

Accepted Date : 22-Sep-2015
Article type : Research Letter

Eczema susceptibility and composition of faecal microbiota at four weeks of age: A pilot study in Chinese infants

M.F. Tang,¹ H.Y. Sy,¹ J.S.L. Kwok,² W.H. Tam,³ K.L. Hon,¹ C.K.C. Tung,² G.W.K. Wong,¹
S.K.W. Tsui,² and T.F. Leung¹

Departments of ¹Paediatrics and ³Obstetrics and Gynaecology and ²School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong

Running title: Eczema and stool microbiome

Funding sources: This study was funded by Research Committee Group Research Scheme (3110087) and Direct Grant for Research (2013.2.033) of The Chinese University of Hong Kong.

Conflicts of Interest: All authors do not have any conflict of interest to declare.

Correspondence:

Ting F. Leung, MD, FRCPCH, Department of Paediatrics, 6/F, Lui Che Woo Clinical Sciences Building, Prince of Wales Hospital, Shatin, N.T., Hong Kong.

Tel: +852 2632 2981. Fax: +852 2636 0020. E-mail: tfleung@cuhk.edu.hk

DEAR EDITOR, Early-life microbial exposure plays an important role in the development of

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bjd.14205

This article is protected by copyright. All rights reserved.

immune system whereby reducing the likelihood of allergy. Several studies investigated alterations in the relative abundance of bacterial species in gut microbiota. *Escherichia coli* were more abundant in infants who developed atopic eczema than healthy infants.¹ Reduced abundance of *Bifidobacterium* has also been reported in allergic patients compared to controls.² Reduced gut microbial diversity in early life was also important in modulating allergy risk.³⁻⁷ The methods being used changed from low-throughput bacterial cultures, terminal restriction fragment length polymorphism^{3,4}, denaturing gradient gel electrophoresis⁵ to the more efficient 16S rRNA pyrosequencing^{6,7}. More recently, next-generation sequencing (NGS) and bioinformatics tools facilitated the non-targeted sequencing and un-biased analysis of the entire gut microbiome.^{8,9} Faecal microbiome is likely to be ethnic-specific and diet-specific, but there is limited metagenomics data in Asians. This prospective pilot study made use of NGS to characterise stool microbiome in Hong Kong Chinese neonates and investigate faecal microbial factors that are associated with the subsequent development of eczema.

149 Chinese term neonates with uneventful perinatal course and born in our hospital in September 2012 were followed for 9 months. The occurrence of allergic diseases was ascertained by Chinese International Study of Asthma and Allergy in Childhood questionnaire.^{10,11} Eczema diagnosis was confirmed according to criteria by Hanifin and Rajka.¹² The Clinical Research Ethics Committee of our University approved this study, and all subjects' parents gave informed written consent to participate.

Fifteen infants with eczema and 15 non-allergic controls at 9 months old were identified, and their frozen random stool samples (~1 g each) collected at 4 weeks of age were retrieved for NGS using Ion 318 Chip v2 by Ion PGM™ System (see online supplement). All these subjects were formula-fed term infants with normal birth weight (≥ 2.5 kg). Besides, they

must not receive antibiotics during the neonatal period. The Shannon-Weaver diversity index was used to measure microbial diversity of stool samples.⁶ Briefly, this index was calculated by the formula $-\sum \log(\pi_i)\pi_i$ where π_i denotes relative abundance of species. It incorporates the richness and evenness of species in the community, typically with a value between 1.5 and 3.5. Differences of this index and different bacterial taxa at genus level between eczema and controls at 9 months were analysed by Mann-Whitney U test.

Table S1 in online supplement describes infants whose stool samples were subjected to NGS. Five controls were excluded due to insufficient DNA, so 15 cases and 10 controls were sequenced. All clinical features were matched between these two groups. Fig. 1 illustrates average relative abundances of dominant bacterial genera in infants' stool samples, whereas Fig. S1 describes the corresponding results in each case and control subject. There was high inter-subject variability in stool microbiome at 4 weeks of age. Table 1 summarises the relative abundance of dominant bacterial genera. No significant association was detected between eczema and any bacteria with $\geq 1\%$ relative abundance, including *Bacteroides*, *Escherichia*, *Klebsiella*, *Bifidobacterium*, *Streptococcus* and *Lactobacillus*. Among the less abundant genera (relative abundance $< 1\%$), *Campylobacter* was more abundant in cases (median 0.008%, IQR 0.003-0.022%) than controls (median 0.001%, IQR 0.001-0.004%) while *Roseburia* was less abundant in eczema (median 0%, IQR 0-0.063%) than controls (median 0.055%, IQR 0.002-0.270%). Nonetheless, Shannon-Weaver diversity index of stool microbiota at 4 weeks was similar between infants with eczema and non-allergic controls at 9 months (median [IQR]: 1.28 [0.94-1.93] versus 1.47 [1.31-1.80]; $P=0.698$).

