



Communication

Differences in Developing Intestinal Microbiota between Allergic and Non-Allergic Infants: A Pilot Study in Japan

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The bacterial compositions of feces were monitored in the first 2 months for 15 infants born in Japan, including eight subjects who developed allergy by the age of 2 years. Primer sets targeting six predominant bacterial groups in the infant intestine, *Bacteroidaceae*, *Enterobacteriaceae*, bifidobacteria, enterococci, lactobacilli, and the *Clostridium perfringens* group, were used for real-time PCR to quantitate each population in the feces. The population of *Bacteroidaceae* was significantly higher in the allergic group at the ages of 1 month ($P = 0.03$) and 2 months ($P = 0.05$) than in the non-allergic group, while no statistically significant difference was observed for the other bacterial populations.

Key words: infant; allergy; gastrointestinal (GI)-tract microbiota; feces; bacteroides

Soon after birth, a variety of bacteria start to colonize the previously germ-free gastrointestinal (GI) tract, and they construct a complex microbial ecosystem step by step.¹⁻³⁾ Generally, a stable anaerobic microbial community including bifidobacteria as dominant, the so-called “bifidus flora,” is established within one or few weeks of birth.¹⁻³⁾ The bacteria in this process vary among individuals, notably at the species level,³⁾ more or less depending on the mode of delivery, mother’s vaginal and skin microbiota, the type of infant feeding, antibiotic therapy, and the environment surrounding the baby.^{1,4)}

The major functions of the mucosal immune system

are also defective at birth and develop under the influence of a number of stimuli from GI-tract colonizing bacteria.⁵⁾ Hence, it is believed that the development of intestinal microbiota in early infancy is crucial for the promotion of a healthy and balanced immune system in later life. An increasing prevalence of childhood allergy diseases during recent decades, especially in developed countries, including Japan is obvious.⁶⁻⁸⁾ This is suspected to be caused by aberrant intestinal microbiota resulting from an improved hygienic environment.⁹⁾ Indeed, the composition of the intestinal microbiota of 1-year-old healthy children differs significantly between Estonia and Sweden,¹⁰⁾ two countries that have a low and a high prevalence of allergies respectively.¹¹⁾ In a prospective study performed in these two countries, it was reported that children who developed allergy during the first 2 years of life were less often colonized by enterococci, bifidobacteria, bacteroides, and had higher stool counts of *Staphylococcus aureus*, coliforms, and clostridia than healthy infants.^{12,13)} These differences were observed before allergic symptoms appeared. These data support the “microflora hypothesis” that developmental GI-tract microbiota has a great influence on the development of the immune system in infancy,⁹⁾ but not many studies have been performed on Japanese infants, even though the prevalence of allergy in them has obviously increased.⁷⁾ Considering differences in genetic background, life style, and living environment between Japanese and European infants, the study of Japanese infants is considered to be indispensable in

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Abbreviations: GI, gastrointestinal; ISAAC, International Study of Asthma and Allergies in Childhood

addressing what happens to the GI tract of Japanese infants with allergy crisis. To achieve this objective, we conducted a pilot study in Japan to relate GI-tract microbiota in early infancy with allergy development prospectively.

We monitored differences in GI-tract microbiota in the first two months between two groups of infants who did and did not develop allergic manifestations later, by the age of 2 years (henceforth in this report, “allergic” and “non-allergic” are used to refer to these groups). All the infant subjects used in this study were born and raised in the first week after the birth in a hospital in Fukuoka, Japan, and were brought up in Japan until the end of follow-up. All were vaginally delivered and were fed both breast and formula milk during the feces-sampling period. Infants who were administered any antibiotic during the sampling period were excluded from the study because the fecal microbial composition of antibiotic-treated infants showed an aberrant profile distinct from those of non-antibiotic-treated infants (data not shown). Fecal samples were collected once daily in the first 5 d and once monthly at the ages of 1 month and 2 months. The fecal samples were stored at -80°C until bacterial composition analysis.

