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Ratio of *Klebsiella/Bifidobacterium* in Early Life Correlates with Later Development of Paediatric Allergy

J.S.Y. Low¹, S.-E. Soh^{2,3,#}, Y.K. Lee⁴, K.Y.C. Kwek^{5,#}, J.D. Holbrook^{2,6}, E.M. Van der Beek^{7,8}, L.P. Shek^{2,3,#}, A.E.N. Goh^{5,#}, O.H. Teoh^{5,#}, K.M. Godfrey^{9,#}, Y.-S. Chong^{2,10,#}, J. Knol^{7,11}, and C. Lay^{1,3,*}

¹Nutricia Research, Danone Nutricia Early Life Nutrition, Matrix Building #05-01B, 30 Biopolis Street, 138671 Singapore, Singapore

²Singapore Institute for Clinical Sciences, Agency for Science and Technology Research (A*STAR), 30 Medical Drive, 117609, Singapore, Singapore

³Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 5 Science drive 2, 117597, Singapore, Singapore

⁴Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 5 Science drive 2, 117597, Singapore, Singapore

⁵KK Women's and Children's Hospital, Singapore, Singapore

⁶Human Development & Health Academic Unit, University of Southampton & NIHR Southampton Biomedical Research

Centre, University of Southampton & University Hospital Southampton NHS Foundation Trust,

Tremona Road, Southampton, SO16 6YD, UK

⁷Nutricia Research, Uppsalalaan 12, 3584 CT, Utrecht, the Netherlands

⁸Department of Pediatrics, University Medical Centre Groningen, University of Groningen,

P.O. Box 30.001, 9700 RB Groningen, the Netherlands

⁹MRC Lifecourse Epidemiology Unit, and NIHR Southampton Biomedical Research Centre, University Hospital

Southampton NHS Foundation Trust and University of Southampton, Tremona Road, SO16 6YD

Southampton, United Kingdom

¹⁰Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 5

Science drive 2, 117597 Singapore, Singapore

¹¹Laboratory of Microbiology, Wageningen University, P.O. Box 7005, 6700 CA, Wageningen, the Netherlands

Abstract

Several studies have reported that intestinal microbial colonization patterns differ between non-allergic and allergic infants. However, the microbial signature underlying the pathogenesis of allergies remains unclear. We aim to gain insight into the development of the intestinal microbiota of healthy infants and infants who develop allergy in early life, and identify potential microbiota biomarkers of later allergic disease. Using a case-control design in a Chinese sub-cohort of a Singaporean birth cohort (GUSTO), we utilized 16S rRNA gene sequencing to assess intestinal

Corresponding author: Christophe Lay, PhD, Nutricia Research, Danone Nutricia Early Life Nutrition, Matrix Building #05-01B, 30 Biopolis Street, Singapore 138671. Tel: +65 6830 9421, Fax: +65 6830 9410, christophe.lay@danone.com.

[#]on behalf of GUSTO Study Group

Conflict of Interest

The authors June S. Y. Low, Eline M. Van Der Beek, Jan Knol and Christophe Lay are employees of Nutricia Research. KMG has received reimbursement for speaking at conferences sponsored by companies selling nutritional products, and is part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone.

microbial composition and diversity of 21 allergic and 18 healthy infants at 3 weeks, 3 months and 6 months of age, and correlated the microbiota with allergy at ages 18 and 36 months. Pronounced differences in intestinal microbiota composition between allergic and healthy infants were observed at 3 months of age. The intestine of healthy infants was colonised with higher abundance of commensal *Bifidobacterium*. Conversely, *Klebsiella*, an opportunistic pathogen, was significantly enriched in the allergic infants. Interestingly, infants with a high *Klebsiella/Bifidobacterium* (K/B) ratio (above the population median K/B ratio) at age 3 months had an odds ratio of developing allergy by 3 years of age of 9.00 (95% confidence interval 1.46 – 55.50) compared to those with low K/B ratio. This study demonstrated a relationship between the ratio of genera *Klebsiella* and *Bifidobacterium* during early infancy and development of paediatric allergy in childhood. Our study postulates that an elevated *Klebsiella/Bifidobacterium* ratio in early infancy could be a potential indicator of an increased risk of allergy development. This line of research might enable future intervention strategies in early life to prevent or treat allergy. Our study provides new insights into microbial signatures associated with childhood allergy, in particular, suggests that an elevated *Klebsiella/Bifidobacterium* ratio could be a potential early-life microbiota biomarker of allergic disease.

Introduction

Over recent decades the prevalence of childhood allergic diseases has been increasing worldwide, with large numbers of children suffering from asthma, rhinitis and/or food allergies (Pawankar *et al.*, 2013). Particularly, the manifestation of allergies in early life, such as atopic eczema, could pose a risk for developing other allergies — which could persist in the individual over years or decades (Bieber *et al.*, 2012).

The rise in allergic diseases has in large been attributed to the hygiene hypothesis. The hypothesis originated from Strachan's observation of a link between the increased incidence of allergic diseases and improved hygiene standards and smaller family size (Strachan, 1989). The hygiene hypothesis was later revisited, and expanded to the 'microbiota hypothesis' which suggests that altered intestinal microbiota colonization in early life may have an impact on the maturation of the immune system and contribute to the increase in allergies (Noverr and Huffnagle, 2005). Support for the microbiota hypothesis comes from a series of epidemiological studies, demonstrating that the microbial colonization dynamics differ between allergic and healthy children (Bjorksten *et al.*, 2001; Kalliomaki *et al.*, 2001; Nakayama *et al.*, 2011; Renz-Polster *et al.*, 2005; Sjogren *et al.*, 2009; Thompson-Chagoyan *et al.*, 2011; Watanabe *et al.*, 2003; Woodcock *et al.*, 2002; Yap *et al.*, 2014). A number of studies also reported an association between reduced intestinal microbial diversity in early life and allergic manifestations in childhood (Abrahamsson *et al.*, 2012; 2014; Bisgaard *et al.*, 2011; Forno *et al.*, 2008). Collectively, these studies suggest that a compromised quality of intestinal microbial diversity and a disturbed equilibrium of microbiota composition in early life may impair host immunity and increase host vulnerability to allergy development.