This pilot study provided high-resolution picture of stool microbiome in Chinese newborns using NGS. Whereas we did not confirm the importance of the more abundant bacterial genera^{1,2}, *Campylobacter* and *Roseburia* (mean relative abundance $< 1\%$) were

associated with the occurrence of eczema by 9 months. *Campylobacter* was more abundant in our eczema patients than controls. Previous studies found that *Campylobacter* disrupted the intestinal epithelial barrier, which allowed the translocation of non-invasive bacteria such as *Escherichia coli*¹³ and primed the intestine for inflammatory responses in susceptible subjects. On the other hand, *Roseburia* was less abundant among our eczema patients. This butyrate-producing bacterium inhibited histone deacetylase activity, leading to hyperacetylation of histones and thus suppression of nuclear factor-kappa B activation.¹⁴ *Roseburia* also reinforced colonic defence barrier by enhancing production of mucins and antimicrobial peptides as well as expression of tight junction proteins.¹⁵

This study found substantial interethnic variation in stool microbiota. Abrahamsson et al. reported using 16S rRNA pyrosequencing that *Bifidobacterium* was more abundant and *Bacteroides* less abundant in their healthy Swedish infants.⁶ These findings were opposite to what we found in Chinese neonates (Table S2). *Clostridium*, *Parabacteroides* and *Lactobacillus* were found only in Chinese eczema and healthy Swedish newborns. *Bifidobacterium*, *Bacteroides* and *Streptococcus* were found among top 5 genera among cases and controls in both populations. We believed that these differences could be explained by our use of cutting-edge NGS that yielded much denser microbial sequences.

Reduced microbial diversity conferred increased eczema risk in Swedish⁶ and Australian infants⁴. On the other hand, we could not detect any association between infantile eczema and neonatal microbial diversity as assessed by Shannon-Weaver diversity index. Whereas this study might be underpowered (15 cases and 10 controls) for stool microbiome analysis, our result could also be due to interethnic difference in gut microbial compositions⁹ or different nature of datasets that was generated from Ion Torrent NGS (metagenomics with all microbes and human) and 16S rRNA pyrosequencing (purely bacterial) platforms.

Small sample size is the major limitation of this exploratory study, which prohibits us to adjust for confounders such as family history and diet that are known to modulate eczema risk. The findings of this pilot study should thus be considered as preliminary and interpreted cautiously. Albeit statistically significant, the small differences in low-abundant bacteria between cases and controls cast doubt on their clinical relevance. We expect that a larger study sample will not alter the relative abundances of *Campylobacter* and *Roseburia* in infants' stool samples, but it will enhance our confidence in concluding about any association between eczema and presence of these bacteria and also allow any significant difference in abundances of dominant bacteria such as *Bifidobacterium* and *Klebsiella* to show up. Besides, we were unable to investigate the relationship between allergy risk and dynamics of faecal microbiome because only one random stool sample was collected when subjects were 4 weeks old. Another limitation is that stool microbiota might not necessarily reflect microbial colonisation in the gut (especially small intestine). We tried to overcome this problem by adopting high-resolution and un-biased NGS technology to capture information about gut microbiome.

In conclusion, eczema susceptibility might be associated with low-abundant bacteria but not microbial diversity in neonatal stool of Chinese infants.

References

- 1 Penders J, Thijs C, van den Brandt PA *et al.* Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. *Gut* 2007; **56**:661-7.
- 2 Watanabe S, Narisawa Y, Arase S *et al.* Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* 2003; **111**:587-91.