Two years after birth, a follow-up was done by means of a questionnaire sent to the parents, including questions about the history of medical diagnosis of allergies and symptoms of atopic dermatitis, asthma, and food allergy during the first 2 years, based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire.¹⁴ Since the ISAAC questionnaire has been tested and validated, this standardized protocol of the ISAAC facilitates investigation of the prevalence of allergy all over the world. Written informed consent was obtained from the parents of subjects, and the Ethics Committee of the Faculty of Medicine of Kyoto University approved this study protocol.

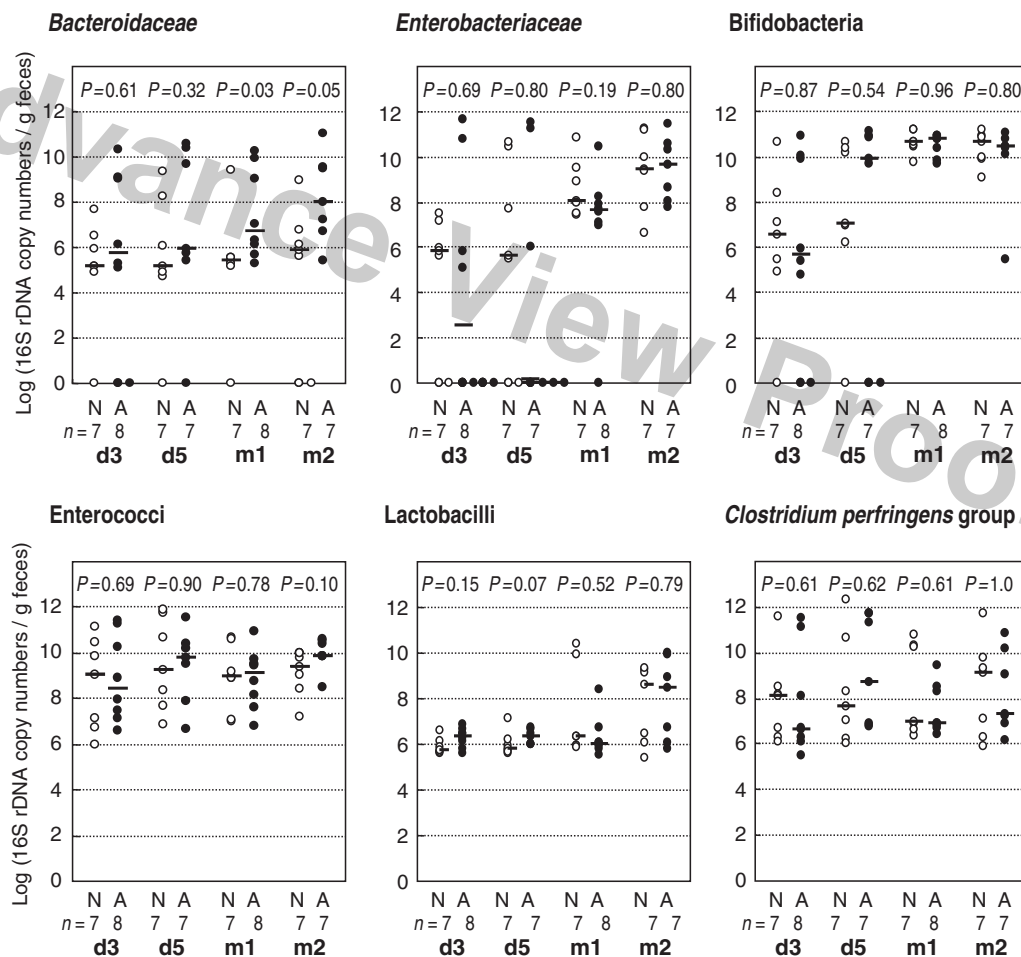
Total bacterial DNA was extracted from each fecal sample by a bead beating method,³⁾ purified with a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and was then used as a template for quantitative real-time PCR. The quantitative real-time PCR targeted six bacterial groups, family *Bacteroidaceae*, family *Enterobacteriaceae*, genus *Bifidobacterium*, genus *Enterococcus*, genus *Lactobacillus* and the *Clostridium perfringens* group, which are commonly found to be predominant in infants from the age of 3 d to 2 months.³⁾ Primer sets specifically targeting 16S rRNA gene (rDNA) of these bacterial groups were used to estimate the bacterial populations (Table 1).^{15–17)} The family *Bacteroidaceae* targeted by the primer set BacP-F and BacP-R, includes major genera of fecal bacteria, *Bacteroides* and *Prevotella*.¹⁵⁾ The target of the primer set for *Enterobacteriaceae*, Ecol457F and Ecol652R, includes *Escherichia coli* and genera *Shigella* and *Salmonella*.¹⁶⁾ The *Clostridium perfringens* group includes *Clostridium butyricum*, which was found to be a

