinese immigrants[197], we currently demonstrate that genera from the dominant phyla (Firmicutes, and Proteobacteria) were positively correlated with the overall innate immune response among newly-arrived Chinese immigrant and conversely with negative correlation in long-term immigrants. These complex interactions, which may occur in either direction, between human microbiomes

and innate and adaptive immune response in a Western versus Eastern environment is expected to account for the uneven distribution of asthma and allergies in Western and Eastern world.

One of the limitations of the study is the sample size. After adjusting for multiple comparisons, only a few taxa were found to have a significant difference between newly-arrived and long-term Chinese immigrants. However, in such a study with a relatively small sample size we have convincingly identified the link between human microbiomes and immune response after we have strictly adjusted for multiple tests. This suggests a robust relationship between the microbiome and immune response. Secondly, this is a cross-sectional study that cannot infer a cause-effect relationship. A longitudinal study on newly arrived Chinese immigrants in a Western country that continually scrutinises the changes in diets, microbiomes, and immune responses for several years would be ideal. Follow-up studies are required to clarify the causal relationship between microbiome modifications and immunity changes in immigrants with the western environment.

7.6 Conclusions

The relative abundance of the genus *Leptotrichia* was higher among long-term immigrants, compared to that of newly-arrived Chinese immigrants. However, the abundance of the genus *Deinococcus* was significantly lower in long-term Chinese immigrants. Specific microbial taxa are significantly associated with immunological parameters but with different association patterns between newly-arrived and long-term Chinese immigrants. In addition, the correlations between the microbiome and the overall innate immune responses are distinctly different in newly-arrived and long-term Chinese immigrants. There is a strong bond between oropharyngeal microbiome and host immune

response. Our study identifies a novel avenue to determine the associations of these changes in human microbiota with common chronic diseases such as asthma and allergy in the Western environment.

7.7 Supplemental information

7.7.1 Supplemental tables

Supplemental table S 7-1. The phylum level of oropharyngeal microbiome compared between newly-arrived and long-term Chinese immigrants

Phylum	Test-Statistic	*FDR_P*[*]	Newly-arrived immigrants	Long-term immigrants
Firmicutes	0.243	0.781	54.32%	53.42%
Actinobacteria	0.344	0.781	17.71%	18.34%
Proteobacteria	0.220	0.781	18.17%	16.81%
Fusobacteria	7.542	0.066	2.63%	4.80%
Bacteroidetes	0.009	0.925	3.95%	3.49%
Saccharibacteria	0.838	0.779	2.19%	2.50%
Synergistetes	1.620	0.745	0.60%	0.39%
SR1 (Absconditabacteria)	0.637	0.779	0.31%	0.20%
Cyanobacteria	0.065	0.878	0.02%	0.04%
Tenericutes	0.808	0.779	0.01%	0.00%
Deinococcus-Thermus	4.065	0.241	0.09%	0.00%

*: The p-values were FDR-corrected to control for multiple testing.

Supplemental table S 7-2. The genus level of oropharyngeal microbiome compared between newly-arrived and long-term Chinese immigrants

Genus	Test-Statistic	*FDR_P**	Newly-arrived immigrants	Long-term immigrants
Streptococcus (Firmicutes; Bacilli; Lactobacillales; Streptococcaceae)	0.014	0.953	29.55%	31.07%
Rothia (Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae)	0.926	0.715	9.76%	9.93%
Veillonella (Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae)	0.600	0.763	9.69%	8.38%
uncultured (Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae)	0.267	0.853	6.16%	5.02%
Leptotrichia (Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae)	6.308	0.398	2.38%	4.61%
Haemophilus (Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae)	0.108	0.895	3.84%	4.25%
Actinomyces (Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae)	0.014	0.953	4.08%	4.22%
Neisseria (Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae)	0.754	0.755	6.56%	3.29%
Gemella (Firmicutes; Bacilli; Bacillales; Family XI)	1.607	0.715	1.79%	3.05%
Ambiguous_taxa (Saccharibacteria; Ambiguous_taxa; Ambiguous_taxa; Ambiguous_taxa)	0.564	0.767	1.99%	2.28%
Oribacterium (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	0.055	0.936	2.07%	2.09%

Ambiguous_taxa (Actinobacteria; Actinobacteria; Actinomycetales; Ambiguous_taxa)	2.548	0.649	0.69%	2.04%
Kingella (Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae)	1.110	0.715	0.21%	1.66%
Granulicatella (Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae)	1.166	0.715	1.91%	1.56%
Porphyromonas (Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae)	1.141	0.715	1.00%	1.29%
Prevotella 7 (Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae)	0.927	0.715	1.89%	1.13%
uncultured (Firmicutes; Clostridia; Clostridiales; Lachnospiraceae)	5.446	0.398	2.04%	0.63%
Corynebacterium 1 (Actinobacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae)	1.237	0.715	1.54%	0.38%
Erysipelotrichaceae UCG-007 (Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae)	1.886	0.715	1.52%	0.92%
Selenomonas 3 (Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae)	0.007	0.957	1.06%	0.29%

*: The p-values were FDR-corrected to control for multiple testing.

Supplemental table S 7-3. Alpha diversity metrics for pharyngeal swab samples collected from newly arrived and long-term Chinese immigrants. Values represent mean ± SD

	Newly-arrived	Long-term	*p* value
Observed OTUs	83.58±11.86	92.03±18.56	0.094
Chao1 richness estimate	121.82±29.06	124.97±31.72	0.732
Faith's phylogenetic diversity	3.60±1.44	3.63±1.45	0.943
Shannon's index	4.27±0.68	4.29±0.64	0.907
Simpson’s index	0.88±0.10	0.89±0.09	0.820

Supplemental table S 7-4. The comparison of taxa correlations with innate immune response between newly-arrived and long-term Chinese immigrants (paired sample t test)

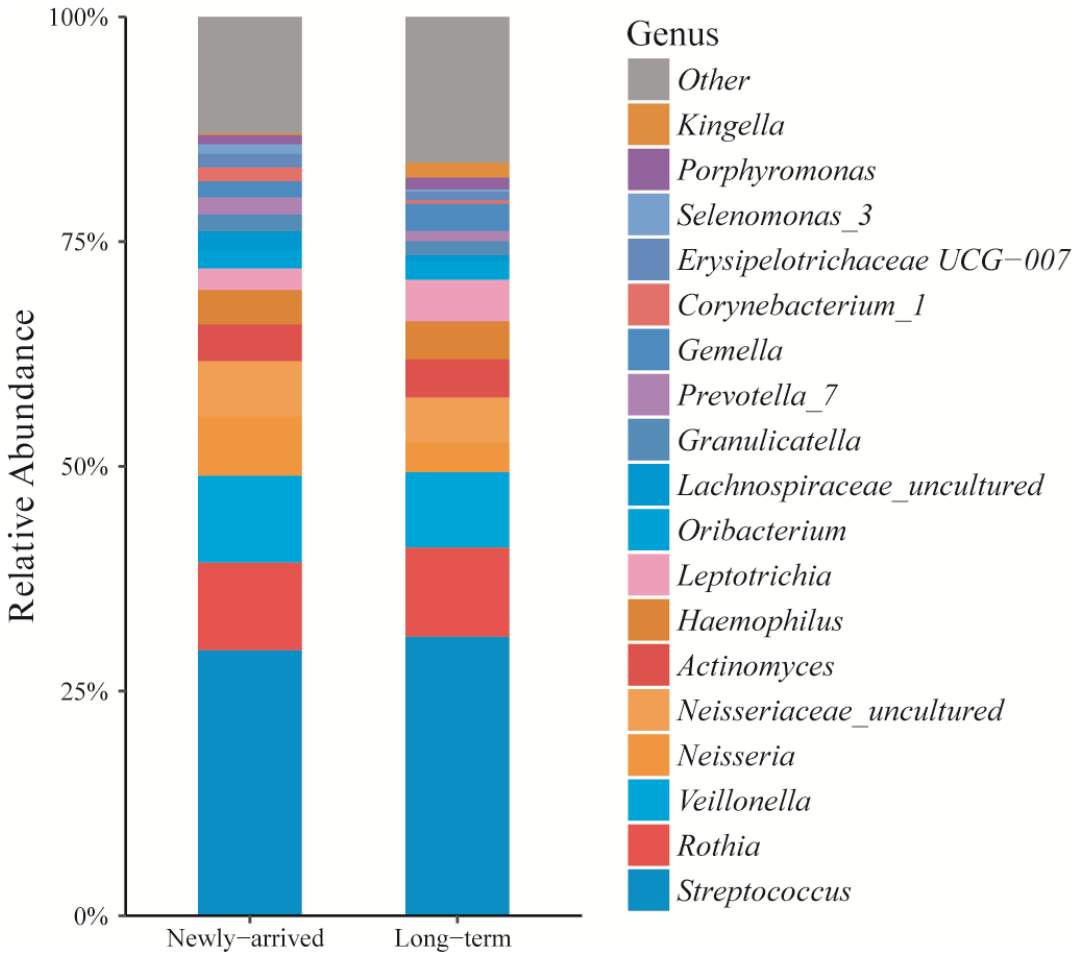
	Taxa	number of test	mean of ρ newly-arrvied Immigrants	mean of ρ long-term Immigrants	mean difference of ρ	*p*	q^*
Phylum	Proteobacteria	5	0.45	-0.11	0.55	<0.01	<0.01
	Bacteroidetes	10	0.37	0.03	0.33	0.01	0.01
	Saccharibacteria	20	-0.11	0.16	-0.27	0.01	0.01
	Firmicutes	8	-0.06	-0.18	0.12	0.21	0.31
	Actinobacteria	11	-0.21	-0.01	-0.19	0.28	0.33
	Fusobacteria	3	0.10	0.19	-0.10	0.77	0.77
Class							
	Bacilli	8	0.05	-0.27	0.32	0.01	0.11
	Betaproteobacteria	10	0.37	0.01	0.37	0.02	0.11
	Actinobacteria	8	0.17	-0.06	0.23	0.30	0.66
	Coriobacteriia	13	-0.23	-0.04	-0.19	0.24	0.66
	Erysipelotrichia	30	-0.29	-0.17	-0.12	0.29	0.66
	Bacteroidia	8	0.02	0.15	-0.13	0.58	0.91
	Clostridia	3	-0.32	-0.04	-0.28	0.53	0.91
	Negativicutes	16	-0.27	-0.26	-0.02	0.90	1.00
	Fusobacteriia	3	0.10	0.19	-0.10	0.77	1.00
Order							
	Bacillales	23	0.34	-0.25	0.59	<0.01	<0.01
	Neisseriales	15	0.34	-0.03	0.38	<0.01	0.01
	Pasteurellales	3	0.04	0.49	-0.44	<0.01	0.01
	Saccharibacteria_Ambiguous_taxa	26	-0.15	0.12	-0.27	<0.01	0.01
	Corynebacteriales	8	0.18	-0.24	0.43	0.03	0.09
	Micrococcales	10	0.31	-0.05	0.35	0.07	0.15
	Coriobacteriales	13	-0.23	-0.04	-0.19	0.24	0.47

	Taxa	number of test	mean of ρ newly-arrvied Immigrants	mean of ρ long-term Immigrants	mean difference of ρ	*p*	q^*
	Lactobacillales	3	-0.05	-0.14	0.08	0.30	0.47
	Erysipelotrichales	30	-0.29	-0.17	-0.12	0.29	0.47
	Clostridiales	3	-0.32	-0.04	-0.28	0.53	0.73
	Bacteroidales	8	0.02	0.15	-0.13	0.58	0.73
	Actinomycetales	14	-0.27	-0.30	0.03	0.79	0.85
	Fusobacteriales	3	0.10	0.19	-0.10	0.77	0.85
	Selenomonadales	16	-0.27	-0.26	-0.02	0.90	0.90
Family							
	Family_XI	21	0.35	-0.25	0.60	<0.01	<0.01
	Neisseriaceae	15	0.34	-0.03	0.38	<0.01	0.01
	Pasteurellaceae	3	0.04	0.49	-0.44	<0.01	0.01
	Saccharibacteria_Ambiguous_taxa	26	-0.15	0.12	-0.27	<0.01	0.01
	Actinomycetaceae	15	-0.36	-0.05	-0.31	0.01	0.05
	Prevotellaceae	17	-0.04	0.28	-0.33	0.02	0.05
	Corynebacteriaceae	8	0.18	-0.24	0.43	0.03	0.08
	Actinomycetales_Ambiguous_taxa	10	-0.04	-0.40	0.36	0.04	0.08
	Micrococcaceae	10	0.31	-0.04	0.35	0.07	0.13
	Carnobacteriaceae	10	0.29	0.10	0.18	0.16	0.29
	Coriobacteriaceae	13	-0.23	-0.04	-0.19	0.24	0.39
	Erysipelotrichaceae	30	-0.29	-0.17	-0.12	0.29	0.44
	Porphyromonadaceae	5	0.17	0.40	-0.23	0.37	0.51
	Streptococcaceae	3	-0.08	-0.14	0.06	0.42	0.54
	Leptotrichiaceae	3	0.24	-0.02	0.26	0.51	0.61
	Lachnospiraceae	6	-0.16	-0.29	0.13	0.60	0.68
	Peptostreptococcaceae	5	0.20	0.28	-0.09	0.72	0.77
	Veillonellaceae	16	-0.27	-0.26	-0.02	0.90	0.90
Genus							
	Gemella	21	0.35	-0.25	0.60	<0.01	<0.01
	Neisseria	14	0.38	-0.40	0.78	<0.01	<0.01
	Prevotella_7	18	-0.12	0.38	-0.50	<0.01	<0.01

Taxa	number of test	mean of ρ newly-arrvied Immigrants	mean of ρ long-term Immigrants	mean difference of ρ	*p*	q^*
Kingella	18	0.18	-0.26	0.43	<0.01	0.01
Saccharibacteria_Ambiguous_taxa	26	-0.15	0.12	-0.27	<0.01	0.01
Rothia	12	0.36	-0.03	0.39	0.02	0.08
Actinomycetales_Ambiguous_taxa	10	-0.04	-0.40	0.36	0.04	0.10
Actinomyces	17	-0.34	-0.09	-0.24	0.04	0.10
Lachnospiraceae_uncultured	12	-0.13	-0.42	0.29	0.09	0.19
Corynebacterium_1	6	0.32	-0.02	0.35	0.19	0.37
Veillonella	17	-0.24	-0.07	-0.17	0.20	0.37
Lachnospiraceae_uncultured	8	0.14	0.32	-0.18	0.30	0.50
Streptococcus	3	-0.08	-0.14	0.06	0.42	0.65
Erysipelotrichaceae_UCG007	28	-0.27	-0.19	-0.08	0.49	0.68
Leptotrichia	3	0.24	-0.02	0.26	0.51	0.68
Haemophilus	3	0.03	0.28	-0.25	0.63	0.79
Atopobium	5	-0.17	-0.25	0.08	0.79	0.83
Porphyromonas	7	0.32	0.36	-0.04	0.83	0.83
Granulicatella	16	0.04	0.08	-0.04	0.74	0.83
Oribacterium	5	0.07	0.17	-0.10	0.75	0.83

*: The *q* values were FDR-corrected *p* values to control for multiple testing.