Although multiple studies have described a link between the role of intestinal microbiota and allergy, to date, the key microbial signature(s) that may underlie allergy development remains unclear. Hence, in this study, we aim to gain insight into the development of

intestinal microbiota of healthy infants and infants who developed childhood allergy, and identified plausible microbiological signatures associated with allergic disease later in life.

Materials and Methods

Study Population

A subset of the Chinese population (HUBBLE cohort) from the GUSTO birth cohort (Soh *et al.*, 2012; Soh *et al.*, 2014) was selected to conduct a case-control study on the link between allergy and microbiota. Written informed consents were obtained from all participants prior to study enrolment, and the study was approved by local ethics committees. Twenty-one allergic cases and eighteen healthy controls (non-allergic) were selected based on availability of stool samples and the assessment of allergy outcomes at 18 months of age, and followed up until 36 months of age. Allergic infants were closely matched to the corresponding healthy controls for birth weight, gestational age, feeding pattern, weaning age and medication. The allergic infants were examined by means of cumulative incidence of allergic manifestations (i.e. allergic eczema, allergic rhinitis and asthma) up to 18 and 36 months of age; according to the study eczema workflow (see **Figure E1** in the Online Repository). The severity of eczema was further assessed using SCORAD if the infant was confirmed as having eczema. Skin prick tests were also conducted in all infants at 18 months of age to assess for sensitization to common food (cow's milk, peanut and egg) and dust mite allergens (Der f/Der p).

Sample Collection

Faecal samples were collected from infants at 3 weeks, 3 months and 6 months of age by the parents using sterile faeces containers. These samples were stored at -80°C until microbiological analysis.

DNA extraction, PCR and 454-Pyrosequencing of the 16S rRNA gene

DNA was extracted from 0.2 gram of faecal sample with QIAamp DNA stool mini kit (Qiagen, Netherlands) according to the manufacturer's instructions, with additional bead-beating step. The V3 - V5 hypervariable regions of the bacterial 16S rRNA gene were amplified using 'bifidobacteria-optimised' primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 926Rb (5'-CCGTCAATTYMTTTRAGT-3') published by Sim *et al.* (Sim *et al.*, 2012). Emulsion PCR and pyrosequencing assays were performed by Beijing Genomics Institute (Shenzhen, China).

Bioinformatics Analyses

A total of 1,141,656 raw sequencing reads were denoised, demultiplexed and filtered using Mothur software (version 1.31.2) to obtain high quality unique reads. The reads were then aligned with NAST-based sequence aligner to a customized reference alignment adopted from SILVA (Pruesse *et al.*, 2007). Identified chimeric sequences via UCHIME algorithm (Edgar *et al.*, 2011) were excluded from the denoised sequences. Classification of high-quality reads were performed utilizing Bayesian classifier with RDP database. The Operational Taxonomic Unit (OTU) numbers were calculated by Mothur software based on 97% nucleotide similarity cut-off. The distribution of OTU in various taxonomic levels were

analysed by QIIME scripts (version 1.50). Indices of alpha diversity for each sample including observed species, Chao1 and ACE were generated using Mothur software. Beta diversity was assessed by weighted and unweighted unifracs distances calculated in QIIME. The differences of species diversity between samples were displayed by Principal Coordinate Analysis (PCoA) generated from unifracs distance matrices. Multivariate statistical analysis applying Principal Component Analysis (PCA) was generated by R package (R-Core-Team, 2014) while the distance based-redundancy analysis (dbRDA) was performed using CANOCO 5 software (Šmilauer and Lepš, 2014) to explain the (dis)similarities in compositional structure of the intestinal microbiota of allergic and healthy infants.

Statistical Analyses

With the aid of biostatistics software GraphPad Prism 6 (GraphPad Software, Inc., USA), differences in population characteristics between the healthy controls and allergic infants were determined by Fisher's exact test (2 categorical data) or Chi-square test (> 2 categorical data). For continuous data, differences between the two groups were performed using non-parametric Mann-Whitney U tests. Similarly, computation of odds ratios were estimated using the GraphPad Prism 6. The statistical tests performed were regarded as significant only if the differences between the two infant groups had a $P < .05$.

To assess differences in relative abundances of bacterial taxa between allergic infants and healthy controls in the first six months of life, two-group Mann-Whitney U tests were applied using R software version 3.2.1 (R-Core-Team, 2014). For all statistical tests performed at phylum, class, order, family and genus level, p-value correction was applied when > 10 taxa (with an average relative abundance above 1% at the same phylogenetic level) were involved. Multiple testing accounting for FDR correction was performed using the "qvalue" package (version 2.0.0) in R (Storey, 2002), with a Q value cut-off of 0.05.

Results

Population Characteristics

A total of 21 allergic and 18 healthy Chinese infants were selected from HUBBLE study, a subset of the Singaporean birth cohort (GUSTO) (Soh *et al.*, 2012; Soh *et al.*, 2014). Some characteristics pertaining to these infants are described in Table I. Several population characteristics including gender, mode of delivery, infant-feeding patterns, gestational age, birthweight, weaning age, infants' medication and pre-pregnancy maternal BMI were well distributed and did not differ between the two groups ($P = .05$). Subjects with parental allergic history was marginally higher in allergic infants compared to the healthy infants ($P = 0.04$) (Table I).

