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Original article

Early-life gut microbiome and cow's milk allergy- a prospective case - control 6-month follow-up study

Ping Dong, Jing-jing Feng, Dong-yong Yan, Yu-jing Lyu, Xiu Xu *

Department of Child Healthcare, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, PR China

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ABSTRACT

Increasing evidence suggests that perturbations in the intestinal microbiota in early infancy are implicated in the pathogenesis of food allergy (FA); existing evidence on the structure and composition of the intestinal microbiota in human beings with FA is limited and conflicting. The main object of the study was to compare the faecal microbiota between healthy and cow's milk allergy (CMA) infants at the baseline immediately after the diagnosis, and to evaluate the changes in the faecal microbiota after 6 months of treatment of CMA infants with hypoallergenic formula (HF), compared with healthy children fed on standard milk formulae. Sixty infants younger than 4 months of age with challenge-proven CMA and 60 healthy age-matched children were investigated in this prospective case - control follow-up study. Faecal samples were collected at baseline and at 6 months of follow-up, microbial diversity and composition were characterized by high-throughput 16S rRNA sequencing. The average age (±SD) of the infants at inclusion was 2.9 ± 1.0 months. Children with CMA have lower gut microbiota diversity and an elevated Enterobacteriaceae to Bacteroidaceae (E/B ratio) in early infancy compared with healthy children (115.8 vs. 0.8, P = 0.0002). After 6 months of treatment with HF, CMA infants had a higher Lactobacillaceae (6.3% vs. 0.5%, P = 0.04) and lower Bifidobacteriaceae (0.3% vs. 8.2%, P = 0.03) and Ruminococcaceae (1.5% vs. 10.5%, P = 0.03) abundance compared with control children. *Conclusion*: Low gut microbiota diversity and an elevated E/B ratio in early infancy may contribute to the development of FA, including CMA. A strict elimination diet may weaken FA by reducing E/B ratio and promoting a gut microbiota that would benefit the acquisition of oral tolerance.

1. Introduction

It has been estimated that the human gut is populated with up to 100 trillion microbes (Ley et al., 2006). Rough estimates are that the microbiota (previously termed flora or microflora) contain on the order of 150-fold more genes than are encoded in the human genome (Qin et al., 2010). A growing body of evidence suggests that gut microbiota plays an essential role in host health by processing energy from food, protecting intestinal epithelial cells from injury, and promoting local and systemic immunity (Hooper et al., 2012; Mazmanian et al., 2005), which has been extensively studied in recent years using culture-independent molecular methods. The next-generation DNA sequencing technologies, including high throughput 454 pyrosequencing, provide a large number of sequencing reads in a single run, resulting in a large sampling depth and the detection of low-abundance taxa. Thus, the results of studies using high-throughput sequencing technologies have revolutionized our understanding of the gut microbiota in healthy and disease conditions.

An increase in the prevalence of allergic diseases has been noted over the past few decades and 2–15% of the population worldwide is estimated to suffer from asthma, which can affect a high percent of children in some countries (Bousquet et al., 2004). A rising prevalence of food hypersensitivity and severe allergic reactions to food has also been reported. Cow's milk allergy (CMA) is the most prevalent type of food allergy in childhood, with an incidence of 2–3% in the first year of life. An elimination diet that avoids the offending cow's milk protein is the only available treatment (De Greef et al., 2012; Fiocchi et al., 2010). Current guidelines

* Corresponding author.
E-mail addresses: dongpingis@163.com (P. Dong), 13788944690@163.com (J.-j. Feng), yandongyong_88@aliyun.com (D.-y. Yan), 504877260@qq.com (Y.-j. Lyu), xuxiu@fudan.edu.cn (X. Xu).
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suggest the use of extensively hydrolyzed formula (eHF) as first-line dietary management of CMA in infancy and an amino acid-based formula (AAF) for management of complex CMA or when an eHF is not tolerated (De Greef et al., 2012), and they collectively known as hypoallergenic formula (HF).

So far a few studies (Azad et al., 2015; Bunyavanich et al., 2016; Ling et al., 2014; Thompson-Chagoyan et al., 2011, Thompson-Chagoyan et al., 2010) have assessed the fecal microbiota for infants with food allergy and/or hypersensitivity and postulated a possible association between allergy and an altered microbiota pattern. Only one Spanish cohort study (Thompson-Chagoyan et al., 2010) compared the faecal microbiota between healthy and CMA infants at the baseline, and evaluated the changes in the faecal microbiota after a period of treatment, but using a conventional bacterial culture method.

With this background, we performed high-throughput sequencing for V3-V4 hypervariable regions of the 16S rRNA gene from gut faecal material to compare faecal microbiota between healthy and challenge-proven CMA infants at baseline, immediately after the diagnosis and after 6 months of treatment with HF, compared with healthy children fed on standard milk formulae.