7.7.2 Supplemental figure



Supplemental figures S 7-1. The relative abundance of identified genera in newly-arrived and long-term Chinese immigrants

8 Study Four—Toll-like receptor signalling has inverted U-shaped response over time with the Western environment

In this chapter, we investigated the time dependence of the Western environment on the expression of toll-like receptor pathway genes, plasma cytokine levels, and total and specific immunoglobulin E (IgE) in the Chinese immigrants cohort. The findings of this chapter provide a potential underlying mechanism for asthma, allergy and other chronic diseases that show an increased prevalence in Western countries.

8.1 Abstract

Immigrants switching from an Eastern to a Western environment experience an epidemiologic transition after living in the new environment for a prolonged period, and gradually approximate the disease morbidity pattern of the local population. In this study, we investigated the time dependence of the Western environment on the expression of toll-like receptor (TLRs) pathway genes, plasma cytokine levels, and total and specific immunoglobulin E (IgE) in Chinese immigrants. We found that TLRs pathway genes had an inverted U-shaped response and total IgE and specific IgE had a quadratic increase as a function of residence time in Australia. The findings of our study provide a potential underlying mechanism for asthma, allergy and other chronic diseases that show an increased prevalence in Western countries.

8.2 Introduction

Asthma, and allergy are distributed disproportionately globally and are more common in industrialized than non-industrialized countries [101, 239]. A mismatch between evolved human physiology and the modern Western lifestyle and environment is believed to contribute to this geographical disease pattern [240]. A systematic review of immigrants concluded that if the prevalence of asthma and allergic diseases is lower in the country of origin than the host country, it will in time gradually approach the prevalence of those in the new location [30]. The disease disparity across the world and the disease assimilation in immigrants indicates that local environment plays an important role in the development of these diseases.

Chronic inflammatory diseases are characterised by dysregulation of innate and adaptive immune responses. How the local environment influences immune response patterns leading to chronic inflammation is a key and unanswered question. We recently found a

marked shift in innate and adaptive immune responses in Chinese immigrants for those with less than versus those with more than five years residency in Australia [154, 197]. Toll-like receptors (TLRs) are a group of transmembrane proteins that play an important role in sensing pathogen invasion. The signalling of TLRs can activate antigen-presenting cells to provoke innate immunity and to establish adaptive immunity[241]. This current study investigated how time in Australia was associated with the expression of TLRs pathway genes in peripheral blood mononuclear cells (PBMC) *ex vivo*, plasma cytokine levels, and total and specific immunoglobulin E (IgE) serum titres among Chinese immigrants.

8.3 Methods

8.3.1 Study recruitment and sample collection

This cross-sectional study included 107 Chinese immigrants recruited from Chinese communities in Perth, Western Australia from 2014 to 2015. Han Chinese immigrants were recruited if they had no serious chronic illnesses and were willing to participate in the study. Each participant completed a comprehensive questionnaire on general health status, allergic symptoms and residency status in Australia. This study was approved by the Human Research Ethics Committee of Western Australia (RGS0000002399), at Curtin University (HR110/2013), and University of Western Australia (RA/4/1/6763). Blood samples were collected via venipuncture for serum, plasma and peripheral blood mononuclear cells (PBMCs) separation. PBMCs were isolated by density gradient centrifugation (Lymphoprep, Nycomed, Norway) as described previously[242] and cryopreserved in 7.5% dimethyl sulfoxide/foetal bovine serum.

8.3.2 RNA extraction, cDNA reverse transcription and Real-time PCR

Total RNA was purified from unstimulated PBMCs using the RNeasy® Mini Kit and RNase-Free DNase Set (Qiagen Pty Ltd., Doncaster, Australia). About 200 ng of total RNA was used to synthesize cDNA using QuantiTect® Reverse Transcription Kit (Qiagen). Real-time amplification reactions were conducted using QuantiFast SYBR® Green PCR Kit (Qiagen) on Applied Biosystems® ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Scoresby, Australia), under the following conditions: 5 min at 95 ºC, 40 cycles of 5 s at 95 ºC, 30 s at different annealing temperatures (Table 8-1). The primers used were designed in house on the specific mRNA sequences (Table 8-1). The fluorescence signal was analysed and normalized against 18S ribosomal RNA (18S rRNA) and beta-2-microglobulin (B2M). Relative expression levels were obtained using $2^{-\Delta\Delta CT}$ method and presented as fold increase relative to group 1 (<1 year residence in Australia).

Table 8-1. Primer sequences designed in the study for real time PCR

Gene		Primer sequence (5'-3')	Accession No.	Annealing temperature (°C)	Product size (bp)
TLR1	F	TTAAGCTGACAGAGCAAGCAA	NM_003263.3	60	176
	R	GAACTGCGACCCGAAGGTAT			
TLR2	F	TCATCATCAGCCTCTCCAAG	NM_001318787.1	62	176
	R	GTCACTGTTGCTAATGTAGGTG			
TLR3	F	TGCCACACACTTCCCTGATG	NM_003265.2	62	192
	R	GCTGCAGTCAGCAACTTCAT			
TLR4	F	GTATTCAAGGTCTGGCTGGT	NM_138554.4	60	157
	R	TCGAGGTAGTAGTCTAAGTATGC			
TLR5	F	GATGCTACTGACAACGTGGCT	NM_003268.5	61	173
	R	GAGGCTCCGACATCTTCCCT			
TLR7	F	GCTGATCTTGGCACCTCTCA	NM_016562.3	60	194
	R	CAGAGTGACATCACAGGGCA			
TLR8	F	CCAGGAGACCTTGAAGGAAG	NM_016610.3	58	167
	R	GGCGCATAACTCACAGGAAC			
TLR9	F	GTAGGCTGTCTGAGAGGGGA	NM_017442.3	57	182
	R	CAGCAGCGGCTCAGAGAATA			
TLR10	F	GCTTGGAGACTTCTCAGCCA	NM_030956.3	62	179
	R	GCATCACCCTCTGCTGTCAT			
NOD1	F	ACCTGGTGGCCAAGTGATTGT	NM_006092.2	60	156
	R	ATTGCTTCTGTCTCTTCCAAGC			
IL2	F	TGCAACTCCTGTCTTGCAT	U25676.1	551	131
	R	AATCATCTGTAAATCCAGCAGT			
IL13	F	CATGTCCGAGACACCAAAAT	NM_002188.2	60	208
	R	CCTCCCTAACCCTCCTTCC			
IFNγ	F	CAGAGCCAAATTGTCTCCTT	NM_000619.2	60	323
	R	TTCAAATATTGCAGGCAGG			
18S rRNA	F	GCTTTGGTGACTCTAGATAACC	HQ387008.1	61	173
	R	TAGCCGTTTCTCAGGCTC			
B2M	F	CGAGATGTCTCGCTCCGT	NM_004048.2	60	123
	R	GACTTTCCATTCTCTGCTGG			

TLR: toll-like receptor;

NOD1: Nucleotide-binding oligomerization domain-containing protein 1;

18S rRNA: 18S ribosomal RNA;

B2M: beta-2-microglobulin;

F: Forward; R: Reverse.

8.3.3 Cytokine measurements

Plasma IL-5, IL-6, IL-13, IL-17, and IFN-γ concentrations were measured using a solid-phase sandwich ELISA assay. The wells in 96-well microtitre plates (**Greiner Bio-One, Frichenhausen, Germany**) were coated with 15 μg of capture antibodies in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. The capture antibodies included ab25034 for IL-5, ab7746 for IL-6, ab9576 for IL-13, ab9565 for IL-17, and ab9657 for IFN-γ (abcam, Melbourne, Australia). The wells were blocked with 3 % skim-milk powder in phosphate buffered saline (PBS: pH 7.2) for 1 h, then washed three times with PBS containing 0.05 % Tween 20. Plasma samples were added and incubated for 2 h at room temperature. After washing as described above, 12 μg detection antibodies were added to react for 2 h. The detection antibodies included ab9624 for IL-5, ab123239 for IL-6, ab25037 for IL-13, sc-53937 for IL-17, and ab25014 for IFN-γ (abcam, Melbourne, Australia). The wells were subsequently washed as above and the bound antigen was detected with either goat anti-rabbit (IL-5) or anti-mouse (IL-6, IL-13, IL-17, and IFN-γ) IgG-HRP diluted in 1: 5000. Colour development was initiated by adding 3,3', 5,5'-tetramethyl-benzidine liquid substrate (Sigma, Castle Hill, Australia) and was stopped after 15 min by adding 0.5 M sulphuric acid. The optical density (OD) was measured at 450 nm on a microplate reader (FLUOstar Omega, BMG Labtech, Mornington, Australia).

8.3.4 IgE testing

Measurements of total IgE and specific IgE to house dust mite extract *Dermatophagoides pteronyssinus* (d1), dog dander (e5), *Alternaria alternate* mould extract (m6), grass pollen mix (gx2) and rye grass (g5) were assessed from serum samples using the Pharmacia CAP system (Pharmacia Diagnostics AB, Uppsala, Sweden) at the PathWest Immunology Department (QEII Medical Centre, Perth, Australia). The CAP score is measured in kU/L where 1 kU/L is equal to 2.42 ng/mL.

8.3.5 Statistical analysis

Gene expression and cytokine level data were log-transformed and compared between different time groups using One-way ANOVA. A line of best fit with a coefficient of determination (r^2) was predicted using linear regression with an optional quadratic term modelled to determine the relative impacts of residence time in Australia groups (1: <1 year, 2: 1 to 3 years, 3: 3 to 6 years, and 4: >6 years) on the gene expression, cytokine and IgE levels. Z-score standardized values (y) were used for the model fitting with the group variable (residence time) as the predictor variable (x). Variable x was included as simple, and quadratic term, and the best fit model was selected with the smallest Akaike information criterion (AIC). The formula $y=ax^2+bx+c$ was employed for model fitting of gene expression and cytokine data and $y=ax^2+c$ for total and specific IgE.

8.4 Results

In total, 107 adult Chinese immigrants were recruited for this study. Among those, PBMCs from 91 participants were used for the gene expression studies and plasma from 86 subjects were used for cytokine measurements due to the availability of samples. Total and specific serum IgE were tested for all the participants. The average age of participants was 30.1±6.9 and 61.7% were female. The participants had a median of 5.8 years residence in Australia with the interquartile range of 8.9 years. Table 8-2 shows the characteristics, allergic symptoms, and skin prick test results comparison between different residence time groups. Participants who have been resident in Australia >6 years were around 3-5 years older than other groups (p= 0.013). The rate of hay fever increased dramatically after living in Australia for more than 3 years, and so did the skin prick tests for mixed rye grass and mixed grass pollen show. We could observe that in participants living in Australia for < 1year, 50.0% were recruited in spring, which is in a high-allergy season. But for other groups, recruitment in autumn has taken the largest proportion. Even

in this situation, the allergies in the first group (<1 year) were still less than those in participants living in Australia for more than three to six years.

Table 8-2. The demographic characteristics, allergic symptoms, and skin prick test for the study population

	<1 year (n= 24)	1~3 years (n= 12)	3~6 years (n= 19)	>6 years (n= 52)	*p*
Characteristic					
Age (y)	27.7±6.3	26.8±7.3	29.4±5.7	32.2±6.9	**0.013**
Sex: male, n(%)	8 (33.3)	5 (41.7)	7 (36.8)	21 (40.4)	0.936
Body mass index	20.6±2.2	22.2±3.4	22.8±4.5	23.3±4.1	0.050
Smoking ever	2 (8.3)	1 (8.3)	3 (15.8)	7 (13.5)	0.893
Current smoking	1 (4.2)	1 (8.3)	3 (15.8)	6 (11.5)	0.629
Season of recruitment					**0.019**
Spring, n(%)	12 (50.0)	1 (8.3)	2 (10.5)	7 (13.5)	
Summer, n(%)	4 (16.7)	1 (8.3)	2 (10.5)	6 (11.5)	
Autumn, n(%)	7 (29.2)	6 (50.0)	10 (52.6)	25 (48.1)	
Winter, n(%)	1 (4.2)	4 (33.3)	5 (26.3)	14 (26.9)	
Allergic symptoms					
Itchy rash	4 (16.7)	5 (41.7)	7 (38.9)	22 (42.3)	0.151
Eczema	5 (20.8)	2 (16.7)	4 (22.2)	20 (38.5)	0.277
Asthma diagnosed	1 (4.2)	0 (0.0)	1 (5.3)	6 (11.5)	0.648
Dry cough	5 (20.8)	2 (16.7)	5 (26.3)	15 (28.8)	0.824
Hay fever	3 (13.0)	0 (0.0)	10 (52.6)	25 (49.0)	**<0.001**
Skin Prick Test (+ve)					
Aspergillus	1 (4.2)	0 (0.0)	4 (21.1)	7 (13.5)	0.271
Alternaria alternata	2 (8.3)	3 (30.0)	2 (10.5)	9 (17.3)	0.387
Dermatophagoides pteronyssinus	7 (29.2)	5 (50.0)	7 (36.8)	24 (46.2)	0.475
Dermatophagoides farinae	8 (33.3)	4 (40.0)	8 (42.1)	25 (48.1)	0.669
Cow's milk	1 (4.2)	1 (10.0)	2 (10.5)	6 (11.5)	0.789
Mixed rye grass	4 (16.7)	1 (10.0)	6 (31.6)	27 (51.9)	**0.005**
Mixed grass	1 (4.2)	0 (0.0)	4 (21.1)	24 (46.2)	**<0.001**
Egg white	1 (4.2)	0 (0.0)	0 (0.0)	3 (5.8)	0.874
Cat pelt	4 (16.7)	0 (0.0)	3 (15.8)	11 (21.2)	0.496
Dog dander	1 (4.2)	0 (0.0)	2 (10.5)	4 (7.7)	0.780
Cockroach	5 (20.8)	1 (10.0)	4 (21.1)	12 (23.1)	0.906

The mean z-score of gene expression levels for TLR pathway and cytokine genes in PBMCs and plasma cytokine levels are shown in Table 8-3. *TLR2*, *TLR7*, *TLR10*, and *NOD1* showed a significant difference between the four groups ($p< 0.05$).