- 3 Wang M, Karlsson C, Olsson C, et al. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol* 2008; **121**: 129-34.
- 4 Ismail IH, Oppedisano F, Joseph SJ *et al.* Reduced gut microbial diversity in early life is associated with later development of eczema but not atopy in high-risk infants. *Pediatr Allergy Immunol* 2012; **23**:674-81.
- 5 Bisgaard H, Li N, Bonnelykke K *et al.* Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol* 2011; **128**:646-52.
- 6 Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012; **129**:434-40.
- 7 Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy* 2014; **44**:842-50.
- 8 Turnbaugh PJ, Hamady M, Yatsunenko T *et al.* A core gut microbiome in obese and lean twins. *Nature* 2009; **457**:480-4.
- 9 Li J, Jia H, Cai X *et al.* An integrated catalog of reference genes in the human gut microbiome. *Nat Biotechnol* 2014; **32**:834-41.
- 10 Leung TF, Yung E, Wong YS, Lam CW, Wong GW. Parent-reported adverse food reactions in Hong Kong Chinese preschoolers: epidemiology, clinical spectrum and risk factors. *Pediatr Allergy Immunol* 2009; **20**:339-46.
- 11 Wang SS, Hon KL, Kong AP *et al.* Eczema phenotypes are associated with multiple vitamin D pathway genes in Chinese children. *Allergy* 2014; **69**:118-24.
- 12 Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980; **2**: 44-7.

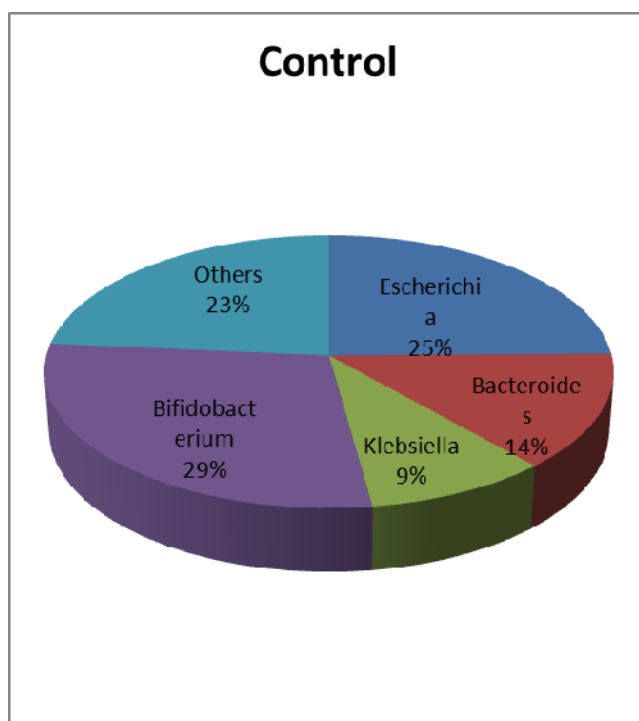
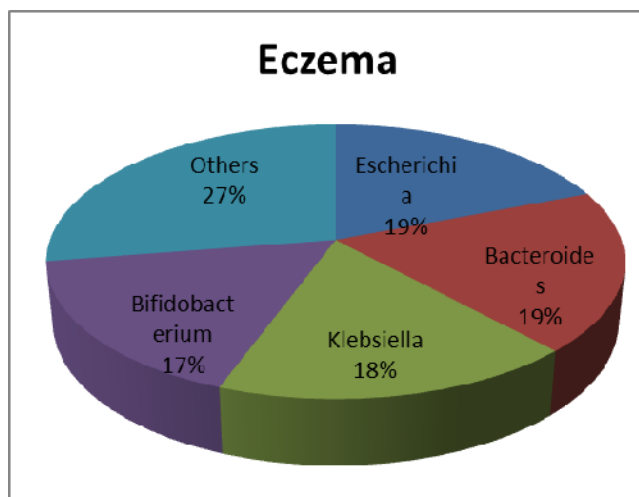
- 13 Lamb-Rosteski JM, Kalischuk LD, Inglis GD, Buret AG. Epidermal growth factor inhibits *Campylobacter jejuni*-induced claudin-4 disruption, loss of epithelial barrier function, and Escherichia coli translocation. *Infect Immun* 2008; **76**:3390-8.
- 14 Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. *J Biol Chem* 2001; **276**:44641-6.
- 15 Wang HB, Wang PY, Wang X, Wan YL, Liu YC. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig Dis Sci* 2012; **57**:3126-35.

Table 1 The relative abundance of the dominant bacterial genera (top 10 genera of any subjects) in stool samples obtained at 4 weeks of age from infants with eczema or without any allergy

