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To cite this article: Ryo Inoue, Toshihiro Sawai, Chihiro Sawai, Masako Nakatani, Gustavo A. Romero-Pérez, Motoyuki Ozeki, Kazuo Nonomura & Takamitsu Tsukahara (2017) A preliminary study of gut dysbiosis in children with food allergy, Bioscience, Biotechnology, and Biochemistry, 81:12, 2396-2399, DOI: [10.1080/09168451.2017.1383849](https://doi.org/10.1080/09168451.2017.1383849)

To link to this article: <https://doi.org/10.1080/09168451.2017.1383849>



Published online: 11 Oct 2017.



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Note

A preliminary study of gut dysbiosis in children with food allergy

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Received August 30, 2017; accepted September 15, 2017
<https://doi.org/10.1080/09168451.2017.1383849>

Gut microbiota of food allergic children was analyzed by high throughput 16S rRNA gene sequencing. Signs of gut dysbiosis, which is likely associated with gut inflammation, was observed in children with food allergies. For example, decreased abundance of genus *Akkermansia* but increased abundance of *Veillonella* was found in children with food allergy in comparison with healthy control children.

Key words: gut dysbiosis; food allergy; high throughput 16S rRNA gene sequencing

The gut microbiota plays many important roles in host's immunity and metabolism as evidenced by studies using germ-free and gnotobiotic animals.¹⁾ In the past decade, the role of the intestinal microbiota in many diseases has been elucidated. For example, many studies suggest an altered composition of the intestinal microbiota, or gut dysbiosis, as a possible cause of allergic diseases.²⁾ Caution should be taken, however, when considering the implication of these results for other allergies such as food allergies, as these studies focused mostly on atopic diseases.^{3–5)} To the best of our knowledge, to date there are only five studies that have assessed the composition of the gut microbiota in children with food allergies using high throughput sequencing techniques, and all demonstrated abnormality in their gut microbiota.^{6–10)} Nonetheless, the number of these studies is thought to be too small to draw unequivocal conclusions. In addition, the age of the experimental subjects was likely a limiting factor too. Indeed, the age of infants who had their gut microbiota analyzed in four of these studies was less than 16 months, an age at which children are usually still lactating. The gut microbiota of lactating children is considered to be “immature”, i.e., with microbial taxa that are very different from those found in the gut microbiota of children consuming foods other than milk.¹¹⁾ This immature condition of the microbiota

likely impeded the acquisition of information needed to assess gut dysbiosis.^{6–8,10)}

A high prevalence of atopic diseases and food allergies are still observed at 5 years of age.¹²⁾ The age of children used in the remaining study of the five experiments mentioned above was 2–8 years.⁹⁾ Thus, we believe its experimental protocol is worth being assessed but only after adjustment of certain parameters. For example, the study in question used 27f as primer, which is known to be unsuitable for amplifying *Bifidobacteria* due to sequence miss-matches.¹³⁾ Moreover, the pyrosequencing method used in the study is known to be less accurate than the widely used Sanger sequencing method.¹⁴⁾

In the present study, to assess a possible relation of gut dysbiosis with food allergies, children aged 18 months to 6 years and found to be allergic to foods were enrolled. Their gut microbiota was analyzed using less biased universal primers 341F and 805R for 16S rRNA gene and a MiSeq platform as the analytical method, which was based on the Sanger sequencing method.

The experimental protocol for this study was approved by the ethics commission of Shiga University of Medical Science (Approval Number: 23-104). Informed consent was obtained from parents of all eight infants whose samples were used in this study. Four children severely affected by food allergies, which were diagnosed by clinical symptoms and using a serum allergen specific IgE test, were enrolled in this study. In addition, four healthy children at *similar* age as that of allergic children were used as control. Information of all infants are listed in Table 1. All children were vaginally delivered at full-term (>37 weeks of gestation) and mixed-fed, except for one control child who was breast-fed. No antibiotic treatment was imposed on any of the infants for at least a month prior to sample collection.

Freshly evacuated feces were aseptically collected into sterilized containers (stool carry: atect, Osaka, Japan) and kept at –20 °C during collection. Next,

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Table 1. Clinical data from eight subjects in this study.

Subject	No.	Age	Gender	Food allergen
Healthy	1	6 years, 7 months	Male	–
	2	4 years, 10 months	Male	–
	3	2 years, 2 months	Male	–
	4	4 years, 1 months	Female	–
Allergic	1	2 years, 4 months	Female	Egg, wheat, soybean, sesame, milk
	2	6 years, 3 months	Female	Egg, wheat, sesame, milk, peanut
	3	4 years, 3 months	Male	Egg
	4	1 years, 6 months	Female	Egg, soybean, sesame, milk, shrimp, crab

within 24 h after evacuation, the fecal samples were transported to our laboratory and stored at -80°C until further use.