Table 8-3. The mean and standard deviation of z-score for gene expression and cytokine levels for different groups stratified by residence time in Australia

	<1 year (n= 21)	1~3 years (n= 12)	3~6 years (n= 12)	>6 years (n= 46)	p^*
Gene expression					
TLR1	-0.47±0.76	-0.01±0.88	0.40±0.98	0.11±1.08	0.066
TLR2	-0.62±0.88	0.10±1.11	0.25±0.99	0.19±0.94	**0.012**
TLR3	-0.38±0.91	0.18±0.96	0.36±0.77	0.03±1.07	0.173
TLR4	-0.44±0.70	0.15±1.04	0.36±0.78	0.07±1.11	0.102
TLR5	-0.05±0.58	0.39±0.56	0.50±0.33	-0.21±1.26	0.073
TLR7	-0.32±0.90	0.43±0.76	0.57±0.74	-0.12±1.08	**0.027**
TLR8	-0.20±0.78	-0.06±1.22	0.37±0.72	0.01±1.09	0.489
TLR9	0.01±1.00	0.30±0.43	0.37±0.21	-0.18±1.18	0.237
TLR10	-0.47±0.76	-0.01±0.88	0.40±0.98	0.11±1.08	**0.019**
IL2	-0.62±0.88	0.10±1.11	0.25±0.99	0.19±0.94	0.307
IL13	-0.38±0.91	0.18±0.96	0.36±0.77	0.03±1.07	0.280
NOD1	-0.44±0.70	0.15±1.04	0.36±0.78	0.07±1.11	**0.010**
IFNγ	-0.05±0.58	0.39±0.56	0.50±0.33	-0.21±1.26	0.420
Cytokine levels					
IL-5	-0.21±0.89	0.70±0.91	0.35±0.80	-0.18±1.04	0.204
IL-6	-0.31±0.94	0.10±1.49	-0.18±0.89	0.16±0.89	0.158
IL-13	-0.33±1.06	0.15±0.90	0.33±0.50	0.03±1.07	0.621
IL-17	-0.29±0.85	0.06±0.92	0.84±0.71	-0.10±1.05	0.708
IFN-γ	-0.23±0.65	0.31±0.90	0.21±0.97	-0.03±1.15	0.391

*: All the gene expression and cytokine levels data were z-score standardized values from log-transformed original values. One-way ANOVA was used to compare different gene expression and cytokine levels between the time groups of residence in Australia.

Table 8-4 has shown the description and comparison of total and specific IgE test result. A significant difference was found between groups in specific IgE, dog dander, grass pollen mix, rye grass. The residence time >6 years group has the highest levels of those specific IgE levels.

Table 8-4. IgE results for different groups stratified by residence time in Australia

	<1 year (n= 24)		1~3 years (n= 12)		3~6 years (n= 19)		>6 years (n= 52)		p^*
	median	(Q1,Q3)	median	(Q1,Q3)	median	(Q1,Q3)	median	(Q1,Q3)	
Total IgE	40.0	(24.7, 108.3)	34.3	(13.2, 143.1)	96.5	(16.8, 222.0)	64.4	(28.7, 161.8)	0.435
Specific IgE†	0.15	(0.09, 0.72)	0.20	(0.10, 1.38)	0.28	(0.12, 29.59)	5.23	(0.23, 29.97)	**0.001**
D. pteronyssinus (d1)	0.06	(0.03, 0.27)	0.12	(0.04, 0.70)	0.06	(0.03, 0.69)	0.16	(0.03, 3.80)	0.465
dog dander (e5)	0.02	(0.01, 0.06)	0.02	(0.01, 0.03)	0.10	(0.02, 0.32)	0.05	(0.02, 0.21)	**0.034**
Alternaria alternate (m6)	0.03	(0.02, 0.05)	0.02	(0.02, 0.03)	0.02	(0.02, 0.04)	0.03	(0.02, 0.05)	0.213
grass pollen mix (gx2)	0.02	(0.02, 0.04)	0.02	(0.01, 0.03)	0.04	(0.02, 0.90)	0.06	(0.02, 5.99)	**0.002**
rye grass (g5)	0.01	(0.01, 0.02)	0.01	(0.01, 0.01)	0.02	(0.01, 0.78)	0.07	(0.01, 5.29)	**<0.001**

*: Kruskal Wallis Test was utilised for group comparison
†: Specific IgE included the sum IgE levels of *D. pteronyssinus*, *Alternaria alternate*, grass pollen mix, and rye grass.
Q1: 25th quantile; Q3: 75th quantile.

Remarkably, we found TLR pathway genes had an inverted U-shaped response as a function of residence time in Australia (Table 8-5). The model was significant for TLR1, TLR2, TLR4, TLR5, TLR7, TLR10 and NOD1, with negative coefficient “a” and positive coefficient “b” indicating an inverted U-shape association between time of residence in Australia and these TLR pathway genes. The inverted U-shaped response was marginally significant for plasma IL-6. These findings remained significant after adjusting for age.

Table 8-5. The models for predicting the association between TLR gene expression, cytokine, IgE levels and residence time in Australia.

$y=ax^2+bx+c$							
	Adjusted R^2 for model	*p* for model	constant	a	*p* for a	b	*p* for b
Gene expression							
TLR1	0.055	**0.031**	-1.454	-0.191	0.109	1.157	0.062
TLR2	0.097	**0.004**	-1.646	-0.193	0.097	1.232	**0.042**
TLR3	0.034	0.082	-1.411	-0.224	0.064	1.255	**0.046**
TLR4	0.047	**0.044**	-1.510	-0.223	0.063	1.288	**0.039**
TLR5	0.054	**0.033**	-1.166	-0.288	**0.017**	1.392	**0.025**
TLR7	0.078	**0.010**	-1.837	-0.361	**0.003**	1.875	**0.003**
TLR10	0.079	**0.010**	-1.594	-0.354	**0.003**	1.768	**0.004**
NOD1	0.067	**0.018**	-1.791	-0.334	**0.006**	1.764	**0.005**
Cytokine levels							
IL-6	0.040	0.074	-1.521	-0.227	0.076	1.300	**0.051**
$y=ax^2+b$ †							
	Adjusted R^2 for model	*p* for model	constant	a	*p* for a		
Total IgE	0.045	**0.036**	0.641	0.033	**0.041**		
Specific IgE ‡	0.187	**<0.001**	0.196	0.073	**<0.001**		
D. pteronyssinus (d1)	0.052	**0.035**	0.483	0.040	**0.022**		
dog dander (e5)	0.158	**0.001**	0.820	0.062	**0.002**		
Alternaria alternate (m6)	0.117	**0.001**	1.188	0.039	**0.012**		
grass pollen mix (gx2)	0.148	**<0.001**	-0.096	0.067	**<0.001**		
rye grass (g5)	0.169	**<0.001**	-0.189	0.072	**<0.001**		

†: age was adjusted for in the model fitting for predicting the assocation between IgE and residence time.

‡: Specific IgE included the sum IgE levels of *D. pteronyssinus*, *Alternaria alternate*, grass pollen mix, and rye grass.

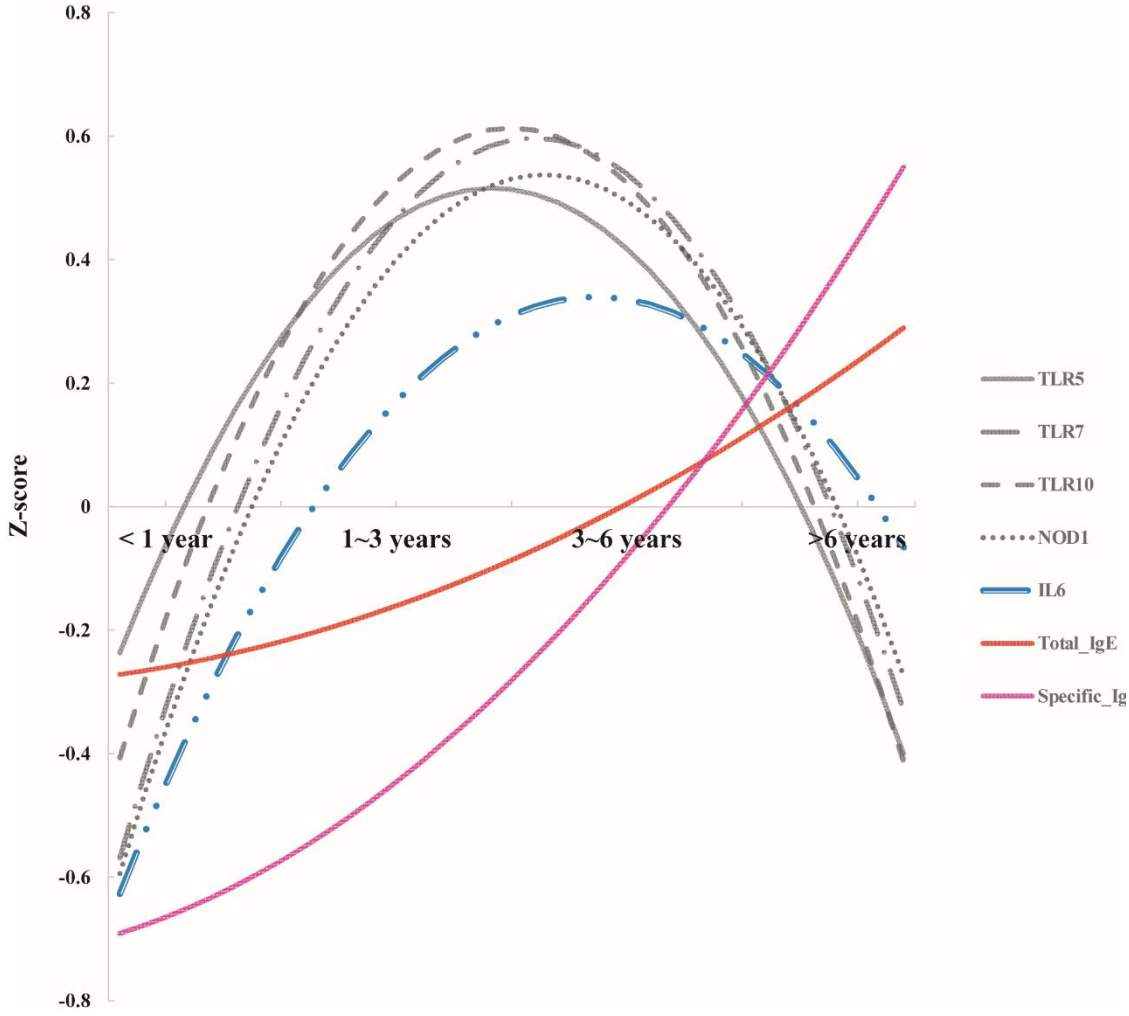


Figure 8-1. The models for predicting the changes of TLR gene expression, cytokine and IgE levels over residence time in Australia

The four inverted U-shape lines with grey colour theme show expression of TLR genes. The changes for cytokine IL6 (blue colour) also show an inverted U-shape. The formula for the inverted U-shape is ax^2+bx+c (x: theoretical residence time change). The total IgE (red) and specific IgE (purple) have been best fitted for $ax^2 + b$ (x: theoretical residence time change).

We observed an accelerated increase in total and specific IgE with increasing residence time in Australia (Table 8-5 & Figure 8-1). However, for the Th2 cytokines IL-5 and IL-13, we did not observe the same trend as IgE. Figure 8-1 was drawn based on the prediction models for the time dependence trends of TLR5, TLR7, TLR10, and NOD1

gene pathways, cytokine IL-6, total and specific IgE with the parameters that are shown in Table 8-5.

8.5 Discussion

The new Western environment has multiple factors that may contribute to these relationships. The combination of exposure to new allergens and microorganisms and other environmental stimuli in Australia may be relevant. Immigrants from China could be expected to receive a reduced signal intensity of environmental microbes when living in Australia. These decreased stimulating signals could modulate responses in TLRs, which are the first line of defence against invading pathogens and play a critical role in innate immune responses [243]. This interaction between the Western environment and innate immune responses in immigrants is poorly understood and may play a critical role in the disease pattern transition of several important chronic inflammatory diseases.

Our findings suggest that immigrants may initially have an upregulated expression of pattern recognition receptors of blood mononuclear cells. After 5 or 6 years, these responses gradually wane to the 'normal' level or even lower. Interestingly, the pro/anti-inflammatory cytokine IL-6 shows the same trend. TLR and MYD88-dependent and -independent pathway signalling is important in the development of innate and adaptive immune responses and the observed TLR upregulation during the first couple of years after migration may play an important role in subsequent atopic sensitization, leading to the assimilation of the higher disease prevalence in local population. Indeed, total and specific IgE also showed an accelerated trend as the residence time increased in Australia, indicating a gradual establishment of atopic sensitization (Figure 8-1). While the TLR function reaches "normal", the allergen sensitisation tendency is established. This is consistent with the well-established epidemiological evidence that the prevalence of

asthma and allergy increases in immigrants from a low risk to a high-risk Western environment after several years [30]. The mechanisms that determine how changes in expression of TLR pathway genes relates to the development of allergic conditions in immigrants requires further study. However, the link between innate immunity, microbial exposure, and development of allergic conditions has been well established in previous studies [187, 244].

This cross-sectional study has shown a distinct TLR gene expression pattern in PBMCs and changes in several important allergic biomarkers associated with residence time in Australia among Chinese immigrants. Tracing the trajectory of the changes of TLRs and allergic biomarkers would contribute to future precise allergy prevention and further improvement of therapeutic strategies. We acknowledge that the convenience sampling strategy is one of the limitations of this study as it may cause selection bias. However, many laboratory measures have been conducted in the population and these objective measurements would significantly increase the validity of the study. Another limitation of this study is the lack of other risk factors for allergic conditions, such as household status, dietary habits. Next, longitudinal studies on newly arrived Chinese immigrants investigating immunological changes and comprehensive risk factors are of interest, ideally with both prospective samples (prior to travel) and follow-up. In addition, a matched Australian control group is required. Such longitudinal studies are essential to understand the time-related effects of the development of chronic diseases in the Western environment.