In this study, the development of allergy typically occurred in infants at the median age of 6 months, and at times as early as in the first month of life (Table 2). The majority of the allergic infants ($n = 15$) developed their first allergy symptom(s) only at or after age 3 months (Supplementary Table S1). Only four infants developed allergy before age 3 months, while the information for age of allergy diagnosis was absent for two infants. Clinical

symptoms of the allergic infants were relatively heterogeneous. Within the allergic group, 18 infants (85.7%) were diagnosed with eczema; with 2 of them experiencing eczema and allergic rhinitis simultaneously by 18 months of age (Table 2; Supplementary Table S1). Only one infant was diagnosed solely with allergic rhinitis at this age. Additionally, some of these allergic infants were sensitized against allergen sources such as cow's milk ($n=1$), peanut ($n=1$), egg ($n=1$) and Der f/Der p ($n=6$). Of interest was also the follow-up of these infants at 36 months of age; all infants with positive allergy diagnosis at 18 months of age remained allergic at age 3 years, suggesting persistency of their allergic symptoms into childhood (Table 2). Moreover, approximately 50% ($n = 10$) of these allergic infants developed multiple allergy phenotypes by 36 months of age, including onset of asthma (Supplementary Table S1).

Faecal Microbiota Profile of Allergic and Healthy Infants

A total of 117 faecal samples were collected from the 39 infants at ages 3 weeks, 3 months and 6 months. Of these 117 samples, 31 samples were excluded from the study due to inadequate amount of sample material or poor quality of DNA. Therefore, the final numbers of samples subjected to 16S rRNA amplicon sequencing were 24 samples at week 3 ($n = 12$ allergic, $n = 12$ healthy), 30 samples at month 3 ($n = 18$ allergic, $n = 12$ healthy) and 32 samples at month 6 ($n = 17$ allergic, $n = 15$ healthy).

Among the 86 samples analyzed, there were 739,575 high-quality filtered reads produced, with an average of 8,600 (range from 3,948 to 17,695) sequences per infant. These sequences were clustered with representative sequences at 97% similarity levels, and generated a total number of 611 OTUs. Relative (to total) abundances of bacterial taxa between allergic infants and healthy controls were compared at each phylogenetic level to investigate differential taxa for allergy development. The faecal microbiota of all the infants was dominated by five abundant phyla: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Verrucomicrobia (Supplementary Figure S2). At 3 weeks of age, the faecal microbiota profiles of allergic infants and healthy controls were highly similar, with no significantly differential abundance in any phylum or genus (Table 3).

The most pronounced differences in the intestinal microbiota composition between allergic and healthy infants were observed at 3 months of age. Phylum Actinobacteria (42.95%, $P = 0.02$) was significantly more abundant in healthy controls compared to the allergic infants (25.73%) at 3 months (Table 4). Analysis at the genus level identified nine genera that differed significantly between the two groups. The predominant distinguishable genera (with average relative abundance above 1%) between allergic and healthy infants included genera *Klebsiella*, *Actinomyces* and *Lachnospiraceae incertae sedis* (Table 4, Figure 1A). Genus *Klebsiella* within the phylum Proteobacteria, was significantly over-represented in allergic infants (12.26% in allergic vs 1.66% in healthy, $P = 0.02$, $Q = 0.04$). Disproportionately high abundances of *Klebsiella* were identified in 50% of allergic infants ($n=9/18$) at 3 months, whereas this genus was almost absent in infants who did not develop allergy ($n=1/12$) (Figure 1B). Interestingly, investigation at species level found highest representative of the OTU corresponding to species *Klebsiella pneumoniae* (not shown). In contrast, genera *Actinomyces* ($P = 0.01$, $Q = 0.03$) and *Lachnospiraceae incertae sedis* ($P = 0.01$, $Q = 0.03$)

were found to be significantly higher in the microbiota of healthy controls compared to allergic infants at 3 months, although it is noteworthy that these genera were identified only in a few healthy subjects. Moreover, there were (non-significant) trends for commensal *Bifidobacterium* and *Akkermansia* to be more abundant in the microbiota of healthy controls (28.84% for *Bifidobacterium*, 4.56% for *Akkermansia*) compared to allergic infants (17.08% for *Bifidobacterium*, 0.06% for *Akkermansia*) at 3 months (Figure 1A).

The compositional dissimilarities observed at 3 months did not persist at weaning age. At 6 months of age, there was no discernably abundant bacterium significantly over or under-represented. Although *Butyricoccus*, a butyrate-producing bacterium was found to be significantly higher in healthy controls at 6 months ($P = 0.03$), this genus existed in low abundance (<1%) (Table 5).

Faecal Microbial Diversity Profile Between Allergic and Healthy Infants

Complexity analysis of a single sample was determined using the observed species metric, Chao1 and ACE. Infants who developed allergy had significantly lower microbial richness at age 3 months ($P = .05$), but not at 3 weeks or 6 months (Figure 2).

Based on unweighted and weighted unifracs PCoA plots, the faecal microbial composition of allergic infants and healthy controls appeared to separate along the first component at 3 months (Figure 3). No definite cluster could be identified in PCoAs of the faecal microbial composition of allergic and healthy infants for week 3 and month 6 samples (not shown). These results were in line with taxonomical results reported earlier, i.e. altered faecal microbiota profiles in allergic infants observed only at 3 months.

Multivariate Analysis of Faecal Microbiota Composition of Allergic and Healthy Infants at 3 Months of Age

Multivariate cluster analysis was performed on entire 3 months faecal samples to gain further insights into microbial factors influencing the clustering pattern observed between allergic and healthy infants. To determine the associations between the abundances of specific bacterial genera and the clinical phenotype, ten genera which showed strongest loading on PC1 and PC2 were plotted on the PCA biplot. Among them, eight unique genera explaining the differences were depicted in Figure 4.