16S rRNA sequencing using a MiSeq™ system (Illumina, San Diego, CA) was conducted according to a previously described method.¹⁵ PCR amplification of 16S rRNA V3-V4 region was conducted using primers 314F and 805R. Processing of sequencing data including quality filtering, chimera check, OTU definition, and taxonomy assignment was carried out using QIIME,¹⁶ USEARCH,¹⁷ and UCHIME¹⁸ exactly as previously described.¹⁵ Statistical difference ($p < 0.05$) in the weighted UniFrac distance calculated by QIIME was evaluated using Wilcoxon's rank sum test. The relative abundance of bacterial genus between groups was analyzed by White's non-parametric t -test, which is a method developed to compare bacterial abundance.¹⁹ Frequency of bacterial genus was statistically analyzed by Pearson's chi-square test using R software (ver. 3.3.2; <https://www.r-project.org/>).

Upon data processing, a total of 205,055 high-quality sequences (average: 25,632 reads) were obtained and 205 OTUs were defined (singleton removed). Principal component analysis of weighted UniFrac distance indicated differences in the gut microbiota between food allergic and healthy children (PC2: $p < 0.05$, Fig. 1). The composition of the gut microbiota in food allergic and healthy children is summarized in Table 2. Abundance of bacterial genus *Dorea* and *Akkermansia* was significantly lower in samples of food allergic children

than in those of healthy control children, while abundance of genus *Lachnospira*, *Veillonella*, and *Sutterella* was significantly higher ($p < 0.05$) in food allergic children than in healthy children. With regard to frequency, although genus *Sutterella* was detected in samples of all food allergic children, it was found only in samples from one control child ($p < 0.05$ by chi-square test). In contrast, although it was detected in all healthy control children, genus *Akkermansia* was rarely detected in food allergic children (1/4 subjects) ($p < 0.05$ by chi-square test). Chao1 index was 106.99 ± 27.43 and 104.86 ± 31.59 in food allergic and healthy children, respectively. In addition, Shannon index was 3.08 ± 0.25 and 3.08 ± 0.35 in food allergic and healthy children, respectively. The alpha diversity (Chao1 and Shannon indices) was not different between groups.

Gut dysbiosis of food allergic children, especially after weaning, has rarely been investigated. In the present study, we conducted an experiment to preliminarily assess gut dysbiosis in food allergic children prior to a larger scale study. Although the number of subjects in this study was relatively small, it yielded interesting data on the features of gut microbiota in food allergic children.

The composition of gut microbiota in food allergic children was different from that in healthy children. Interestingly, the gut microbiota of food allergic children seems to possess characteristic features that are common with that of children suffering from Crohn's disease (CD). For example, we detected a decrease in abundance of genus *Dorea* and *Akkermansia* and an increase in abundance of *Veillonella*, which Lewis et al.²⁰ also detected in pediatric CD. Similarly, Tana et al. also reported *Veillonella* to be significantly higher in the fecal microbiota of irritable bowel syndrome patients than in that of healthy, control patients.²¹ Although the association of genus *Dorea* with gut inflammation remains unclear, *Akkermansia* has been suggested to have anti-inflammatory properties.²² In addition, it was found that *Veillonella* lipopolysaccharides induce a similarly strong inflammatory response as *Salmonella* lipopolysaccharides do.²³ Finally, although it still remains controversial,²⁴ *Sutterella wadsworthensis* has been suggested as one of the predominant bacterial strains in the microbiota of CD patients.²⁵ Taken together, our results suggest that children with food allergy may experience gut inflammation, at least to some extent. An inflamed gut then prevents the establishment of oral tolerance to food antigens and promotes hypersensitivity to antigens.²⁶ In addition, gut

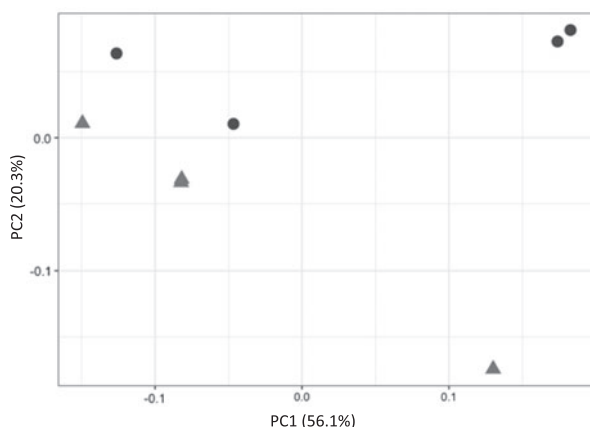


Fig. 1. Principal component analysis plot of weighted UniFrac distance for the gut microbiota in food allergic and healthy children.