9 General Discussion and Conclusion

9.1 Summary of findings

The prevalences of asthma and allergy are more common in Westernised developed countries than in developing countries. Among the risk factors for asthma and allergy, environmental species rich in microbial diversity and the non-harmful commensals from the skin, gut and respiratory tract play a crucial role in priming and regulating the immune system, thereby leading to decrease/increase risk of these allergic diseases in humans. Currently, there is a paucity of studies looking into the role of the microbiome in immigrant populations, in asthmatic children in developing countries, and between children living in contrasting environments such as Eastern/developing country versus Westernised/developed. The work presented in the thesis aimed to clarify this knowledge gap and sought to elucidate the microbiome link in allergic symptoms and immunological parameters among a genetically homogeneous population (Han Chinese).

Study one (Chapter 3-5) is the first study conducted in a developing country environment to investigate both the oropharyngeal and gut microbiome, and their association with serum IgE levels for asthmatic and non-asthmatic children. Compared to non-asthmatics, children with asthma had significantly higher rates of allergic comorbidities (rhinitis, eczema and allergic conjunctivitis) and higher levels of total and specific IgE. When comparing the diversity and abundance in microbiome between asthmatic and non-asthmatic children, we found the alpha diversity of

oropharyngeal bacteria was lower in asthmatic children, but no difference was evident in gut bacteria. To date, several studies have shown the inverse correlation between bacterial diversity and allergic conditions [130, 202], consistent with the findings of this thesis. However, the reason the alpha diversity in gut microbiome did not show a significant difference needs further investigation.

Significant bacterial composition differences were observed between asthmatic and non-asthmatic children. In oropharyngeal microbiome comparison, the phylum Bacteroidetes and its genus *Bacteroides* were significantly lower in asthmatic children. When comparing faecal microbiome abundance, genus *Lachnospira*, and *Ruminococcus 1* were significantly higher in asthmatic children. The association patterns of microbial abundance and serum IgE levels were distinct between asthmatic and non-asthmatic children. These differences in microbiome profiles in asthmatic and non-asthmatic children might provide some indications for future candidate microbiota functional studies.

The distinct pattern of correlation of bacterial relative abundance and serum IgE levels in asthmatic and non-asthmatic children might be due to the different relative abundance of some of the taxa in individual children. In addition, as the asthmatic children had a tendency to Th2 response activation, the cross-talk between commensals and mucosa immunological response could also be affected [245].

Studies involving immigrants provide an important opportunity to investigate the role of environmental factors in the pathogenesis of allergic conditions. It was hypothesised that immigrants to Westernised/developed countries would adopt the higher prevalence of allergic conditions of their new home countries [30]. In the study population in Australia, Chinese immigrant children and adults were found to have a gradual increase in allergic symptoms and diseases, a Westernised/developed country. Human microbiome adaptation of Chinese immigrants to this environment was expected to contribute to the development of allergic conditions. Study two (Chapter 6) investigated the oropharyngeal and gut microbiome difference between Australian Chinese (AC) children in Australia and China-born Chinese (CC) children in mainland China.

As hypothesised, AC children had more allergic conditions than CC children. These children demonstrated a lower richness in microbial diversity and higher diversity evenness compared to CC children in both oropharyngeal and gut microbiome. This suggests that the Westernised environment of Australia might have influenced the microbiome to have less richness in diversity, and reduced diversity in bacterial richness may be related to the increased allergic conditions in AC children [246, 247].

In comparing microbial composition, an apparent difference in microbial abundance was observed for many bacteria between the two groups of children. AC and CC children appear to have distinctly different microbial profiles in the oropharyngeal and

gut microbiomes. Our results are consistent with the notion that bacteria that are more abundant in AC children, relative to CC children, are more likely to be associated with asthma. Specifically, the genus *Ruminococcus 1* and *Lachnospira* of the gut microbiome were more abundant in AC children, and they also showed a higher relative abundance in asthmatic children in China (Chapter 4). Moreover, the relative abundance of genus *Escherichia-Shigella* was both significantly higher in CC children and in non-asthmatic children (Chapter 4). These bacterial genera may contribute to the development of allergic conditions and have the potential as a target for further research on probiotic treatments and diet/environmental interventions to prevent asthma and allergy.

To further examine this notion, in AC children we sought to establish consistent trends in bacterial relative abundance that were either higher or lower in AC versus CC children, and higher or lower in children with allergy versus those without allergy. The majority of oropharyngeal taxa showed a consistent trend while the majority of faecal taxa did not. This is probably because the environments of upper respiratory tract and gastrointestinal tract differ considerably in the way the microbial component interacts with the immune system. The attempt to find a relationship of commonly dominant bacteria in AC children and children with allergic conditions has the promising potential to ascertain whether some bacteria are associated with the development of asthma and allergy.

In study three (Chapter 7), we compared the oropharyngeal microbial profiles between newly-arrived (< 6 months) adult Chinese immigrants and long-term (> 5 years) adult Chinese immigrants, and investigated the correlation between the oropharyngeal bacterial composition and innate immune response, adaptive immune response and atopic indices (serum IgE levels). Some differences in microbiomic profiles between newly-arrived and long-term Chinese immigrants were found, which partly may be attributed to the Westernised versus Eastern environmental influence. The relative abundance of the genus *Leptotrichia* was higher in long-term immigrants than in newly-arrived Chinese immigrants, while the genus *Deinococcus* was significantly lower in long-term Chinese immigrants. This finding in adult Chinese immigrants and the results of AC and CC children's microbiome comparisons supports the primary hypothesis that the Westernised environment of Australia has resulted in modification of the microbiome in Chinese immigrants.

Chapter 7 also found that specific microbial taxa were significantly correlated with immunological parameters, such as toll-like receptor (TLR) pathway cytokines, specific IgE and bacterial or virial antibody response. This indicates that the human microbiome may have an influence on host immune response. As this was a cross-sectional study, we could not clarify a causal relationship between the microbiome and dynamic changes in the human immune system. It should be highlighted that these interactions of the microbiome and the overall innate immune responses were distinctly different in newly-arrived and long-term Chinese immigrants. Considering

our previous findings that a stronger positive correlation between innate and adaptive responses were apparent among newly-arrived Chinese immigrants [197], we demonstrated that genera from the dominant phyla (Firmicutes and Proteobacteria) were positively correlated with the overall innate immune response among newly-arrived Chinese immigrants and, conversely, with negative correlation in long-term immigrants. This indicates that the human microbiome may have a different influence on host immune response in people living in a contrasting environment, and these distinct environmental and microbiome interactions may significantly contribute to the uneven distribution of asthma and allergy in the Westernised and Eastern environments. Moreover, the finding also highlights challenges of microbiome studies, given the possible complex interaction between the microbiome and disease susceptibility with regard to different environmental risk factors.

In study four (Chapter 8), we investigated the time dependence of the Westernised environment on the expression of TLR pathway genes, plasma cytokine levels and total and specific IgE in the Chinese immigrant cohort. We found the TLRs pathway genes had an inverted U-shaped response, and total IgE and specific IgE had a quadratic increase as a function of duration of residence in Australia. As reported in Chapter 6 and Chapter 7, Chinese immigrants living in Australia and interacting with the Westernised environment had an altered microbiome profile relative to their peers in China. These microbiome modifications should be time-dependent and may contribute to the time-dependence effects of TLR pathway genes and total and specific

IgE in Chinese immigrants. Accumulating evidence has shown that human microbiomes could stimulate the regulatory circuits via TLR and other antigen-recognising receptors to prevent inappropriate inflammatory responses in relation to the development of asthma and allergy [248]. Collectively, the primary hypothesis of the thesis has been supported, and the Westernised environment–microbiome interaction may be the underlying mechanism for the increased prevalence of asthma and allergy in Westernised developed countries over the past 70 or 80 years, and the continuing increase of these conditions in developing countries.

It is posited that the Westernised environment of Australia has reshaped the microbial composition in Chinese immigrants, and that these altered microbiomes are the cause of high rates of asthma and allergy in Australia. It is important to understand the contribution of the microbiome in a Westernised environment to the development of asthma and allergy. These findings provide invaluable data to improve understanding of the aetiology of asthma and allergy in Westernised countries such as Australia.

9.2 Key findings

- The bacterial diversity was lower in asthmatic children in China, as was that in AC children. This finding provided supporting evidence for the proposition that lower diversity of human microbiome is associated with asthma and allergy.

- Distinct microbiome profiles existed between asthmatic and non-asthmatic children in China; between AC children and CC children, and between newly-arrived and long-term adult Chinese immigrants living in Australia.
- The Westernised environment of developed countries is likely to produce a complex TLR response pattern in immigrants, resembling an inverted U-shape as a function of duration of residence. The shift in microbiome could contribute to the interaction between the Westernised environment and innate immune response.
- The correlation patterns of microbiome and serum IgE were distinct between children with or without asthma in China, as were the correlation patterns of microbiome and immunological parameters between newly-arrived and long-term Chinese immigrants.

9.3 Study limitations and future research directions

The studies included in this thesis are novel and provided insights into the correlation of human microbiome, asthma and allergy in a developing country environment and in immigrants living in a Westernised environment. However, several limitations and the future directions should be considered.

First, 16S rRNA gene amplicons were utilised in the studies of this thesis for taxonomy profiling. Although this sequencing method is simple, cost-effective and able to describe the bacterial community composition, the resolution of 16S rRNA sequencing

is only reliable to the genus level, with difficulty in defining bacterial species [249]. Shotgun metagenomics is able to sequence all given genomic DNA, and the data yielded from shotgun sequencing can enable a more specific taxonomic and community functional profiling [250]. Thus, in the future, studies in combination of these two sequencing methods are of interest to further investigate differences in the species and strain-level microbiome between the Westernised/developed and Eastern/developing environment, as well as to help improve the establishment of a mechanistic model to elucidate the development of asthma and allergy.

Second, in this thesis we have identified several bacterial taxa that are significantly different between asthmatic and non-asthmatic children, between AC children and CC children, and between newly-arrived and long-term immigrants living in Australia. However, the analyses used a computational approach and could not determine causality for asthma and allergy. The nature of the cross-sectional study design in Chinese immigrants also limited our ability to establish a possible causal relationship. The bacteria 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 bacteria [251, 252].

Third, our definition of a ‘Westernised environment’ included differences in diet, less air pollution, exposure to new allergens and better hygiene than in the Eastern/developing country environment, all of which can lead to differences in

microbiota composition and diversity [212, 213]. However, in this thesis we could only focus on the overall influence of Westernised environments on the microbiome and its relationship with allergy. For future studies, it would be worthwhile to investigate the effects of different environmental factors inherent in the Westernised environment on the microbiome modification individually and, ideally, utilising a longitudinal study design in a larger population.

Only improved understanding of the influence factors and mechanism of asthma and allergy will assure better disease management from primary and secondary prevention to treatment. This thesis enlarges the knowledge of microbiome in asthma and allergy and helps to pave the way for the development of potential probiotics in preventing or treating asthma and allergy. It also emphasizes the impact of biodiversity on health and the public health sectors should pay more attention on advocation for a closer connection to nature.

9.4 Thesis Conclusion

Asthmatic children in China showed a different microbiome profile than children without asthma and allergy. Immigrants in Australia had an increased prevalence of allergic conditions. The lower bacterial diversity in children with asthma and AC children, relative to their peers, and the shifts in microbiome, which were influenced by the Westernised environment, may have contribute to the increased prevalence of asthma and allergy in developed countries in the second half of the twentieth century,

as well as currently in developing countries. The time-dependent microbiome adaptations may be associated with the time-dependence effects of innate immune response and the asthma and allergy hallmarks of total and specific IgE in Chinese immigrants. Additionally, the distinct correlation patterns of the microbiome and immunological parameters reflected that these interactions of the microbiome and the immune responses are markedly different and complex when comparing the Westernised versus Eastern environment.

The overall findings of this thesis provide supporting evidence for the role of the microbiome in asthma and allergy in the context of contrasting environments. These invaluable data provide a significant contribution to furthering understanding of the underlying mechanisms for asthma and allergy, and our findings could assist in the intervention, management, treatment and possible prevention of asthma and allergy.

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Appendices

Appendix A: The published paper for Chapter 6

DOI: https://doi.org/10.1016/j.waojou.2019.100051

World Allergy Organization Journal 12 (2019) 100051

Contents lists available at ScienceDirect

World Allergy Organization Journal

journal homepage: www.sciencedirect.com/journal/wao-journal

Western oropharyngeal and gut microbial profiles are associated with allergic conditions in Chinese immigrant children

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ARTICLE INFO

Keywords:
Microbiome
Immigration
Western environment
Atopy
Allergy

ABSTRACT

Background: The allergy epidemic resulting from western environment/lifestyles is potentially due to modifications of the human microbiome. Therefore, it is of interest to study immigrants living in a western environment as well as their counterparts in the country of origin to understand differences in their microbiomes and health status.

Methods: We investigated 58 Australian Chinese (AC) children from Perth, Western Australia as well as 63 Chinese-born Chinese (CC) children from a city in China. Oropharyngeal (OP) and fecal samples were collected. To assess the microbiomes, 16s ribosomal RNA (rRNA) sequencing for variable regions V3 and V4 was used. Skin prick tests (SPT) were performed to measure the children's atopic status. Information on food allergy and wheezing were acquired from a questionnaire.

Results: AC children had more allergic conditions than CC children. The alpha diversity (mean species diversity) of both OP and gut microbiome was lower in AC children compared to CC children for richness estimate (Chao1), while diversity evenness (Shannon index) was higher. The beta diversity (community similarity) displayed a distinct separation of the OP and gut microbiota between AC and CC children. An apparent difference in microbial abundance was observed for many bacteria. In AC children, we sought to establish consistent trends in bacterial relative abundance that are either higher or lower in AC versus CC children and higher or lower in children with allergy versus those without allergy. The majority of OP taxa showed a consistent trend while the majority of fecal taxa showed a contrasting trend.