The healthy controls cluster closely towards higher PC2 scores, populating the upper quadrants of the PCA biplot (Figure 4). The principal coordinate analysis revealed that the intestinal microbiota of healthy infants was predominantly characterized by commensal *Bifidobacterium*, followed by *Enterococcus* and to a slight extent by *Lachnospiraceae incertae sedis* (Figure 4). Indeed, *Bifidobacterium* was the most abundant bacterial genus in healthy controls at 3 months of age, which accounted for 28.84% of total genera (Figure 1A).

In contrast, most of the allergic infants were widely clustered towards lower PC2 scores and higher PC1 scores (Figure 4). The segregation of the intestinal microbiota of allergic infants towards lower PC2 scores was mostly governed by *Klebsiella*, and to some degree by *Bacteroides*. Conversely, the subset of allergic infants clustered on the high PC1 and PC2

scores (upper right quadrant) were positioned in between the genus component loadings of *Klebsiella* and *Escherichia/Shigella*. In fact, allergic infants in this cluster shared common microbial community with some of the healthy controls, and were driven mainly by facultative anaerobes *Escherichia/Shigella*.

Among the predominant genera described, *Klebsiella* and *Bifidobacterium* were identified as the most dominant genera that seemed to drive the separation of allergic and healthy infants at 3 months. After adjustment for potential confounder (i.e. mode of delivery), *Klebsiella* and *Bifidobacterium* remained as major taxa (relative to other bacteria measured) governing the segregation of allergic and healthy infants at this age. (Supplementary Results and Supplementary Figure S3). To validate this hypothesis, we compared the abundance ratio of *Klebsiella* to *Bifidobacterium* (K/B ratio) between allergic and healthy infants at age 3 weeks, 3 months and 6 months respectively. Using Mann-Whitney U test, we observed that the differential ratio of relative abundances of *Klebsiella* to *Bifidobacterium* were significant ($P = 0.01$) between the two groups only at 3 months, but not at 3 weeks or 6 months (Supplementary Table S2).

Relationships Between K/B Ratio and Risk of Developing Allergy

To explore further whether K/B ratio could be utilized to discriminate between allergic and healthy infants at 3 months, the K/B ratio was compared across all infants in this study, independently of their clinical phenotype. The K/B ratio was derived from subjects whose stool samples were available at 3 months ($n = 18$ out of 21 allergic infants, $n = 12$ out of 18 healthy infants). Analysis at individual level showed that most allergic infants had a high K/B ratio value - with twelve of the allergic infants ranking above the median K/B ratio of this population (Table 6, Figure 4).

Consequently, we analyzed the association of K/B ratio at 3 months in relation to the risk of developing childhood allergy by ages 18 months and 36 months. Using the median K/B ratio as a cut-off, infants who had a high K/B ratio had a 6-fold increased odds of developing allergy by age 18 months (odds ratio 6.00, 95% confidence interval 1.17 – 30.74) compared to those with a low K/B ratio (Table 7). Similarly, infants with a high K/B ratio had a 9-fold increased odds of developing allergy by age 3 years (odds ratio 9.00, 95% confidence interval 1.46 – 55.50) compared to those with a low K/B ratio.

Discussion

The present study investigated the development of intestinal microbiota of non-allergic and allergic infants during the first 6 months of life, with the aim of identifying potential microbiota biomarkers of allergic disease in children. We found that distinct differences of intestinal microbiota composition between non-allergic and allergic infants were evident at the age of 3 months.

To our knowledge, this is the first study to describe the inverse correlation between *Bifidobacterium* and *Klebsiella*, and its association with paediatric allergy. Particularly noteworthy was that infants who harbored a high *Klebsiella/Bifidobacterium* (K/B) ratio at age 3 months had higher odds of developing allergy by ages 18 months and 3 years,

suggesting potential clinical relevance of the K/B ratio in prediction of allergy risk later in life.

At age 3 months, genus *Klebsiella* was significantly over-represented in allergic infants compared to the healthy controls. About 50% of the allergic infants remained persistently colonized with *Klebsiella* at age 3 months, while the abundance of *Klebsiella* decreased substantially in all healthy infants at this age. On the contrary, the allergic infants were colonized with a lower abundance of *Bifidobacterium*, *Akkermansia* and *Lachnospiraceae incertae sedis* group at age 3 months. These results supported the findings of previous studies (Bjorksten *et al.*, 2001; Candela *et al.*, 2012; Fujimura *et al.*, 2016; Ismail *et al.*, 2016; Nakayama *et al.*, 2011; Sjogren *et al.*, 2009; Watanabe *et al.*, 2003; Yap *et al.*, 2014); and confirmed results reported by Nakayama *et al.* (Nakayama *et al.*, 2011), which showed higher abundance of *Klebsiella* in the first 2 months of life before the onset of allergic symptoms. Arrieta and colleagues also recently demonstrated that infants with a depleted abundance of certain bacterial taxa, including genus *Lachnospira* at age 3 months, were more likely to subsequently develop asthma (Arrieta *et al.*, 2015).