dominant *Clostridium* species in our subjects.³⁾ Quantitative real-time PCR was performed with the Mx3000P QPCR System (Stratagene, La Jolla, CA). Each reaction was performed in 20 μl of reaction mixture containing 10 μl of SYBR Green Premix Ex Taq (Takara Bio, Shiga, Japan), 0.2 μM of each primer set, 0.4 μl of ROX Dye II (50x, Takara), and 2 μl of the template DNA solution containing extracts from 0.1 to 1 mg of feces. For the negative control, 2 μl of milli-Q water was added to the reaction solution instead of the template DNA solution. The copy number of 16S rDNA of the target bacteria in the template DNA solution was estimated using standard curves made from known concentrations of the corresponding DNA fragment. The standard curves were prepared in the same PCR assay as for samples. The real-time PCRs were performed in duplicate and average values were used for enumeration. PCR conditions were optimized based on those described in references 15–17, except for lactobacilli, whose primers and condition were optimized originally in this study (Table 1). In all assays, the amplification efficiency was higher than 70%, and the standard curve showed a linear range across at least 5 logs of DNA concentrations with a correlation coefficient higher than 0.99. The lower detection limits of all assays were as low as 10–100 copies of specific bacterial 16S rDNA per reaction, corresponding to 10^4 – 10^5 copies per gram of wet-weight feces.

Figure 1 shows the distribution of the number of 16S rDNA of each bacterium in the allergic and non-allergic groups. Since the counts of 16S rDNA of each bacterium might have been non-normally distributed, the non-parametric Mann-Whitney *U*-test (Analyze-it statistical software add-in for Excel, Microsoft Windows) was employed to evaluate the statistical significance of difference between the two groups. Except for *Bacteroidaceae*, there were no statistical differences in numbers between the allergic and non-allergic groups. This disagrees with some previous studies^{12,13,18)} showing differences in the numbers of bifidobacteria, enterococci, lactobacilli, clostridia, or *Enterobacteriaceae* between allergic and non-allergic infants. Notably, differences in the *Bifidobacterium* population between allergic and non-allergic infants have often been commonly found in previous studies,^{12,13,18)} in which lower numbers and lower prevalence of *Bifidobacterium* in allergic infants were observed; inadequate bifidobacteria in GI tract microbiota has been suggested to be a risk factor for the development of allergy. On the other hand, our allergic subjects gained normal bifidus flora, except for one subject who had a low number of bifidobacteria less than 10^6 at the age of 2 months. This suggests that the establishment of bifidus flora is alone not sufficient to avoid the development of allergy. The lack of allergic subjects less colonized by bifidobacteria in this study might be due to the mode of delivery and the growth condition of our subjects: all of our subjects were vaginally delivered, were fed both breast and

Table 1. Primers and PCR Conditions Used for Quantitative Real-Time PCR in This Study