Abbreviations: AC, Australian Chinese; CC, China-Born Chinese; rRNA, ribosomal RNA; OP, oropharyngeal; SPT, skin prick test; BMI, body mass index; LDA, The linear discriminant analysis; LEfSe, The linear discriminant analysis effect size; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG, Kyoto Encyclopaedia of Genes and Genomes; FDR, false discovery rate.

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https://doi.org/10.1016/j.waojou.2019.100051
Received 1 April 2019; Received in revised form 9 July 2019; Accepted 19 July 2019

Conclusion: Distinct differences in microbiome compositions were found in both oropharyngeal and fecal samples of AC and CC children. The association of the OP microbiome with allergic condition is different from that of the gut microbiome in AC children. The microbiome profiles are changed by the western environment/lifestyle and are associated with allergies in Chinese immigrant children in Australia.

Introduction

The rising prevalence of asthma and allergies has become a global public health concern, and there are wide variations between countries.[1,2] The prevalence of adult asthma is highest in developed countries, such as Australia (21.0%), while it is the lowest in developing countries, such as China (0.2%).[3] The substantial difference in allergy prevalence indicates that environmental factors play a vital role in the development of these conditions.[4] Immigrant populations in industrialized countries represent a unique opportunity to examine western environmental influences.[5] Immigrants moving from less affluent countries (asthma-low risk) to more affluent countries (asthma-high risk) experience a gradually increased prevalence of allergies and asthma, correlated with the length of residence in the more affluent country.[6] For example, a cross-sectional survey of school-age children reported that compared to a residence in Australia from zero to 4 years, residence for 5 to 9 years after migration was associated with a two-fold increase in reported wheezing, and this increased to a three-and-a-half-fold for 10–14 years after migration.[7] This time-dependent effect points to a gradual change of individual homeostasis, potentially related to ongoing modifications of the human microbiome due to western environmental risk factors.

Studies have shown that perturbations in the human microbiome are associated with an increased risk of allergic disease.[8,9] This agrees with the well-known "hygiene hypothesis" that suggests early exposure of children to high microbial abundance and increased biodiversity protects against development of allergic diseases.[10,11] Our recent studies showed that Chinese immigrants in Australia had a significant shift in the innate and adaptive immune response.[12,13] Chinese immigrants living in Australia for more than 5 years had reduced innate immune cytokine production and weaker adaptive antibody responses to pathogen-associated antigens relative to recently-arrived Chinese immigrants.[12,13] We presume that the human microbiome inherent to the western environment may regulate the priming of immune response and modulate the susceptibility to allergic disorders.[14–16] However, there is a lack of knowledge about the difference in human microbiome between immigrants and their counterparts in the country of origin. Chinese immigrant children in Australia with matched Chinese children in China are a relatively homogeneous population, yet living in an industrialized or non-industrialized environment. Therefore we compared the oropharyngeal (OP) and gut microbiome of Australian Chinese (AC) children in Australia and China-born Chinese (CC) children in mainland China. The two cohorts were strictly matched for age-range, gender-frequency, and season of recruitment to control for potential confounders.

Methods

Study design and recruitment

This study is a cross-sectional investigation in which the participants are living in Australia and China. First we recruited AC children from the local Chinese community living in Perth, Western Australia by advertisements through Chinese media such as radio and newspaper (from March to May 2015). Chinese children aged 3 to 18 and residing in Australia were recruited. Second we recruited CC children from cluster randomly selected students from kindergartens, primary and high schools in Hebi City in the northern Henan Province. Gender frequency and age range were matched with the AC children (from September to October 2015). The recruitment took place during autumn taking into account the countries are in opposite hemispheres. Hebi city is a relatively less affluent (prefecture-level) city in China, where agriculture has traditionally been a pillar of its economy. In total we recruited 58 AC children (aged 3–18) and 63 CC children (aged 2–17), all of whom were of Han Chinese descent.

OP swabs and fecal samples were collected from the participants and one of parents/guardians was asked to fill out a questionnaire for their child. The questionnaire collected demographic information, delivery method (Vaginal delivery/Caesarean section), breastfeeding history, self-reported food allergic history and current wheezing status. At recruitment, skin prick tests (SPT) were performed to measure the child's atopic status. The SPT results were evaluated after 15–20 min exposure, and positive atopy was defined as a wheal size >3 mm diameter in reaction to at least at one allergen (details in Supplemental Notes).[17]

This study was approved by the Human Research Ethics Committee (HREC) at the University of Western Australia. All parents provided informed consent on behalf of their children.

16S rRNA gene sequencing, bioinformatics and statistical analysis

Amplicons of the 16S rRNA gene V3–V4 region were sequenced on an Illumina HiSeq 2500 platform. The paired-end reads were merged, then filtered, and the sequences were assigned into Operational Taxonomic Units (OTUs) against the SILVA reference database (128 release). Bioinformatics and statistical analysis were carried out within the Quantitative Insights Into Microbial Ecology (QIIME 1.9.1) pipeline or using RStudio (Version 1.0.153). Alpha diversity, which describes the number of taxa in sites or habitats at a more local scale, was estimated using the chao1 richness estimate and Shannon index. Beta diversity, which indicates the extent of similarity between microbial communities, was measured using weighted and unweighted UniFrac. To infer the microbiome phenotypes and functional pathways associated with the bacterial taxa, we used Bugbase and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. The predictions of functional pathways were collapsed into Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups and shown by linear discriminant analysis effect size (LEfSe) plots.

Mann-Whitney U tests were used to compare the group difference between AC and CC children, and we selected taxa with relative abundance over 1.0% to illustrate with figures and tables. Linear regression was used to investigate the associations of bacterial relative abundance and AC/CC groups, after adjusting for age, gender, BMI, breastfeeding percentage, and antibiotic usage. If the taxa that show a change in abundance in AC children relative to CC children play a role in the occurrence of allergic conditions, we expect that these taxa will show a similar change in children with allergic conditions compared to children without allergic conditions. To examine such trend, we selected taxa with significant differences (at two significance levels: $p < 0.05$ and $p < 0.01$) in relative abundance between AC and CC children at 5 taxonomic levels (phylum, class, order, family, and genus). Subsequently the mean difference of taxa abundance between children with positive and negative allergic conditions (atopy, food allergy, and wheezing) was calculated in AC children, and those with mean difference over 0.01% and 0.1% were selected for further analysis respectively. If the mean relative abundance of a bacterium is higher or lower in AC children (compared to CC children) and also higher or lower in children with allergic conditions (compared to children without allergic conditions), a "1" was assigned to the bacterium, otherwise a "0" was assigned. A new variable with these binomial values (1 and 0) was created for all the selected taxa and used to test for consistency using a binomial probability test. The null hypothesis

for the binomial probability tests is that the proportion of 0 (inconsistent) or 1 (consistent) is equal to 50% which indicates that there is no consistent trend. We used this consistency test of the major distinct taxa to infer the influence of the western environment on the human microbiome and its relation with allergic conditions. The full methods and related references are available in Supplemental Notes.

Results

Characteristics of the study population

As shown in Table 1, there were no significant differences in gender, age, body mass index (BMI), delivery method, breastfed percentage and antibiotic usage between the AC and CC children. Forty-two (72.4%) of the AC children were born in Australia, and 16 (27.6%) were born in China and had been living in Australia with a median duration of 4.6 years. The percentages of atopy, food allergy, and current wheeze were all significantly higher among AC children than among CC children.

Microbial diversity and composition

The microbiome composition between the AC children born in China or Australia was similar and therefore grouped for further analysis. Two AC and 8 CC participants had used antibiotics 2 weeks prior to sample collection, and we performed a sensitivity test without those subjects which gave a consistent result.

Microbial diversity

The Chao1 richness estimates are consistently lower in AC children for both OP (257.70 ± 43.22) and fecal samples (330.24 ± 41.65) compared to CC children (288.62 ± 43.03, 345.00 ± 33.39, $p = 0.002$, 0.046) (Fig. 1 a and d). Conversely, the Shannon indices were significantly higher in AC children (OP: 4.46 ± 0.59, fecal: 5.58 ± 0.73) than in CC children (OP: 3.99 ± 0.95, fecal: 5.06 ± 0.67, $p = 0.009$, 0.002).

A distinct clustering was observed of the OP and fecal bacteria communities between the AC and CC children using both the unweighted and weighted UniFrac matrix presented by Principal Coordinate Analysis (PCoA) plots (Fig. 1 b, c, e, f). Additionally, ANOSIM and Adonis statistical model analyses further showed a significant difference between both OP and fecal bacterial communities of AC and CC children (Supplemental Table1).

Oropharyngeal sample bacterial composition

A total of 16 bacterial phyla were detected from OP swabs (Fig. 2a, Supplemental Table2). Phylum-level taxonomical assignment showed that *Firmicutes* and *Proteobacteria* were dominant in both AC (49.6%, 19.8%) and CC children (46.2%, 26.6%). At the genus level, 12 genera accounted for 80.8% of the abundance in AC, and 15 genera accounted for 82.3% of the abundance in CC children, using a minimum relative abundance of 1.0% (Supplemental Figure 1a, Supplemental Table2).

Among the total of 16 bacterial phyla and 193 genera, 6 (37.5%) phyla and 113 (58.5%) genera were significantly different between AC and CC children. AC children had a lower proportion of phylum Deinococcus-Thermus ($p < 0.001$), and higher proportions of Actinobacteria ($p < 0.001$), Fusobacteria ($p < 0.001$), and Bacteroidetes ($p = 0.013$) (Fig. 2b, Supplemental Table2). The differences between the two groups of children are shown for the 10 most abundant genera in Supplemental Figure 1b. These differences remained significant after further adjustment for confounders (age, gender, BMI, breastfed percentage, and antibiotic use) using linear regression.

Table 1
The characteristics of participants.

Characteristic	AC (n = 58)	CC (n = 63)	*p*
General Information			
Females: n (%)	26 (44.8%)	30 (47.6%)	0.758
Age (y): mean (SD)	8.6 (3.5)	7.7 (3.7)	0.196
BMI (kg/m^2): mean (SD)	17.1 (2.6)	17.6 (4.3)	0.391
Delivery method			
Vaginal delivery n (%)	34 (58.6%)	32 (50.8%)	0.701
Caesarean section n (%)	22 (37.9%)	24 (38.1%)	
Breastfed: n (%)	46 (79.3%)	53 (84.1%)	0.584
Clinical Information			
Antibiotic used (past 2 weeks): n (%)	2 (3.4%)	8 (12.7%)	0.179
Atopy: n (%)	36 (62.1%)	8 (12.7%)	**0.000**
Food allergy: n (%)	15 (25.9%)	5 (7.9%)	**0.017**
Wheezing: n (%)	16 (27.6%)	2 (3.2%)	**0.000**

Fecal sample bacterial composition

We observed 10 distinct phyla in the fecal microbiomes of AC and CC children (Fig. 2c, Supplemental Table3). The phylum Firmicutes (49.4%) was dominant in AC children, whereas the phylum Bacteroidetes (47.5%) had the highest proportion in CC children. These 2 phyla made up the vast majority of OTUs, namely 89.3% in AC and 88.4% in CC children. Seven (70.0%) phyla and 91 (62.8%, 91/145) genera were significantly different between AC and CC children, with the major differences shown in Fig. 2d, Supplemental Figure 2. After adjusting for confounders, the relative abundance of phyla Firmicutes, and Bacteroidetes, genera *Ruminococcus1*, *Lachnospira*, *Eubacterium*, *Peptoclostridium*, *Barnesiella*, *Parasutterella*, and *Escherichia-Shigella* remained significantly different between AC and CC children.

Trend consistency of taxonomic abundance with the western environment and allergy

We identified taxa that were different between the AC and CC children across the 5 taxonomic levels. We selected 204 OP taxa with a significance level $p < 0.05$ and 141 OP taxa with a significance level $p < 0.01$. For fecal samples this was 123 and 81 taxa, respectively. Combined with the mean difference of taxa abundance between positive and negative allergic conditions (>0.01% and >0.1%) we performed 4 sets of binomial tests each for atopy, food allergy, and wheezing among AC children (Supplemental Table5).

We discuss the binomial test results for the analysis of $p < 0.05$ and difference >0.01% (Supplemental Table5). In OP samples, 84.0% (63/75) of the taxa showed a consistent trend for food allergy that is significantly higher than 50% ($p < 0.001$). Such trend was also found for the fecal taxa and atopy (61.0% (36/59)). In contrast, only 23.6% (13/55) and 29.8% (14/47) of the fecal taxa showed a consistent trend for food allergy and wheezing, significantly lower than 50% with a p value of <0.001 and 0.008, respectively. This indicates that the trend is inverse, an increase in abundance of the fecal taxa in AC children corresponds to a decrease in these taxa in children with food allergy or wheezing. These findings are observed for all the 4 analyses presented in Supplemental Table5 albeit different cut-off points of significance and difference. Fig. 3 shows the proportion of consistency in OP and fecal samples for food allergy and wheezing.

Discussion

This is the first study that compares the diversity and composition of human OP and fecal microbiomes in a single ethnic (Han) group of children living in either a Western (Australia) or Eastern (China) environment. Here, we use the term *western environment* as a phrase to collectively denote the socio-cultural, lifestyle, and geographical environment in industrialized countries, such as Australia, and the *eastern environment* as a collective term to indicate non-industrialized counties, such as China. The children were matched in age and gender, and we also matched the recruitment season in Australia and China. We selected this

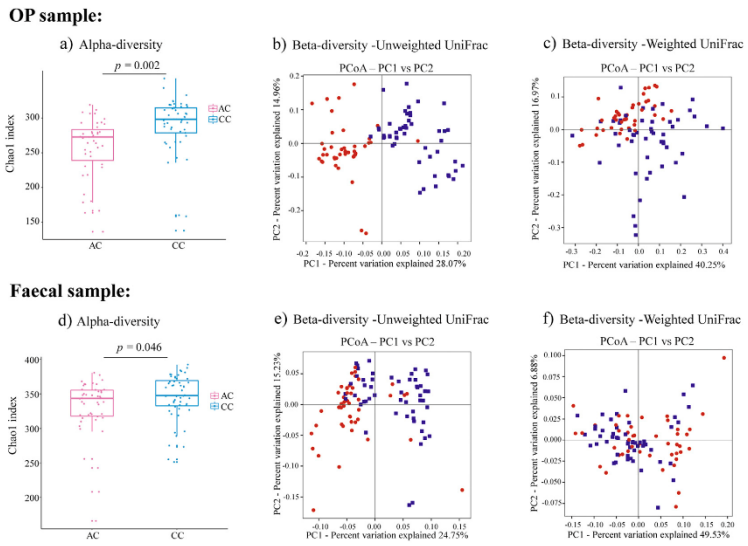

Fig. 1. – The alpha and beta diversity of OP and fecal samples from AC and CC children: Alpha diversities are exemplified by the Chao1 index for oropharyngeal (OP) samples in panel **a)**, as well as fecal samples in panel **d)**. The top and bottom lines of box plots showed the interquartile range, and lines inside the boxes represented medians. Beta diversities for OP samples are represented by Principal coordinate analysis (PCoA) plots of **b)**, unweighted and **c)**, weighted UniFrac matrix, whereas those for fecal samples are similarly represented in panels **e)**, unweighted and **f)**, weighted UniFrac matrix. For beta diversity analyses, data points represent either AC samples (red) or CC samples (blue), and the 2 major principle components are respectively represented on the x- and y-axes.

homogenous population to control the genetic influence so that only environmental exposure is varied, which is a main advantage of this study.