Genus	Eczema (n=15)		Controls (n=10)		P-value by Mann-Whitney U test
	Mean (SD)	median % (IQR)	Mean (SD)	median % (IQR)	
<i>Bacteroides</i>	19.04 (27.71)	4.423 (0.001-31.039)	13.67 (17.06)	5.855 (1.788-29.175)	0.723
<i>Escherichia</i>	19.08 (24.38)	7.959 (1.741-35.408)	24.78 (21.14)	20.286 (8.022-36.906)	0.177
<i>Klebsiella</i>	17.51 (28.28)	0.681 (0.214-38.444)	9.37 (18.00)	0.743 (0.100-13.155)	0.428
<i>Bifidobacterium</i>	16.89 (26.62)	4.407 (0.014-26.743)	28.60 (24.78)	20.813 (6.578-44.074)	0.091
<i>Streptococcus</i>	3.08 (4.00)	1.505 (0.751-3.163)	2.00 (1.91)	1.343 (0.770-2.731)	0.723
<i>Lactobacillus</i>	2.31 (4.70)	0.015 (0.004-0.590)	1.26 (2.48)	0.031 (0.018-1.641)	0.367
<i>Veillonella</i>	1.98 (3.00)	0.450 (0.034-2.210)	2.23 (3.09)	0.230 (0.016-5.288)	0.935
<i>Parabacteroides</i>	1.61	0.825	0.89	0.161	0.531

	(2.55)	(0.001-2.458)	(2.15)	(0.041-0.496)	
<i>Alistipes</i>	0.72 (2.04)	0.083 (0.002-0.426)	0.15 (0.19)	0.112 (0.008-0.199)	0.935
<i>Eggerthella</i>	0.49 (1.71)	0.016 (0.000-0.117)	0.08 (0.10)	0.052 (0.009-0.117)	0.605
<i>Enterobacter</i>	0.91 (1.84)	0.124 (0.028-1.157)	0.09 (0.14)	0.029 (0.014-0.110)	0.216
<i>Salmonella</i>	0.42 (0.62)	0.180 (0.061-0.299)	0.25 (0.21)	0.199 (0.070-0.393)	0.892
<i>Enterococcus</i>	1.30 (3.47)	0.129 (0.028-0.924)	0.46 (0.59)	0.245 (0.016-0.735)	1.000
<i>Lactococcus</i>	0.40 (0.74)	0.024 (0.002-0.416)	0.86 (2.19)	0.149 (0.008-0.420)	0.238
<i>Shigella</i>	0.16 (0.23)	0.049 (0.017-0.273)	0.20 (0.17)	0.123 (0.083-0.320)	0.129
<i>Clostridium</i>	2.35 (4.94)	0.074 (0.048-0.380)	0.35 (0.76)	0.100 (0.024-0.255)	0.935
<i>Staphylococcus</i>	0.18 (0.28)	0.043 (0.010-0.218)	0.11 (0.09)	0.076 (0.036-0.173)	0.683
<i>Prevotella</i>	0.52 (1.04)	0.034 (0.001-0.081)	0.04 (0.04)	0.021 (0.004-0.070)	0.807
<i>Citrobacter</i>	0.10 (0.27)	0.005 (0.001-0.071)	0.01 (0.02)	0.004 (0.001-0.017)	0.311
<i>Cronobacter</i>	0.12 (0.26)	0.026 (0.010-0.080)	0.03 (0.02)	0.018 (0.007-0.038)	0.461
<i>Tannerella</i>	1.13 (2.32)	0.023 (0.005-0.038)	0.02 (0.02)	0.011 (0.004-0.036)	0.723
<i>Campylobacter</i>	0.10 (0.32)	0.008 (0.003-0.022)	0.00 (0.01)	0.001 (0.001-0.004)	0.019
<i>Acidaminococcus</i>	0.03 (0.05)	0.003 (0.000-0.033)	0.39 (1.15)	0.009 (0.002-0.074)	0.495
<i>Roseburia</i>	0.06 (0.10)	0.000 (0.000-0.063)	0.12 (0.13)	0.055 (0.002-0.270)	0.036

Fig 1. Average relative abundances of dominant bacterial genera in 15 cases and 10 controls.



ONLINE SUPPLEMENT

Eczema susceptibility and composition of faecal microbiota at four weeks of age: A pilot study in Chinese infants

M.F. Tang,¹ H.Y. Sy,¹ J.S.L. Kwok,² W.H. Tam,³ K.L. Hon,¹ C.K.C. Tung,² G.W.K. Wong,¹
S.K.W. Tsui,² and T.F. Leung¹

Departments of ¹Paediatrics and ³Obstetrics and Gynaecology and ²School of Biomedical
Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong

Correspondence:

Ting F. Leung, MD, FRCPCH, Department of Paediatrics, 6/F, Lui Che Woo Clinical
Sciences Building, Prince of Wales Hospital, Shatin, N.T., Hong Kong.