Specific alterations of intestinal microbiota have been associated with inflammatory related processes. Inflammation has been described as an important contributing factor for non-communicable diseases such as cardiovascular diseases, metabolic diseases and allergic diseases (Prescott, 2013). In the context of allergic diseases, inflammation in early life should not be trivialized as it has been perceived as the first step in the progression of “atopic march” (Bantz *et al.*, 2014). Although the implications of intestinal colonization of *Klebsiella* for allergy disease are limited, it is known that bacteria belonging to genus *Klebsiella* are associated with induction of pro-inflammatory responses in the host. For instance, *Klebsiella pneumoniae* was shown to be highly correlated with coligenotic phenotype in IBD mice model (Garrett *et al.*, 2010). A similar connection between *Klebsiella* and intestinal inflammation was also described in studies in infants with colic (de Weerth *et al.*, 2013; Rhoads *et al.*, 2009). In contrast, the intestinal microbiota of healthy infants at the age of 3 months was dominated by *Bifidobacterium*. This is in accordance with several previous studies in which high levels of bifidobacteria were associated with reduced risk of allergic diseases (Kalliomaki *et al.*, 2001; Renz-Polster *et al.*, 2005; Sjogren *et al.*, 2009; Watanabe *et al.*, 2003; Yap *et al.*, 2014). Past *in vitro* studies have shown that *Bifidobacterium* species contributed to colonization resistance to several intestinal pathogens (Kondepudi *et al.*, 2012). Also, it was demonstrated in murine models that an infant gut-associated strain of *Bifidobacterium breve* is associated with anti-inflammatory and anti-allergic effects by downregulating the expression of inflammatory molecules (Ohtsuka *et al.*, 2012).

At age 6 months, these compositional differences in intestinal microbiota between allergic infants and healthy controls were not sustained. It is known that at weaning age, changes in consumption of complex carbohydrates and dietary fibres in infants induce a major transition in the intestinal microbiota composition and diversity (Edwards and Parrett, 2002). Therefore, due to the increased complexity of microbial configuration during weaning, it is conceivable that our findings at age 6 months could be limited by the sample size.

Intestinal colonization by *Klebsiella* is not an unfamiliar occurrence in early life, and is usually held in check by the groups of commensal bacteria in the intestinal ecosystem (Kamada *et al.*, 2013). However, in the event of disturbance of intestinal microbial community due to antibiotic usage or pathological factors, some of the commensal bacteria may be eliminated or outcompeted by the opportunistic colonizers. Although the exact mechanism underlying the possible effects of *Bifidobacterium* against *Klebsiella* remains to be elucidated, it is hypothesized that the suppression of pathogen colonization is dictated by direct or indirect mechanisms of colonization resistance (Kamada *et al.*, 2013). A past *in vitro* study supported the antimicrobial activity of *Bifidobacterium* strains against members of coliforms, including of the genus *Klebsiella* (Aloisio *et al.*, 2012; Simone *et al.*, 2014). Correspondingly, in our present study, allergic infants were characterized by depletion of commensal *Bifidobacterium*, in conjunction with proliferation of *Klebsiella*.

Several past studies also underscored the association between reduced intestinal microbial diversity and increased risk of allergy development (Abrahamsson *et al.*, 2012; 2014; Bisgaard *et al.*, 2011; Forno *et al.*, 2008). Abrahamsson *et al.* (Abrahamsson *et al.*, 2012; 2014) claimed that low microbial diversity in the first month of life could play a more influential role than changes of specific bacterial taxa in predicting risk of atopic eczema and asthma development later in life. Another recent study by Nylund *et al.* (Nylund *et al.*, 2014) also showed that improvement of eczema symptoms in infants were correlated with higher microbiota diversity. In the current study, allergic infants had significantly lower microbial richness compared to the healthy controls at age 3 months, but not at age 3 weeks or age 6 months. This observation corresponds to compositional differences in intestinal microbiota between the two groups reported at age 3 months. However, the association between decreased microbial diversity and allergy development remains controversial. For instance, several studies support that exclusively breast-fed infants were protected against allergy (Dogaru *et al.*, 2014; Gdalevich *et al.*, 2001), despite lower intestinal microbial diversity in these infants (Azad *et al.*, 2013; Praveen *et al.*, 2015). Hence, the diversity concept still warrants further investigation, and it might be more relevant to gain insight into infants' gut microbiomes at a functional level.

Our findings demonstrate, for the first time, the correlation of the ratio of *Klebsiella*/*Bifidobacterium* and the risk of developing early childhood allergy. The presence of specific group of intestinal microbiota before weaning appears to be critical for allergy development, although it is debatable whether the contrasting observations between allergic and healthy infants at the age of 3 months were the cause or consequence of allergic development and/or an aberrant immune response. The present study sheds light on the association between early life gut microbiota development and childhood allergic disease, and may offer further perspective on nutritional intervention strategies to manage the disease burden.

Alongside its strengths, the present study has a number of limitations. For instance, two allergic infants (Case 6 and Case 18, Table 6) who developed allergy at age 3 months had a moderate low K/B ratio of 0.04 and 0.05 (slightly below the population median K/B ratio). We acknowledge that the predictive power of the K/B ratio is modest in terms of sensitivity and specificity. The ratio of genera *Klebsiella* and *Bifidobacterium* at 3 months accurately predicted allergy in 12 out of the 18 infants who developed allergy by ages 18 months and

36 months, giving a test sensitivity (true positive rate of allergic infants) of approximately 67%. On the other hand, the specificity of this test (true negative rate of non-allergic infants) is approximately 75% (predicted non-allergy in 9 out of 12 non-allergic infants) for allergy at age 18 months and 82% (predicted non-allergy in 9 out of 11 non-allergic infants) for allergy at age 36 months. While our results suggest an association of the *Klebsiella/Bifidobacterium* ratio during early infancy with development of allergy in later life, larger studies are needed to confirm our putative microbial biomarker in predicting the odds. It is noteworthy to mention that there is evidence that early onset of allergy in infancy may have a different pathogenesis, for instance, the evidence for differences in eczema phenotypes from the GUSTO birth cohort (Loo *et al.*, 2015). In further research, other risk factors of allergy could be incorporated to improve the predictive value with the *Klebsiella/Bifidobacterium* ratio.