As expected, AC children had higher rates of atopy, food allergy, and wheezing compared to CC children. We hypothesized that the Australian environment has modified the human microbiome in Chinese immigrant children (microbiome modification by the western environment), thereby leading to more allergy in AC children (the western microbiome causing allergy). Consistent with the first part of our hypothesis, we found significant differences in microbial diversity, composition, and functional pathway expression in both OP and fecal microbiota between Chinese children living in Australia and China. We designed the study to measure significant differences in the microbiome between AC and CC children, rather than identifying specific taxa associated with allergic conditions in the population. To examine the hypothesis of the western microbiome relating to allergy we investigated if the change in relative abundance of taxa in AC children (compared to CC children) is also apparent in children with allergic conditions (relative to children without allergies) among the AC population to examine the hypothesis of the western microbiome relating to allergy. Such a consistent trend was significant (>50%) for food allergy and wheezing in the OP microbiome. This means that OP taxa which increase/decrease in the western environment are likely to show the same increase/decrease in children with food allergy and wheezing. In contrast, we found that such trend was significantly lower than 50% for fecal samples with these two phenotypes. fecal taxa which increase/decrease in the western environment likely show an opposite effect, namely decrease/increase, in children with food allergy or wheezing. Conceivably, the environment of upper respiratory tract and gastrointestinal tract are very different, and the way the microbial component interacts with the immune system differs considerably. The gut has numerous immunogenic regions, where microbial elements interact rapidly with regulatory T cells.[18] The segmented filamentous bacteria have the function of promoting intestinal T helper type (Th17) responses.[19] Therefore, the mechanism of interaction could be very different between the two sites as is the diversity and taxonomic groups in those two distinct areas.

This is a cross-sectional study and these trend consistencies do not indicate a cause-effect relationship. Children with food allergy and wheezing may have a changed immune status that changes the abundance of taxa. This may partly explain the inverse trend mentioned above. We think that the observed trends are unlikely to be false findings as they are consistently significant in all 4 analyses. Our study shows that western oropharyngeal and gut microbial flora are associated with allergic conditions in Chinese immigrant children. More studies are required to clarify the opposite trend that is observed for OP and gut microbiomes with food allergy and wheezing in industrialized countries.

Environmental biodiversity is important for human health. Lynch et al. reported that healthy children were exposed to richer and more diverse bacterial communities in the first year of life, compared to those children that developed either atopy or recurrent wheeze.[20] Another recent study compares the prevalence of asthma between Amish and Hutterites schoolchildren (similar genetic ancestries and lifestyle). It revealed that Amish children, living on a traditional farm, have been exposed to a more enriched microbiota environment and demonstrate low rate of asthma, compared to Hutterites children whose farming practice is industrialized.[21] There is emerging evidence that the environmental influence (environmental microbiomes) on shaping human microbiomes is a key element in tuning immune system and development of allergy. Several studies have shown that a reduced diversity of the human microbiome may be a risk for asthma and allergy.[22] A low microbial diversity in early infancy can potentially predict atopic dermatitis.[23] A longitudinal study demonstrated a lower oral bacterial diversity

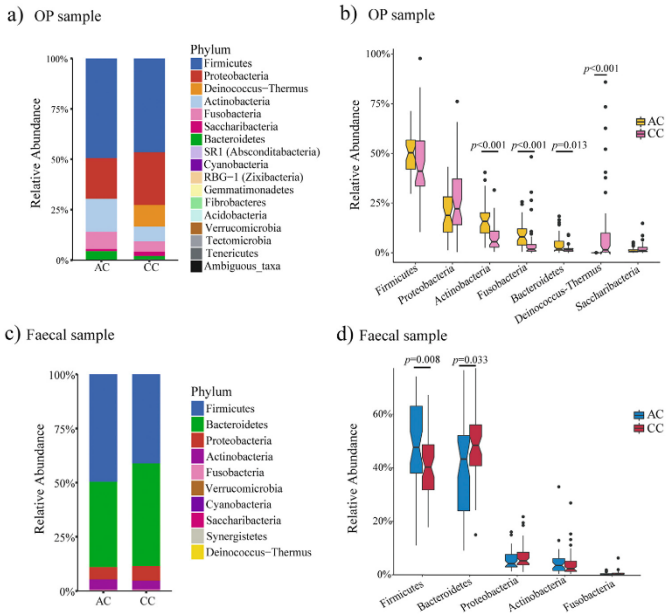

Fig. 2. – Bacteria relative abundance composition and comparison of oropharyngeal and fecal samples at the phylum level: The composition of bacterial relative abundance are shown in bar plots: panel **a)** oropharyngeal (OP) samples and **c)** fecal samples. The major phyla (relative abundance >1.0%) comparisons between AC and CC children are shown on box plots in panel **b)** OP samples and **d)** fecal samples. The top and bottom lines of box plots show the interquartile range, and lines inside the boxes represent medians, and black dots represent outliers.

among children who developed allergic disease, particularly asthma at an age of 7 years.[24] There are different indexes that estimate microbial diversity such as the Chao1 index as a richness estimator and the Shannon index for the bacterial evenness.[25] In our study both OP and fecal samples in AC children had a lower Chao1 index but a higher Shannon index. This indicates that the western environment has shaped the microbiome to have less richness and more evenness. In another population comparison study it was found that the alpha-diversity of the fecal microbiome

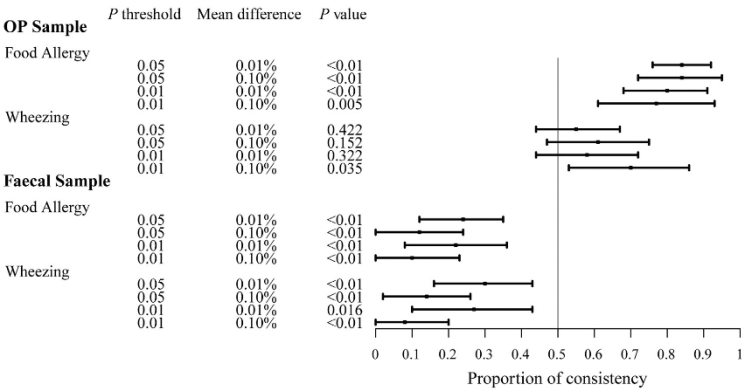

Fig. 3. – Consistency in trends of taxa abundance between the oropharyngeal (OP) and fecal microbiome: Left columns indicate the taxa selection thresholds *p* values (0.05 and 0.01) for the abundance comparison between AC and CC children, and the mean difference between with or without allergic conditions (over 0.01% or 0.10%). The "P value" column represents the significance of each binomial probability test. The horizontal axis of the plot represents the proportion of consistency of taxa abundance that is higher or lower in AC children (compared to CC children) and in children with allergic conditions (relative to children without allergies) among AC children. At the vertical "line of null effect" there is no consistent trend. Each horizontal line on the plot represents a consistent trend under certain thresholds. The black box indicates the mean value of the proportion of consistency, and the horizontal line represents the 95% confidence intervals.

(Chao1 and Shannon indexes) was higher in African children (non-industrialized) compared to those of European (industrialized environment like Australia) children.[26] The findings of these studies are largely consistent with our study. Microbial diversity variations in the human microbiome related to the western environment may provide a mechanistic explanation for the allergy epidemic in the past 60 or 70 years.

The microbiome profiles in AC children are significantly different from CC children. This indicates that western and eastern populations may be living with a different genus and species group of commensal microorganisms. This present study is not designed to ascertain which bacteria that are commonly present in western populations cause allergy, as it is likely a combination effect of many. Rather we analyze the difference between microbiome profiles in western and eastern populations to aid further studies to clarify their causal effects on asthma and allergy.

We discuss a few dominant taxa and compare our findings with recent literature. The genus *Streptococcus* (Firmicutes), a Gram-positive bacterium, has the largest abundance in both AC and CC children in OP samples but in a significantly higher proportion among AC children compared to CC children. Studies have shown that *Streptococcus* is associated with allergic symptoms. A 234 children cohort study revealed that early colonization of *Streptococcus* in the nasopharyngeal microbiota was a strong predictor for asthma during the first year of life, and its colonization was linked to atopy by the age of two years and chronic wheeze at age five.[27] Similarly, another study of neonatal oropharynx bacteria showed that a high burden of *Streptococcus* within the first month of life increased the risk for recurrent wheeze and asthma development.[28] That the gut microbiota is critical for immune development has been well documented.[29] The majority of genera that showed a significantly higher abundance in AC children compared to CC children were in the class Clostridia of phylum Firmicutes. Interestingly, a recent study found that the same class and phylum were enriched in fecal microbiome of food-allergic children compared to siblings and healthy children, but other *Clostridium* species were enriched in non-food-allergic subjects.[30] Class Clostridia has been associated with immune tolerance in mouse models of allergy and aids protection from allergic inflammation.[31] Our inverse association between the western fecal microbiome and food allergy and wheezing partly supports the association of the class Clostridia with food allergy reported in the literature.

The cell walls of Gram-negative bacteria contain lipopolysaccharide (LPS), which contribute to innate immune tolerance and help to prevent inappropriate immune stimulation through the microbiota-epithelial crosstalk.[32] Indeed, we found Gram-negative bacteria were higher among CC children in both OP and fecal samples using BugBase. Although the KEGG pathways provide limited understanding of the actual bacterial potential functions, differences in the expression of certain pathways can indicate potential associations.

One limitation is that this study is cross-sectional, and the results cannot determine causality. To Chinese migrants, the change to a western environment is the combination of a different diet, less air pollution, exposure to new allergens and, greater hygiene, all of which can lead to different microbiota composition/diversity, and contribute to the increased allergies in AC children.[33,34] However, in this study we could only focus on the overall influence of a western environment on the microbiome and the relation with allergy. Thirdly, recent antibiotic usages are known to have a significant impact on the human microbiome. Antibiotic use during the two-week period prior to the recruitment was 3 to 4 times more common in CC than in AC children. The disparity of antibiotic use in the two population may confound the findings in this study. Unfortunately, we did not collect a detailed history of antibiotic use in this population. In addition, the resolution of 16s rRNA sequencing is reliable down to the genus level. Studies utilizing whole-genome sequencing or real-time PCR, are of interest to further investigate the species and strains of bacteria that are different between the industrialized and non-industrialized environment, as well as to understand how the western microbiome shapes the immune system, leading to the development of asthma and allergy. Moreover, a comparison of microbiota and allergies present before and after immigration is worthy of investigating for future study.

Conclusion

We found evident differences in the compositions of the OP and gut microbiome between AC and CC children. The AC children demonstrated a lower microbial diversity richness and higher diversity evenness compared to CC children. The association of the OP microbiome with food allergy and wheezing is different from the gut microbiome in Chinese immigrant children in Australia. The western environment/lifestyle promotes a different human microbiome profile that may significantly contribute to the increased prevalence of asthma and allergy in industrialized countries.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This study was approved by the Human Research Ethics Committee (HREC) at the University of Western Australia. All parents provided an informed consent on behalf of their child.

Authors' contributions

J.G. was responsible for the data analysis, interpretation, and writing and revising the manuscript. Q.L., X.W., and A.S. contributed to the study design, recruitment, and data acquisition. A.A., X.Z., C.S.P, Y.S., P.E.M., G.A.D., and E.K.M verified the analytical methods, and contributed to manuscript revision. G.Z., F.L., and P.N.L. designed and directed the study, provided critical revision of the manuscript. G.Z. supervised all aspects of the study. All authors read and approved the final manuscript.

Funding

The study was funded by Telethon Perth Children's Hospital Research Funds.

Acknowledgements

We cordially thank Dr. Emilija Filipovska-Naumovska, Dr. Shu Chen, Dr. Yuchun Li, and Ms. En Nee Schultz for their contribution to data collection. We sincerely thank all the participants of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2019.100051.

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Appendix B: Additional paper published during candidature

This appendix contains one first-authorship paper published during candidature. This paper was a collaboration work with Children's Hospital of Capital Institute of Paediatrics.

Guo et al.
Allergy Asthma Clin Immunol (2019) 15:38
https://doi.org/10.1186/s13223-019-0351-4

Allergy, Asthma & Clinical Immunology

RESEARCH Open Access

Risk factors and prognosis of recurrent wheezing in Chinese young children: a prospective cohort study

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Abstract

Background: Nearly all the investigations into the risk factors for wheezing and asthma were conducted in developed countries with a high prevalence rate of asthma and allergy, but the studies in developing countries are limited. In this study, we aimed to investigate the risk factors for different wheezing phenotypes in Chinese young children and to explore the prognosis of recurrent wheezing.

Methods: This cohort study contained the recruitment stage and the follow-up stage conducted by phone questionnaire survey. According to the information collected at the follow-up for wheezing episodes and remission age, our cohort was divided into transient wheezing, persistent wheezing and late-onset wheezing. The wheezing symptoms and potential risk factors were compared between these three wheezing groups.