Tel: +852 2632 2981. Fax: +852 2636 0020. E-mail: tfleung@cuhk.edu.hk

Next-generation Sequencing of Stool Samples

Bacterial DNA was extracted from 0.2-0.4 g of these stool samples by Powersoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to manufacturer's protocol. DNA concentration was measured by Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, CA), with 1.25 ng/μl being the minimum DNA concentration for sequencing. Extracted DNA was fragmented with BioRuptor® UCD-200 TS Sonication System on low power level, at intervals of 0.5 minutes, for a total 75 minutes. Library preparation and barcoding was done using Ion Plus Fragment Library Kit (Ion Torrent™, Life Technologies, CA) and Ion Xpress™ Barcode Adapters 1-16 Kit. The Agilent 2100 Bioanalyzer and the associated High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) were used to determine quality and concentration of the libraries. The amount of library required for template preparation was calculated using the Temple Dilution Factor calculation. Emulsion PCR and enrichment steps were performed using Ion PGM™ Template OT2 200 Kit on Ion OneTouch™ 2 System, and sequencing done using Ion PGM™ Sequencing 200 Kit v2, Ion 318 Chip v2 and Ion PGM™ System. Reads from each patient were filtered for low quality (Phred quality < 20). The taxonomic classification of the reads was assigned by BLASTn.

Table S1 The clinical features of 9-month-old infants whose stool samples were subjected to NGS and microbiome analyses

Feature	Eczema (n=15)	Controls (n=10)
Male	9 (60.0)	5 (50.0)
Birth weight, kg	3.25 ± 0.37	3.17 ± 0.50
Born by vaginal delivery	12 (80.0)	9 (90.0)
With older siblings	5 (33.3)	3 (30.0)
Parental history of asthma/eczema	9 (60.0)	5 (50.0)
Family history of asthma/eczema	9 (60.0)	5 (50.0)
Breastfeeding ever	12 (80.0)	9 (90.0)
Exclusive breastfeeding at 1 month	4 (26.7)	2 (20.0)
Wheeze ever	3 (20.0)	1 (10.0)
Physician-diagnosed asthma	0 (0)	0 (0)
Physician-diagnosed allergic rhinitis	2 (13.3)	0 (0)
Physician-diagnosed food allergy	0 (0)	0 (0)
Atopy	2 (13.3)	3 (30.0)
NESS score	6.5 ± 1.5	NA
Objective SCORAD score	8.2 ± 9.3	NA

NESS, Nottingham Eczema Severity Score; NGS, next-generation sequencing; SCORAD, SCORing Atopic Dermatitis.

Results expressed in number (percentage) or mean ± standard deviation.

No significant difference between two groups was found for any parameter.

Table S2 Comparison of relative abundances of dominant genera in stool samples obtained at 4 weeks between our Chinese and Swedish infants

Genus	South Eastern Sweden ^{Ref *}		Hong Kong Chinese (this study)	
	Healthy (n=20), %	Eczema (n=20), %	Healthy (n=10), %	Eczema (n=15), %
<i>Bifidobacterium</i>	29 (22)	41 (35)	29 (25)	17 (27)
<i>Bacteroides</i>	21 (22)	7 (13)	14 (17)	19 (28)
<i>Parabacteroides</i>	2 (4)	<1	1 (2)	2 (3)
<i>Streptococcus</i>	9 (9)	11 (14)	2 (2)	3 (4)
<i>Enterococcus</i>	1 (3)	3 (6)	<1 (1)	1 (3)
<i>Lactobacillus</i>	2 (4)	<1	1 (2)	2 (5)
<i>Veillonella</i>	2 (2)	3 (6)	2 (3)	2 (3)
<i>Clostridium</i>	2 (8)	2 (6)	<1 (1)	2 (4)

Results expressed in mean (standard deviation) for relative abundance values.

* Abundance data was not reported for *Campylobacter* and *Roseburia*.

Ref: Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012; **129**:434-40.

Genus-Level Microbial Distribution of Stool Microbiome

This stacked bar chart illustrates the relative abundance of various bacterial genera in stool samples. The y-axis shows the percentage of each genus, ranging from 0% to 100%. The x-axis lists 27 samples, identified by IDs such as NM14_001, NM142_002, and NM127_007. The legend identifies 14 genera: Staphylococcus, Enterococcus, Lactobacillus, Streptococcus, Parabacteroides, Clostridium, Homo, Klebsiella, Alistipes, Veillonella, Escherichia, Bifidobacterium, Uncultured, and Bacteroides. The chart shows significant variation in microbial composition between samples, with some dominated by Bacteroides (e.g., NM14_001) and others by Escherichia (e.g., NM127_007).