Besides, due to the small sample size, the influence of potential confounding factors in allergy such as family environmental exposures, antibiotic exposures, maternal microbiota, mode of delivery and infant feeding pattern on the compositional structure of the intestinal microbiota could not be fully explored. The hypothetical contribution of the parent's microbiota and especially the maternal microbiota legacy in predisposing their offspring to develop allergy is a relevant scientific hypothesis to address. Unfortunately, the GUSTO birth cohort was not designed to collect microbiota samples from the parents. Thus, we cannot exclude the possibility that these factors may influence the infants' susceptibility to allergy development. We also acknowledge that the clinical characteristics of allergic subjects in the present study are quite heterogeneous, with various allergy phenotypes. In addition, faecal samples were collected from infants of Chinese ethnicity; therefore those results may not be generalized to infants of other ethnicity. To gain more conclusive answers, future studies could focus on increasing the sample size and investigate the microbiota profile of allergic infants based on well-characterized allergic phenotypes. A system biology approach encompassing microbiota/ microbiome, human genetics/ epigenetics would also be a great direction for future studies aiming to disentangle complex diseases such as allergy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

95% CI	95% confidence interval
ACE	abundance-based coverage estimators
BMI	body mass index
GUSTO	Growing Up in Singapore Towards healthy Outcomes
K/B ratio	<i>Klebsiella/Bifidobacterium</i> ratio
NAST	Nearest Alignment Space Termination
OR	odds ratio
OTU	operational taxonomic unit
PC	principal component
PCA	principal component analysis
PCoA	principal coordinate analysis
QIIME	Quantitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
rRNA	ribosomal ribonucleic acid
SCORAD	SCORing Atopic Dermatitis

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Figure 1.

(A) Relative abundance (%) of bacterial genus between allergic and healthy infants at 3 weeks, 3 months and 6 months of age as determined by 16S rRNA sequencing. Only genera with relative abundance above 1% were represented in the graph above (B) Scatter plots show the $\log_{10}$ relative abundance (%) of genus *Klebsiella* between allergic infants and healthy controls at 3 weeks, 3 months and 6 months of age as determined by 16S rRNA sequencing. Each dot represents the $\log_{10}$ -transformed relative abundance of *Klebsiella* harbored by each infant, and lines indicate medians.

Figure 2.

Boxplots show comparison of microbial richness of allergic and healthy infants at 3 weeks, 3 months and 6 months of age based on (A) Observed species (B) Chao1 (C) ACE. The boxplots are shown as median, with whiskers represent minimum and maximum values. Single asterisk (*) indicates $P < .05$ as evaluated by Mann-Whitney U test. The differences in microbial richness are regarded as significant if $P < .05$.

Figure 3. Principle coordinates analysis based on (A) Unweighted and (B) Weighted Unifrac metrics to compare intestinal microbiota profiles of allergic and healthy infants at 3 months of age. (Red – Healthy controls, Blue – Allergic Infants). PC1 and PC2 explain the percentage variation observed within the data.

Bac : *Bacteroides*

Bif : *Bifidobacterium*

Col : *Collinsella*

Ent : *Enterococcus*

Esc-Shi :

Escherichia/Shigella

Kle : *Klebsiella*

Lac : *Lachnospiraceae*

incertae sedis

Vei : *Veillonella*

▲ : Allergic infants with > median K/B ratio

Figure 4.

PCA biplot based on the compositional structure of the intestinal microbiota of allergic infants and healthy controls at 3 months of age. The PCA biplot is explained by two principal components (PC) i.e. PC1 and PC2 which could explain 27.56% variation observed within the data. Each circle represents the composition of intestinal microbiota of a subject (Red – Healthy controls, Blue – Allergic Infants). The allergic infants who had levels above median K/B ratio (K/B ratio > 0.09) are represented by blue triangle. The eight unique genera explaining the differences are represented by the arrows. There were 2 boxes with

“Bac” and “Vei” as there are two loading vectors representing similar OTUs belonging to the *Bacteroides* and *Veillonella* genera, respectively.

Table 1

Detailed characteristics of infants enrolled in this study.

PARAMETERS	HEALTHY CONTROLS (n = 18)	ALLERGIC INFANTS (n = 21)	P value
Gender, no. (%)			
Male	12 (66.7)	17 (81.0)	a0.46
Female	6 (33.3)	4 (19.0)	
Mode of Delivery, no. (%)			
Vaginal Delivery	13 (72.2)	18 (85.7)	a0.43
Caesarean Section	5 (27.8)	3 (14.3)	
Gestational age in weeks, median (range)	39.0 (37.0 - 40.0)	39.0 (37.0 - 40.0)	c0.67
Birthweight in grams, median (range)	3219.0 (2740.0 - 3820.0)	3045.0 (2205.0 - 3850.0)	c0.27
Infant-Feeding Pattern at 1 month, no. (%)			
dExclusive/ePredominant Breastfeeding	5 (29.4)	7 (35.0)	b0.73
fMixed feeding	10 (58.8)	12 (60.0)	
gExclusive Formula Feeding	2 (11.8)	1 (5.0)	
Missing	1	1	
Infant-Feeding Pattern at 3 months, no. (%)			
dExclusive/ePredominant Breastfeeding	5 (27.8)	5 (23.8)	b0.69
fMixed feeding	7 (38.9)	11 (52.4)	
gExclusive Formula Feeding	6 (33.3)	5 (23.8)	
Infant-Feeding Pattern at 6 months, no. (%)			
dExclusive/ePredominant Breastfeeding	5 (27.8)	4 (19.1)	b0.09
fMixed feeding	2 (11.1)	9 (42.8)	
gExclusive Formula Feeding	11 (61.1)	8 (38.1)	
Weaning age in months, median (range)	6.0 (4.0 - 7.0)	6.0 (4.0 - 8.0)	c0.28
Antibiotic exposure by 3 months, no. (%)			
Yes	1 (5.6)	3 (14.3)	a0.61
No	17 (94.4)	18 (85.7)	
Antibiotic exposure by 6 months, no. (%)			
Yes	4 (22.2)	4 (19.0)	a1.00
No	14 (77.8)	17 (81.0)	
Pre-pregnancy Maternal BMI, median (range)	20.9 (18.7 - 30.3)	22.0 (18.1 - 30.8)	c0.52