Results: From the initial 109 participants, 78.0% completed the follow-up survey. The frequency of current wheezing at followup was significantly reduced in all three groups compared to the recruitment stage ($p < 0.01$). We observe a trend that the rhinovirus (RV) and respiratory syncytial virus (RSV) infection rates were higher in the persistent wheezing group, and the overall infection rates appear to be the lowest in late-onset wheezing group at recruitment. At follow-up stage, the rates of rhinitis ever and current rhinitis were both higher in the persistent wheezing (63.0%, 50.0%) and late-onset wheezing groups (88.2%, 58.8%), compared to the transient wheezing group (14.3%, 14.3%). The incidence of current wheezing episodes increased cumulatively if the participant had concomitant risk factors of rhinitis ever, aeroallergens sensitization at recruitment, either alone or together with previous RV infection at the time of recruitment.

Conclusion: While the incidence of wheezing declined overall with age, but in addition to transient wheezers, additional subsets of children manifest persistent wheeze or late onset wheeze, and moreover the risk factors for wheezing display phenotypic variability between these subgroups. Rhinitis ever and aeroallergens sensitization, either alone or together with previous RV infection, were the most significant predictors for persistent wheezing in children in an eastern environment, such as in China.

Keywords: Preschool children, Wheezing, Phenotype, Risk factor

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Background

Recurrent wheezing among infant/preschoolers is very prevalent. This not only has an impact on the affected children and their families but also on the society due to increased utilization of ED visits and hospitalizations [1]. Although many young wheezers outgrow their symptoms, a significant proportion of these young children will keep wheezing throughout school age years, or even into adulthood [2]. Studies have shown that the years at preschool are crucial for asthma development as lungs and immune system develop and mature functionally [3, 4]. However, it is very difficult to identify which children with wheezing symptoms will develop asthma in later years. Early identification of recurrent wheezing in children could help physicians to improve the secondary preventive measures and suitable treatments.

To date, multiple risk factors have been identified that contribute to the development of persistent wheezing and susceptibility to asthma. These are: a family history of asthma or atopy, childhood eczema, allergic rhinitis, allergic sensitization (skin prick test and specific IgE), and early life infection with viruses [5, 6]. Notably, viral respiratory infections have been strongly associated with wheezing and susceptibility to asthma. Especially human rhinoviruses (RVs) are gaining recognition as an important risk factor for wheezing and asthma [7]. Jackson et al. found that wheezing RV illness in infancy is the most significant predictor of the development of pre-school wheezing at the age of 3 years, and also a predictor of the development of asthma at age of 6 years [8, 9]. Another recent study showed that sensitization, eczema, and RV infection are predictors for atopic asthma at the school age [10].

Nearly all the investigations into the risk factors for wheezing and asthma were conducted in developed countries with a high prevalence rate of asthma and allergy [11]. In contrast, there have been limited studies in developing countries. Although the prevalence of childhood asthma and allergy is increasing in developing countries, like China, it is still relatively low compared to that of the Western developed countries, like Australia, the UK, etc. [11, 12]. We hypothesize that the risk profiles for the prognosis of recurrent wheezing are different in developing countries, compared to developed countries. In this study, we aimed to investigate the risk factors for different wheezing phenotypes in Chinese young children and to explore the prognosis of recurrent wheezing.

Methods

Study design

Recruitment

This longitudinal cohort study was undertaken at the Children's Hospital of the Capital Institute of Pediatrics, Beijing, China. Figure 1 shows the flow of the study. During phase 1 (Oct 2013–May 2014) a total of 109 participants were recruited at the asthma outpatient clinic during their wheezing exacerbation.

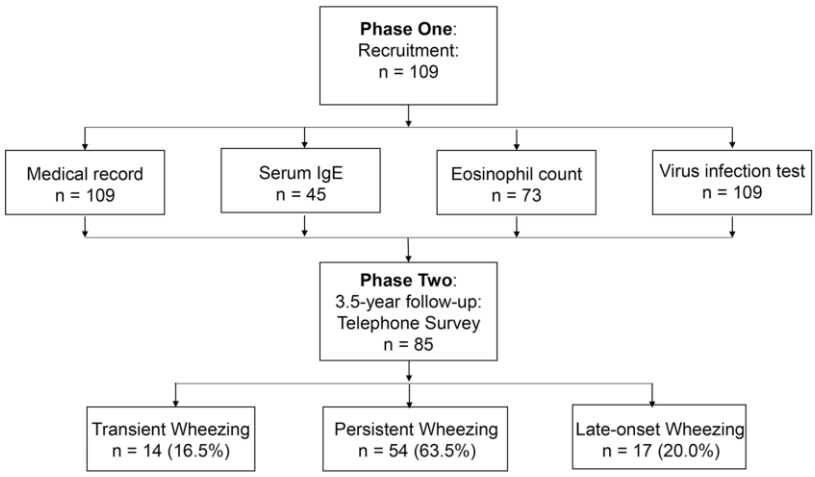

Fig. 1 The flow chat of the study design: a total number of 109 participant were recruited at phase 1, with all of them having medical record, and virus infection test, but 45 and 73 of them had got serum IgE and EOS count tested. 85 of the participants had finished the telephone survey at follow-up stage

The criteria of recruitment were aged from 6 months and up to 6 years old with 3 or more episodes of doctor-diagnosed wheezing. The exclusion criteria were: history of congenital pulmonary airway malformation, bronchopulmonary dysplasia, trachea cannula, severe pneumonia, severe immunodeficiency disease, and cardiovascular disease. The parents/guardians were asked to fill in a questionnaire for their children during the recruitment. Blood was taken to measure serum total and specific IgE (sIgE) and eosinophil (EOS) count in peripheral blood. Nasopharyngeal aspirate (NPA) specimens were collected to detect virus infection, which included the detection of RV, Human Metapneumovirus (hMPV), Bocavirus, respiratory syncytial virus (RSV), parainfluenza viruses type I, II and III (PIV), influenza types A and B (Flu).

Follow-up stage

Phase 2 (Oct–Nov 2017) was the follow-up of the phase 1 cohort. A specialist physician at the asthma clinic conducted a telephone questionnaire survey on the child's current wheezing status, etc.

This study was approved by the Ethics Committee of the Capital Institute of Pediatrics (SHERLL-2013072). All parents of the recruited children gave informed consent.

Questionnaire survey

The questionnaire at phase 1 captured baseline information on the child's date of birth, gender, birth history, siblings, breastfed history, pets ownership (at birth, and current), mother and other family members smoking history, onset age of wheezing, past-year wheezing episodes, rhinitis history, eczema history and parental history of asthma.

The telephone questionnaire during phase 2 recorded the following items, [1] current wheezing: Wheezing episodes in the past 12 months, [2] history of doctor-diagnosed rhinitis and eczema after recruitment, and [3] current rhinitis and eczema: Symptoms of doctor-diagnosed rhinitis and eczema in the past 12 months.

Serum IgE and peripheral blood eosinophil count

Serum was isolated for total IgE and specific IgE (sIgE) measurements using the ImmunoCap™ assays (Phadia, Sweden) on Phadia® 250 System (Thermo Fisher Scientific). Any sensitization was defined as positive sIgE results against common food allergens FX5 (egg white, cow's milk, codfish, wheat, peanut, soybean), and aeroallergens: Phad, mould mix (MX1), and house dust mite (HDM: Der p, Der f) with cutoff level of 0.35 kU/L [10]. Peripheral blood eosinophil count was obtained from XN-1000™ Hematology Analyzer (Sysmex Corporation).

Virus infection detection

The NPA specimens were processed within 3 h after collection. RV, hMPV and Bocavirus were screened by a reverse transcription-polymerase chain reaction. Direct detection of viral antigens by fluoroimmunoassay was carried out using UltraTMDFA Respiratory Virus Screening & ID Kit (Diagnostic Hybrids, USA) for RSV, PIV type I, II and III, Flu types A and B.

Grouping strategy

After the telephone survey of phase 2, our participants were assigned into 3 groups according to the study of Martinez et al. [5]:

1. Transient wheezing group: wheezing symptom started and remitted before age 3.
2. Persistent wheezing group: wheezing symptom started before age 3-year and persisted until age 6 or after.
3. Late-onset wheezing group: wheezing symptom started after age 3.

Statistical analysis

Results of the continuous variables (normal distribution) were expressed as mean ± SD. Non-normal distribution data were expressed as the median and inter-quartile range. The Mann–Whitney U test was utilized to compare the past-year wheezing episodes between recruitment and follow-up stages and the Kruskal–Wallis H test was used for comparing the current wheezing episodes and the onset age of wheezing between the three wheezing phenotype groups. Variance analysis was utilized for the three groups' comparison of EOS and logarithmic transformation of total serum IgE. Categorical variables were compared using χ^2 test. If the expected values in any of the cells of the contingency table were below 5, Fisher's exact test was used. Poisson regression was used to determine the incidence rate ratio (IRR) of current wheezing episodes at the follow up and its association with concomitant risk factors adjusted for the confounders. SPSS 17.0 was used for the statistical analysis and RStudio (Version 1.0.153) was used for creating figures.

Results

Characteristics of the participants and the wheezing status comparison

The average age of the 109 participants at the start of the study (phase 1) was 2.6 ± 2.3 year and 80 (73.4%) were males. At phase 2, 85 (78.0%) of the participants' parents completed the telephone survey, and 66 (77.6%) of these children were boys. Forty-three (50.6%) of the

participants were initially recruited in spring, while 24 (28.2%) and 18 (21.2%) were recruited in autumn and winter, respectively. The demographic information are shown in Table 1. There was no significant difference in the gender frequency, birth history, recruitment season, siblings, breastfed history, childcare, pet ownership, mother and other family member smoking history between the different wheezing phenotypes.

The number of current wheezing episodes had decreased in all groups, including those with persistent and late onset wheezing (both of the $p<0.01$) (Fig. 2). However, there was no significant difference in current wheezing between the different phenotypes of wheezing groups, both at recruitment ($p=0.925$) and the follow-up stage ($p=0.323$) (Table 1). Comparison of the onset

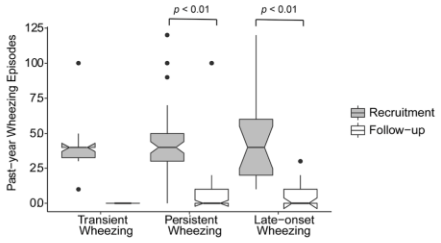

Fig. 2 The current wheezing episodes comparison between recruitment and follow-up stages in three different phenotypes: Notched boxes showed the inter quartile ranges, lines inside the boxes represented medians, and dots were outliers

Table 1 Patients' characteristics and wheezing episode

	Transient wheezing (n = 14)	Persistent wheezing (n = 54)	Late-onset wheezing (n = 17)	χ^2	*p*
Age at recruitment (mean ± SD)	1.32 ± 0.62	2.61 ± 1.44	4.57 ± 0.79	–	–
Age at follow-up (mean ± SD)	4.95 ± 0.70	6.28 ± 1.26	8.19 ± 0.75	–	–
Gender [n (%)]				0.62	0.734
Male	11 (78.6)	43 (79.6)	12 (70.6)		
Female	3 (21.4)	11 (20.4)	5 (29.4)		
Birth history [n (%)][a]				3.60	0.134
Full-term	11 (78.6)	47 (87.0)	17 (100.0)		
Preterm	3 (21.4)	7 (13.0)	0 (0.0)		
Recruitment season [n (%)][a]				1.24	0.891
Spring	9 (64.3)	26 (48.1)	8 (47.1)		
Autumn	3 (21.4)	16 (29.6)	5 (29.4)		
Winter	2 (14.3)	12 (22.2)	4 (23.5)		
Siblings [n (%)][a]					
No-siblings	8 (57.1)	37 (68.5)	14 (82.4)	2.32	0.321
Breastfed history [n (%)][a]					
Yes	14 (100.0)	50 (92.6)	15 (88.2)	1.39	0.496
Childcare [n (%)][a]					
Yes	0 (0.0)	1 (1.9)	1 (5.9)	1.54	0.605
Pets kept when the child was born [n (%)][a]					
Yes	2 (14.3)	8 (14.8)	2 (11.8)	0.17	1.000
Current pet ownership [n (%)][a]					
Yes	2 (14.3)	6 (11.1)	2 (11.8)	0.40	0.892
Mother smoking history [n (%)][a]					
Yes	1 (7.1)	1 (1.9)	1 (5.9)	2.14	0.300
Other family member smoking history [n (%)][a]					
Yes	9 (64.3)	31 (57.4)	10 (58.8)	0.25	0.949
Onset age of wheezing (months)[b]	6.0 (5.8, 12.0)	12.0 (6.0, 24.0)	36.0 (36.0, 48.0)	43.66	*< 0.001*
Current wheezing episode at recruitment[b]	4 (3, 4)	4 (3, 5)	4 (2, 6)	0.16	0.925
Current wheezing episode at follow-up[b]	0 (0, 0)[c]	0 (0, 1)	0 (0, 1)	0.98	0.323

Italic values indicate the significance of *p* value ($p < 0.05$)

[a] Fisher's exact test was used for groups' comparison

[b] Variables were expressed as median (inter-quartile)

[c] Children in Transient wheezing group stopped wheezing symptom at follow-up

age of wheezing showed that the transient wheezing group started earlier than the persistent wheezing group ($p=0.039$).

Risk factors for the three different wheezing phenotypes

We have compared the risk factors between the three different wheezing phenotypes (Table 2). The total virus infection rates were 42.9%, 51.9%, and 29.4% for the transient, persistent, and late-onset wheezing groups, respectively. The RV infection rate was the highest among the tested viruses in all three groups, followed by the RSV positive rate. Although there was no significant difference between the three wheezing phenotype groups, we did observe a trend that the RV and RSV infection rates were higher in the persistent wheezing group, and the overall infection rate appear the lowest in late-onset wheezing group.

There was a significant difference for 'rhinitis ever' between the three groups ($p<0.01$). The rhinitis rates of the persistent and late-onset wheezing groups were both higher than the transit wheezing group (both $p<0.01$). We found that the current rhinitis rate of the persistent wheezing and late-onset wheezing groups was also higher than transient wheezing group at the follow-up stage ($p=0.016$, $p=0.011$).

The total IgE of the transient wheezing group was significantly lower than the other two groups ($p<0.05$). However, there was no significant difference of total IgE level between the persistent and late-onset wheezing groups. The prevalence of a positive specific IgE to aeroallergen was highest in the late-onset wheezing group, but no difference was observed in the prevalence of mould mix, house dust mite (HDM) and food allergen sIgE positive. Significant difference was detected for the percentage of EOS in peripheral blood ($p=0.045$) between the three wheezing groups, with the highest EOS percentage in late-onset wheezing group.