2. Methods

2.1. Subjects and study design

Sixty infants diagnosed with CMA from the Gastroenterology and Immunology clinics at the Children's Hospital of Fudan University were recruited between January 2014 and September 2015. The children were less than 4 months of age and received no human milk. CMA (IgE or non-IgE-mediated) were confirmed by family allergic history, feeding history, clinical manifestation (acute severe reaction after cow's milk ingestion combined with either cow's milk-specific IgE > 5 kU/l or with SPT wheal diameter $\geq$ 3 m m, no confirmed history of cow's milk protein reaction but with either SPT wheal diameter $\geq$ 6 mm, or cow's milk associated allergic eosinophilic gastroenteritis or other non-IgE-mediated symptoms) and positive result of double-blind placebo-controlled food challenge with cow's milk, of which the food challenge test was the main method to make definite diagnosis (Koletzko et al., 2012). The choice of an eHF or AAF was determined by the pediatrician in charge according to the severity of the infant's symptoms, and a switch from eHF to AAF was considered if an eHF was not tolerated. The control group comprised 60 healthy age-matched infants recruited from our health care clinic.

These children had been exclusively breast fed until introduced to milk formulae at the same age as the paired CMA infants. The recommendations on solid food introduction were given to all the recruited children from 4 to 6 months old. The following exclusion criteria were established: allergic rhinitis; asthma; use of antibiotics, probiotics, prebiotics, or synbiotics in the previous month; and known active bacterial, fungal, and/or viral infection (s).

Written informed consent was obtained from the children's parents. The study was approved by the Ethics Committee of the Children's Hospital of Fudan University (Children's Hospital of Fudan University Ethics Protocol 2013–033).

2.2. Sample collection and DNA extraction

At baseline and at 6 months of follow-up, faecal samples were collected at the hospital or by the parent at home. Samples were refrigerated during transport and stored at −80 °C until analysis.

DNA was extracted from 300 mg of feces using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of DNA was determined using a Qubit® 2.0 Fluorometer (Life Technologies, USA); the integrity and size were checked by 1.5% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. All DNA was stored at −20 °C until further analysis.

2.3. PCR and pyrosequencing

The bacterial genomic DNA was amplified with 343F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′- AGGGTATCTAATCCT-3′) primers specific for the V3-V4 hypervariable regions of the 16S rRNA gene (Nossa et al., 2010). Barcodes that allowed sample multiplexing during pyrosequencing were incorporated between the 454 FLX Titanium adapter and the 5′ end of the forward primer. The thermocycling steps were as follows: 95 °C for 5 min, followed by 20 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension step at 72 °C for 10 min. Each PCR reaction was performed in a 50 μL system, and the products were extracted with the QIAquick gel extraction kit (Qiagen) and quantified on a NanoDrop 2000C spectrophotometer and Qubit® 2.0 Fluorometer (Life technologies). Pooled PCR amplicons were subjected to paired-end sequencing by Illumina MiSeq. Using a QIIME pipeline (v 1.6.0, qiime.org), forward and reverse reads were assembled using PandaSeq for a final length of 144 bp (unassemblable sequences discarded), demultiplexed and filtered against the GREENGENES reference database (v12.10) to remove all sequences with <60% similarity. Remaining sequences were clustered with Usearch61 at 97% sequence similarity against the GREENGENES database (closed-picking algorithm), and taxonomic assignment was achieved using the RDP classifier constrained by GREENGENES. Operational taxonomic units (OTUs) with overall relative abundance below 0.0001 were excluded from subsequent analyses.

2.4. Bioinformatics and statistical analysis

Using default settings in QIIME, OTU relative abundance was summarized at the phylum, family and genus levels of taxonomy. The Shannon diversity index was used to measure the biodiversity in samples. Briefly, it is a test that takes into account the number of species and the evenness of the species, typically with a value between 1.5 and 3.5. It was calculated as $-\sum \log (p_i)\, p_i$, where p_i denotes the frequency of OTU $_i$, and differences in this index were tested with the Mann-Whitney U test by using R software (http://www.r-project.org/). Microbiota community differences between samples (beta diversity) were tested by permutational multivariate analysis of variance (PERMANOVA) comparison of unweighted UNIFRAC (Lozupone and Knight, 2005) distance matrices, with 500 permutations. Median relative abundance of dominant taxa were compared by nonparametric Kruskal–Wallis test and Spearman rank correlation. As gut microbiota coexist in functional communities, ratios of specific taxa are commonly evaluated. We evaluated the ratio of Enterobacteriaceae to Bacteroidaceae (E/B ratio) as a measure of gut microbiota maturity as Proteobacteria (mainly Enterobacteriaceae) are prevalent in the early gut microbiota, while Bacteroidetes (mainly Bacteroidaceae) become dominant as the community matures towards an adult-like profile (Matamoros et al., 2013).

3. Results

3.1. Demographic and clinical data

The average age (±SD) of the infants at inclusion was 2.9 ± 1.0 months. The baseline characteristics of the enrolled infants are presented in Table 1. The symptoms that occurred most often in

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