PARAMETERS	HEALTHY CONTROLS (n = 18)	ALLERGIC INFANTS (n = 21)	P value
Subjects with Parental Allergic History, no. (%)			
Paternal and/or Maternal Allergic History	3 (16.7)	11 (52.4)	* ^a 0.04
None	15 (83.3)	10 (47.6)	

For categorical data, differences between the two groups were determined by ^aFisher's exact test (2 categorical data) or ^bChi-square test (> 2 categorical data). For continuous data, differences between the two groups were performed using ^cnon-parametric Mann-Whitney U test. Single asterisk (*) indicates $P < .05$.

^d Infants who were solely-fed with human milk

^e Infants who received breast milk and liquids (including water) other than formula

^f Infants who were fed entirely with formula milk

^g Infants who received the mixtures of human milk, formula and liquids

Table 2

Clinical characteristics of infants who developed childhood allergy (n=21) by 18 months and 36 months of age.

Age of first allergy diagnosis (in months), median (range)	6.0 (1.0 – 17.0)
Allergy Diagnosis by 18 months of age, no. (%)	
Eczema	18 (85.7)
Allergic Rhinitis	3 (14.3)
Asthma	0 (0.0)
Allergy Diagnosis by 36 months of age, no. (%)	
Eczema	18 (85.7)
Allergic Rhinitis	10 (47.6)
Asthma	3 (14.3)
Positive Skin Prick Test by 18 months of age, no. (%)	
Cow's Milk	1 (4.8)
Peanut	1 (4.8)
Egg	1 (4.8)
Der f/Der p	6 (28.6)
SCORAD measurement by 18 months of age, no. (%)	
SCORAD < 25 (mild eczema)	8 (38.1)
SCORAD from 25-50 (moderate eczema)	7 (33.3)
SCORAD > 50 (severe eczema)	0 (0.0)
SCORAD not available	6 (28.6)

Table 3

Relative abundance (%) of bacterial genus (*italicized*) which differs significantly between allergic infants and healthy controls at 3 weeks of age.

	Healthy Controls		Allergic Infants		P value
	% Relative abundance	% Standard error	% Relative abundance	% Standard error	
<i>Actinobacteria</i>	26.26	6.03	21.74	5.52	0.76
<i>Bacteroidetes</i>	12.64	6.69	11.40	5.33	0.71
<i>Firmicutes</i>	33.82	4.29	33.07	7.26	0.48
<i>Proteobacteria</i>	27.27	6.71	33.79	6.64	0.44

[^] No significant differences were detected at bacterial genus level between healthy controls and allergic infants at 3 weeks of age

Table 4

Relative abundance (%) of bacterial genus (*italicized*) which differs significantly between allergic infants and healthy controls at 3 months of age.

	Healthy Controls		Allergic Infants		P value	Q value
	% Relative abundance	% Standard error	% Relative abundance	% Standard error		
Acidobacteria	0.00	0.00	0.01	0.01	0.17	-
Actinobacteria*	42.95	6.03	25.73	6.11	0.02	-
<i>Actinomyces**</i>	1.45	0.66	0.68	0.52	0.01	0.03
Bacteroidetes	7.15	4.24	11.51	6.00	0.19	-
Firmicutes	28.14	4.60	33.82	5.65	0.85	-
<i>Clostridium_XIVa*</i>	0.78	0.27	0.06	0.04	0.00	-
<i>Finegoldia*</i>	0.05	0.04	0.01	0.00	0.04	-
<i>Gemella*</i>	0.05	0.02	0.03	0.02	0.01	-
<i>Lachnospiraceae_incertae_sedis**</i>	2.91	2.14	0.02	0.02	0.01	0.03
<i>Peptoniphilus*</i>	0.04	0.02	0.00	0.00	0.02	-
Proteobacteria	17.17	4.48	28.57	5.81	0.13	-
<i>Blifphila*</i>	0.04	0.02	0.00	0.00	0.01	-
<i>Klebsiella**</i>	1.66	0.86	12.26	4.10	0.02	0.04
<i>Lysobacter*</i>	0.01	0.01	0.01	0.01	0.03	-
TM7	0.01	0.01	0.00	0.00	0.38	-
Verrucomicrobia	4.56	3.13	0.06	0.03	0.42	-
Unknown	0.01	0.01	0.29	0.27	0.67	-

Table 5

Relative abundance (%) of bacterial genus (*italicized*) which differs significantly between allergic infants and healthy controls at 6 months of age.

	Healthy Controls		Allergic Infants		P value
	% Relative abundance	% Standard error	% Relative abundance	% Standard error	
Actinobacteria	26.50	5.70	28.93	6.47	0.79
Bacteroidetes	20.13	8.20	14.33	4.83	0.55
Firmicutes	40.39	6.90	36.24	4.07	0.85
<i>Butyrivibrio</i> *	0.06	0.03	0.00	0.00	0.03
Fusobacteria	0.05	0.05	0.16	0.12	0.08
Proteobacteria	9.13	3.27	19.58	4.87	0.26
Spirochaetes	0.01	0.01	0.00	0.00	0.32
Verrucomicrobia	3.75	2.40	0.74	0.68	0.85
Unknown	0.02	0.01	0.01	0.01	1.00

Their respective phylum is highlighted in bold. The differences between the two groups were performed using non-parametric Mann-Whitney U test. For bacterial genera with relative abundances above 1%, P values were corrected for multiple testing (Q value). The differences are regarded as significant if P .05 and/or Q .05. Single asterisk (*) indicates P .05, and double asterisks (**) indicate P .05 and Q .05 for the differentially abundant bacterial genus between the two groups.