The concomitant risk factors for the incidence of current wheezing episodes

The incidence of current wheezing episodes increased cumulatively if the participant had concomitant risk

Table 2 The comparison of wheezing risk factors between the three wheezing phenotypes

	Transient wheezing (n = 14)	Persistent wheezing (n = 54)	Late-onset wheezing (n = 17)	Statistics	p
Information from recruitment					
Total virus positive [n (%)]	6 (42.9)	28 (51.9)	5 (29.4)	$\chi^2=2.68$	0.281
RV positive [n (%)][a]	2 (14.3)	20 (37.0)	3 (17.6)	$\chi^2=3.85$	0.150
RSV positive [n (%)][a]	1 (7.1)	8 (14.8)	1 (5.9)	$\chi^2=0.91$	0.703
PIV positive [n (%)][a]	2 (14.3)	3 (5.6)	0 (0.0)	$\chi^2=2.45$	0.239
hMPV positive [n (%)][a]	0 (0.0)	0 (0.0)	1 (5.9)	$\chi^2=3.43$	0.365
Bocavirus positive [n (%)][a]	0 (0.0)	1 (1.9)	0 (0.0)	$\chi^2=1.12$	1.000
Flu positive [n (%)][a]	1 (7.1)	0 (0.0)	0 (0.0)	$\chi^2=3.82$	0.165
Serum IgE test (n = 45)	n = 7	n = 29	n = 9		
Total IgE (ku/L)	59.8 ± 35.1	193.7 ± 206.4	237.7 ± 202.7	–	–
Log transformed total IgE	0.7 ± 0.4	2.1 ± 0.4	2.3 ± 0.3	$F=5.03$	*0.011*
Atopy	4 (57.1)	24 (82.8)	9 (100.0)	$F=4.36$	0.076
Aeroallergens positive (Phad) [n (%)][a]	1 (14.3)	11 (37.9)	7 (77.8)	$\chi^2=6.71$	*0.031*
Mould mix positive (MX1) (n = 38)[a]	1 (16.7)	6 (25.0)	5 (62.5)	$\chi^2=4.16$	0.128
House dust mite positive (HDM) (n = 37)[a]	0 (0.0)	7 (30.4)	4 (50.0)	$\chi^2=3.88$	0.167
Food allergens positive [n (%)][a]	2 (28.6)	15 (51.7)	5 (55.6)	$\chi^2=1.39$	0.623
EOS in peripheral blood (n = 68)	n = 10	n = 44	n = 14		
Percentage (%)	2.60 ± 2.54	3.65 ± 2.41	5.06 ± 2.27	$F=3.24$	*0.045*
Absolute count ($\times 10^9$/L)	0.27 ± 0.24	0.39 ± 0.30	0.49 ± 0.30	$F=2.56$	0.085
Parental asthma history [n (%)][a]	1 (7.1)	5 (9.3)	1 (5.9)	$\chi^2=0.23$	1.000
Information from follow-up					
Rhinitis ever [n (%)]	2 (14.3)	34 (63.0)	15 (88.2)	$\chi^2=18.00$	*0.000*
Current rhinitis [n (%)]	2 (14.3)	27 (50.0)	10 (58.8)	$\chi^2=7.14$	*0.028*
Eczema ever [n (%)]	8 (57.1)	36 (66.7)	10 (58.8)	$\chi^2=0.64$	0.727
Current eczema [n (%)][a]	1 (7.1)	7 (13.0)	3 (17.6)	$\chi^2=0.74$	0.804

Italic values indicate the significance of p value ($p<0.05$)

[a] Fisher's exact test was used for groups' comparison

factors (Table 3). The significance did not change after adjustment for the confounders we investigated in this study, including gender, birth history, number of siblings, breastfed history, childcare, pets ownership, mother and other family members' smoking history, onset wheezing age (months), past-year wheezing episode at recruitment and parental asthma history. Four confounders were finally included in the regression model. The incidence rate ratios (IRRs) of wheezing increased from with either rhinitis or RV infection (3.61, $p<0.01$) to both (4.54, $p=0.01$), compared to neither, respectively. The IRRs were also significantly high with either aeroallergens positive or RV infection (2.54, $p=0.05$), and marginally significant with both (3.44, $p=0.07$). In addition, children with both rhinitis and aeroallergens positive were 4.36-fold higher of IRR than children with none of the risk factors ($p<0.01$). Poisson regression showed concomitant rhinitis and/or RV infection strongly indicated an increased risk of wheezing at the follow up and Fig. 3 visualizes the overlapping characteristics of these two risk factors among the three different wheezing phenotypes. The overlap of rhinitis and RV infection was 14 (25.9%) in persistent wheezing group compared to 0 (0.0%) and 2 (11.8%) in the transient and late-onset wheezing groups, respectively.

Table 3 The incidence rate ratios (IRRs) of concomitant risk factors for the incidence of current wheezing episodes

	IRRs	95% CI		*p*
		Lower	Upper	
No rhinitis and no RV	–	–	–	–
Rhinitis or RV	3.61	1.46	8.94	*0.005*
Rhinitis and RV	4.54	1.43	14.46	*0.010*
No eczema and no RV	–	–	–	–
Eczema or RV	1.50	0.69	3.25	0.307
Eczema and RV	2.17	0.76	6.19	0.149
No aeroallergens positive and no RV	–	–	–	–
Aeroallergens positive or RV	2.54	1.00	6.45	*0.050*
Aeroallergens positive and RV	3.44	0.89	13.29	0.073
No rhinitis and no aeroallergens positive	–	–	–	–
Rhinitis or aeroallergens positive	2.25	0.86	5.87	0.097
Rhinitis and aeroallergens positive	4.36	1.56	12.14	*0.005*

Italic values indicate the significance of *p* value ($p < 0.05$)

Poisson regression model was employed after adjusting for gender, onset wheezing age (months), past-year wheezing episode at recruitment and parental asthma history

95% CI 95% confidence intervals

Discussion

The prevalence of wheezing has been increasing recently in China [13] but the prognosis of recurrent wheezing during young age is unknown in developing countries. To the best of our knowledge, this is the first longitudinal study conducted in China that investigates the prognosis of recurrent wheezing in young children and identifies risk factors for different wheezing phenotypes.

The strength of our study is that it includes the comprehensive collection of medical records, viral etiology, serum IgE test, and EOS count at study entry, and is combined with a 3.5-year follow-up by an experienced asthma clinic specialist practitioner. We found that rhinitis and aeroallergen sensitization, either alone or together with early RV infection, were the most significant predictors for subsequent persistent and late onset wheeze in China. Previously, an observational study conducted in Turkey investigated the short-term prognosis and risk factors of recurrent wheezing in the first 3 years of life [14]. Maternal smoking during

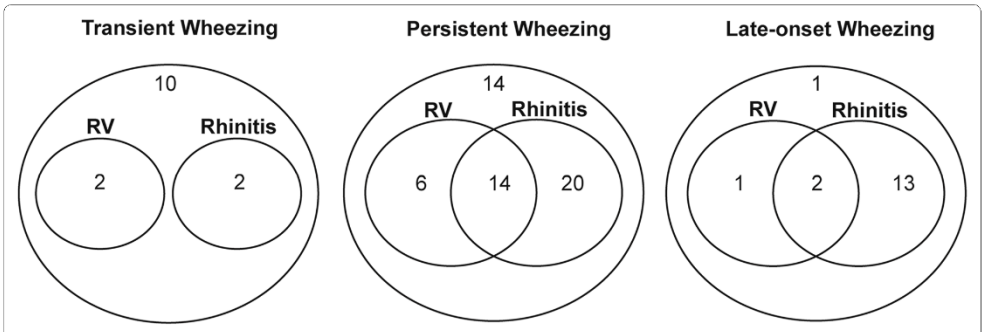

Fig. 3 Patient numbers of wheezing phenotypes with sole and overlapping risk factors (RV and rhinitis): Venn diagrams were draw to show the concomitant of RV infection and rhinitis in the three different wheezing phenotypes

pregnancy and emergency room admissions were significant risk factors for the persistence of wheezing. A study in Mozambique by O'Callaghan-Gordo et al. showed that an initial episode of lower respiratory infection (LRI) with RV during infancy could increase the risk of wheezing [15].

We found that the number of wheezing episodes had decreased significantly during the follow-up stage and the onset age of wheezing was earlier in the transient wheezing than in the persistent wheezing group. The findings are consistent with earlier studies from Martinez et al. [5] and Matricardi et al. [16] showing that the incidence of wheezing declined with age. The respiratory system gradually undergoes development with age which may be an important reason for the decrease in the number of wheezing episodes [17]. In addition, our findings echo the previous reports that the risk factors of wheezing also show phenotypic variability [18, 19]. A large population-based cohort in the UK showed that episodic viral wheeze decreased with age, but multiple trigger wheeze (mainly due to exercise or aeroallergen-associated wheeze) increased [20]. Martinez suggested that transient wheezing in young children is not associated with an atopic predisposition [21].

It is well known that respiratory tract virus infection is an important cause for early childhood wheezing and asthma [22]. Jackson et al. revealed that RV and RSV are the main viruses that cause wheezing, and RV infection can affect the long-term prognosis of children with wheezing [23]. Early life RV-induced wheezing can increase the asthma risk at school age [24]. We observed 37% of RV in children with persistent wheezing and less than 20% of RV in children with the other wheezing phenotypes, although these differences were not statistically different due to the relatively small sample size. To date, studies on the mechanisms of respiratory virus-induced wheeze in children has surged, driven by efforts to find better interventions to reduce wheezing exacerbations. A recent review recapitulated that RV type C was associated with a decrease in expression of a cellular receptor specific for this virus-CDHR3, and a decrease in interferon-β expression [25]. The shift to focus on the mechanisms and new intervention methods is promising.

Allergic rhinitis (AR) is often associated with asthma and asthma is present in 15% to 38% of the patients with AR, and nasal symptoms are found in 6% to 85% patients with asthma [26]. Indeed we found that the incidence of rhinitis ever and current rhinitis were more common in the persistent and late-onset wheezing groups compared to the transient wheezing group. The sIgE results showed that a positive allergy response to aeroallergens was lower in the transient wheezing groups. Serum IgE levels has long been associated with asthma and allergic sensitization is an independent predictor for the persistence of wheezing [27, 28]. Interestingly, when we analyzed the concomitant risk factors we found that the increase of wheezing episodes in the year preceding follow up was associated with overlapping RV and/or rhinitis, RV and/or aeroallergens sensitization, rhinitis and aeroallergens sensitization. This confirms RV, aeroallergens and rhinitis, more likely working together are dominant risk factors for persistent wheezing in China. As noted above, this constellation of risk factors closely resembles those reported for developed countries, despite the fact that as a developing country China has different social/economic contexts and lower allergy prevalence compared to industrialized countries.

In this regard, a community-based birth cohort study demonstrated that viral infections interact with atopic sensitization in infancy to enhance the susceptibility to asthma development and the occurrence of both of the two factors is associated with maximal risk for subsequent asthma [29]. Jackson and colleagues also showed that asthma rate was highest in infants with both sensitization and RV-related wheezing, but RV-related wheezing was the most significant predictor of subsequent asthma in the Childhood Origins of ASThma (COAST) study [8]. Consistently, a recent cohort study revealed that first RV-induced wheezing alone or together with sensitization could predict atopic school-aged asthma [10]. Notably, in our result, children in persistent and late-onset wheezing groups are approximately equivalently "atopic" relative to the children in transient wheezing group. We speculate that the reason why onset of asthma is faster in the persistent wheezing group is because the early impact of viral infections on them was higher, whereas it took longer time for the late-onset wheezing group to accumulate enough viral-associated tissue damage to catch up and start expressing symptoms. On the other hand, we suggest that the late-onset wheezing group could be driven by atopy alone and in the absence of high level viral infection, it takes longer for lung function to deteriorate. However, the wheezing symptom in transient wheezing group is simple and mainly due to the congenitally smaller airways not being able to manage viral inflammation and children in this group grow out of that because they don't have the "second hit" from atopy.

This study has limitations that should be taken into account. Firstly, we acknowledge the small sample size of this study. Nonetheless, the conclusion should be interpreted with caution. Secondly, not all the participants had serum IgE and EOS count measured at recruitment. This is because that not all patients were covered by health insurance for serum IgE test due to

policy (e.g. outpatient clinic attendance in non-local tertiary hospitals). However, this novel Chinese cohort provided insights into the risk factors and prognosis of early life wheezing in an eastern environment. The focus for future researches should be larger, preferably multicentre, study cohorts with extensive follow-up to elucidate the risk factors in the development of asthma in children living in developing countries.

In conclusion, we show that while the incidence of wheezing declined overall with age, but in addition to transient wheezers, additional subsets of children manifest persistent wheeze or late onset wheeze, and moreover the risk factors for wheezing display phenotypic variability between these subgroups. Rhinitis ever and aeroallergens sensitization, either alone or together with previous RV infection, were the most significant predictors for persistent wheezing in children in China.

Abbreviations
RV: rhinovirus; RSV: respiratory syncytial virus; hMPV: human metapneumovirus; PIV: parainfluenza virus; Flu: influenza; EOS: eosinophil; IRR: incidence rate ratio; HDM: house dust mite; LRI: lower respiratory infection; AR: allergic rhinitis.

Acknowledgements
We sincerely thank Prof. Peter Le Souëf (School of Paediatrics and Child Health, the University of Western Australia) for his assistance of the questionnaire design.

Authors' contributions
JG and CL contributed to the study design, data analysis and interpretation, and drafting and revising the manuscript. WZ and HW contributed to the study design, recruitment, and data acquisition. PGH and GZ contributed to the data analysis, interpretation, and critical revision of the manuscript. All authors read and approved the final manuscript.

Funding
This research was supported by Special Scientific Research Fund of Public Welfare Profession of China from National Health and Family Planning Commission of the PRC [Grant Number 201502025] and Key Medical Specialty Development Plan of Beijing Hospital Administration [ZYLX201829].

Availability of data and materials
The data that support the findings of this study are not publicly available due to the undergoing of more analysis, but are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Capital Institute of Pediatrics (SHERLL-2013072). All parents of the recruited children gave informed consent.

Competing interests
The authors declare that they have no competing interests.

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Received: 4 October 2018 Accepted: 5 June 2019
Published online: 13 June 2019

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