Notes: Solid triangles indicate data of food allergic children. Solid circles indicate data of healthy children.

Table 2. Composition of gut microbiota of healthy and food allergic children.

Phylum	Class	Order	Family	Genus	Healthy		Food allergic	
					Abundance (%)	Frequency (%)	Abundance (%)	Frequency (%)
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	<i>Bifidobacterium</i>	14.90 ± 10.50	100	6.80 ± 4.50	100
Actinobacteria	Coriobacteria	Coriobacteriales	Coriobacteriaceae	<i>Collinsella</i>	1.50 ± 1.90	50	0.20 ± 0.20	100
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	<i>Bacteroides</i>	18.00 ± 12.70	100	31.80 ± 9.40	100
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	<i>Parabacteroides</i>	1.60 ± 2.10	75	1.40 ± 1.30	75
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	<i>Streptococcus</i>	2.80 ± 4.90	100	1.60 ± 1.50	100
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium</i>	2.50 ± 2.40	100	0.90 ± 0.40	100
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified	1.30 ± 0.60	100	1.60 ± 1.30	75
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Blautia</i>	7.60 ± 8.30	100	3.50 ± 3.60	100
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Clostridium</i>	2.30 ± 2.20	100	1.60 ± 0.50	100
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Coprococcus</i>	1.60 ± 1.20	100	1.30 ± 0.90	100
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Dorea</i>	6.60 ± 4.90	100	0.60 ± 0.60*	100
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Lachnospira</i>	0.10 ± 0.10	100	1.70 ± 1.30*	75
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Roseburia</i>	1.20 ± 1.60	100	2.00 ± 1.30	75
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[<i>Ruminococcus</i>]	5.20 ± 3.30	100	3.30 ± 1.60	100
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unclassified	0.30 ± 0.30	100	1.10 ± 2.20	50
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Butyrivibrio</i>	0.80 ± 0.80	100	1.50 ± 0.60	100
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Faecalibacterium</i>	9.50 ± 9.20	100	8.50 ± 5.60	100
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Gemmiger</i>	3.30 ± 4.80	100	2.00 ± 1.60	75
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Oscillospira</i>	1.00 ± 0.90	100	0.90 ± 0.60	100
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcus</i>	3.00 ± 2.60	100	2.80 ± 5.40	100
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Dialister</i>	<0.01	25	1.20 ± 2.40	75
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Megasphaera</i>	<0.01	50	1.00 ± 1.80	75
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Veillonella</i>	2.40 ± 2.70	75	11.60 ± 8.80*	100
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Unclassified	2.10 ± 4.20	25	0.50 ± 0.60	50
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[<i>Eubacterium</i>]	0.80 ± 1.00	100	0.10 ± 0.10	75
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Sutterella</i>	0.20 ± 0.40	25	1.50 ± 1.00*	100†
Proteobacteria	Gamma proteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Escherichia</i>	1.40 ± 1.80	100	1.80 ± 2.40	100
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Akkermansia</i>	3.50 ± 2.70	100	<0.01*	25†

Notes: Only taxonomy of which the mean relative abundance in either group is above 1% is listed. Data are shown as the mean ± SD. Significant differences ($p < 0.05$) in mean relative abundance between groups are indicated with an asterisk (*). Significant differences in frequency between groups ($p < 0.05$) are indicated with a dagger (†). Abundance and frequency with significant differences are shown in bold letters.

inflammation also seems to increase gut permeability,²⁷⁾ which consequently results in higher antigenic loads from food.²⁸⁾

In the present work, we demonstrated that gut dysbiosis, which likely associated with gut inflammation, occurs in children with food allergy. Based on our results, we propose that, using larger sample sizes, further studies focusing on gut dysbiosis in food allergic subjects and its relation with gut inflammation are worth being conducted.

Author contribution

R.I., C.S., T.S., and T.T. designed research, T.S., C.S., and K.N. collected clinical samples, R.I., M.N., and T.T. performed the experiments, R.I. and M.O. analyzed data, and R.I. and G.A.R. wrote the manuscript. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Morinaga Foundation for Health & Nutrition and SUMS President's Grant for Encouragement of Young Researchers.

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