Table 6

Ranking of infants at age 3 months based on abundance of K/B ratio in descending order, independently of their clinical phenotype (Control – Healthy Controls, Cases – Allergic Infants).

Subject	3 months				Allergy Diagnosis by 18 Months				Allergy Diagnosis by 36 months
	Feeding pattern	<i>Klebsiella/Bifidobacterium</i> (K/B) ratio	Relative abundance of <i>Klebsiella</i> (%)	Relative abundance of <i>Bifidobacterium</i> (%)	Allergy Phenotype	Skin Prick Test Results (positive against allergens)	Average SCORAD Measurement		
Case 19	Mixed feeding	2281.33	63.22	0.03	eczema only		37.9	eczema & allergic rhinitis	
Case 8	Exclusive Formula Feeding	11.69	4.13	0.35			18.23	information not available	
Case 5	Mixed feeding	8.91	22.06	2.48	eczema & allergic rhinitis		8.25	eczema & allergic rhinitis	
Case 14	Mixed feeding	5.96	45.73	7.67	eczema only	Der p	11.63	eczema & allergic rhinitis	
Case 13	Exclusive/Predominant Breastfeeding	2.20	24.63	11.18	eczema only			eczema and asthma	
Case 4	Mixed feeding	1.34	7.02	5.25	eczema & allergic rhinitis		17.35	eczema & allergic rhinitis	
Case 20	Mixed feeding	0.68	3.63	5.35	eczema only			eczema only	
Case 12	Exclusive/Predominant Breastfeeding	0.66	5.77	8.76	eczema only	Cow's milk, Der f and Der p	29.5	eczema only	
Case 10	Exclusive Formula Feeding	0.54	9.05	16.79	eczema only		19.15	eczema only	
Case 16	Mixed feeding	0.51	12.89	25.25	eczema only	Der p	26.73	eczema & allergic rhinitis	
Case 7	Exclusive/Predominant Breastfeeding	0.43	17.46	40.84	eczema only			eczema only	
Control 3	Exclusive Formula Feeding	0.39	10.84	27.50					
Case 17	Exclusive/Predominant Breastfeeding	0.31	2.41	7.84	eczema only		20.18	eczema & allergic rhinitis	
Control 18	Mixed feeding	0.27	2.01	7.46					
Control 10	Mixed feeding	0.12	0.22	1.88				allergic rhinitis only	
Control 13	Exclusive Formula Feeding	0.06	1.22	21.72					

Subject	3 months				Allergy Diagnosis by 18 Months			Allergy Diagnosis by 36 months
	Feeding pattern	<i>Klebsiella/Bifidobacterium</i> (K/B) ratio	Relative abundance of <i>Klebsiella</i> (%)	Relative abundance of <i>Bifidobacterium</i> (%)	Allergy Phenotype	Skin Prick Test Results (positive against allergens)	Average SCORAD Measurement	
Case 18	Mixed feeding	0.05	1.20	22.24	eczema only	Der f and Der p		eczema & allergic rhinitis
Control 6	Mixed feeding	0.05	1.79	38.19				
Case 6	Exclusive Formula Feeding	0.04	0.39	9.33	eczema only		9.8	eczema and asthma
Control 11	Mixed feeding	0.03	1.23	38.22				
Control 15	Exclusive/Predominant Breastfeeding	0.02	1.42	69.22				
Control 14	Exclusive Formula Feeding	0.02	0.35	19.60				
Control 12	Exclusive Formula Feeding	0.01	0.74	55.26				
Case 15	Mixed feeding	0.01	0.76	60.98	eczema only	Der f and Der p	26.8	eczema only
Case 21	Exclusive Formula Feeding	0.01	0.25	34.04	allergic rhinitis only			allergic rhinitis only
Case 2	Exclusive Formula Feeding	0	0.07	31.62	eczema only		25.4	eczema only
Control 17	Mixed feeding	0	0.04	30.75				
Case 1	Mixed feeding	0	0.01	8.10	eczema only		43	eczema only
Control 9	Exclusive Formula Feeding	0	0.02	18.72				
Control 5	Exclusive Formula Feeding	0	0.00	13.94				

The computed median K/B ratio (range) for all infants regardless of clinical phenotype at age 3 months is 0.09 (0.00 – 2281.33). Infants who had levels above median K/B ratio (K/B ratio > 0.09) were highlighted in bold. The characteristics of infants were included i.e. feeding pattern at 3 months, average SCORAD measurement, and allergy development by 18 months and 36 months of age.

Table 7

Computation of odds of infants developing childhood allergy by (A) 18 months and (B) 36 months of age based on K/B ratio values at age 3 months.

(A)			
	Diagnosis by 18 months		OR (95% CI)
	Developed allergy	No allergy	
Infants with value > median K/B ratio	12	3	6.00 (1.17 – 30.74)
Infants with value < median K/B ratio	6	9	

(B)			
	Diagnosis by 36 months*		OR (95% CI)
	Developed allergy	No allergy	
Infants with value > median K/B ratio	12	2	9.00 (1.46 - 55.50)
Infants with value < median K/B ratio	6	9	

Median K/B ratio was used as a cut-off for computation of odds ratio to measure likelihood of allergy development. "OR" represents odd ratio and "95% CI" represents the 95% confidence interval.

* Allergy information was not available from 1 case at 36 months of age.