Target	Primer name	Primer sequence (5'-3')	Size ^a (bp)	PCR program ^b	Ref.
<i>Bacteroidaceae</i>	BacP-F BacP-R	GGTGTGCGCTTAAGTGCCAT CGGA(C/T)GTAAGGGCCGTGC	140	95 °C (20s)—58 °C (10s) —72 °C (30s)	15)
<i>Enterobacteriaceae</i>	Ecol457F Ecol652R	CATTGACGTTACCCGAGAAGAAGC CTCTACGAGACTCAAGCTTGC	195	95 °C (15s)—58 °C (15s) —72 °C (30s)	16)
<i>Bifidobacteria</i>	Bif-F Bif-R	TCGCGTC(C/T)GGTGTGAAAG CCACATCCAGC(A/G)TCCAC	243	95 °C (20s)—56 °C (20s) —72 °C (30s)	15)
<i>Enterococci</i>	Enc-F Enc-R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCATTGT	144	95 °C (20s)—56 °C (10s) —72 °C (25s)	15)
<i>Lactobacilli</i> ^c	LactoR'F LBFR	CACAATGGACG(A/C)AAGTCTGATG CGCCACTGGTGTCTTCCAT	358	95 °C (30s)—65 °C (15s) —72 °C (20s)	This study
<i>Clostridium perfringens</i> group ^c	Cl-per-F Cl-per-R	ATGCAAGTCGAGCGA(G/T) G TATGCGGTATTAATC T(C/T)CCTTT	120	95 °C (15s)—55 °C (15s) —72 °C (20s)	17)

^aExpected size of amplicon.^bTemperature and time (in parenthesis) in one PCR cycle are shown.^cThis primer set can also amplify 16S rDNAs of genera *Leuconostoc*, *Pediococcus*, and *Weissella* in addition to genus *Lactobacillus*.**Fig. 1.** Distribution of the Number of 16S rDNAs of Each Bacterium in Non-Allergic (N, open circle) and Allergic (A, closed circle) Groups.

Horizontal bars represent median values. The number of 16S rDNAs was enumerated by quantitative real-time PCR, and was expressed as copy numbers per wet gram feces. Values less than the detection limit were plotted at 10^0 . A nonparametric Mann-Whitney *U*-test was used to compare the 16S rDNA copy numbers between allergic and non-allergic groups. d3, 3 d old; d5, 5 d old; m1, 1 month old; m2, 2 months old.

formula milk, and were free from antibiotic therapy. These conditions are known to be favorable to the establishment of bifidus flora.⁴⁾

Although there was no statistically significant difference in the numbers of *Enterobacteriaceae*, it was remarkable that some allergic subjects were colonized

by high numbers of *Enterobacteriaceae* in the first week. On the contrary, in some other allergic subjects, no colonization was detected until the age of 5 d. Previously, both positive and negative effects of *Enterobacteriaceae* on allergy development have been reported, and it has been suggested that the immunological consequences of lipopolysaccharide (LPS)-exposure depend on a variety of chemical structures and conditions, *e.g.*, timing and dose.^{19–21} It is necessary to analyze the bacterial composition of the enterobacterial community on the genus or species level with a much higher number of subjects.

The number of *Bacteroidaceae* differed statistically between the allergic and non-allergic groups at the ages of 1 month ($P = 0.03$) and 2 months ($P = 0.05$). Although the difference was not statistically significant, it should be also noted that some allergic subjects already had high numbers of *Bacteroidaceae* in the first week, which was not observed in the non-allergic group. Our unpublished data with a higher number of subjects analyzed by terminal-restriction fragment length polymorphism also showed a higher prevalence of *Bacteroidaceae* during the first 2 months in the allergic group (73%, 8/11) than that in the non-allergic group (38%, 8/21) (S. Tanaka *et al.*, unpublished results). These data agree with those of Kirjavainen *et al.*, who found that allergic infants before weaning were more colonized with bacteroides than healthy infants of the same age.^{22,23} On the other hand, Björkstén *et al.* reported that allergic infants at the age of 1 year were less colonized with *Bacteroides*.¹³ This difference may be due to the difference in sampling ages before and after weaning. Indeed, it is known in general that bacteroides gains in population and becomes dominant after weaning. The higher colonization of *Bacteroidaceae* may be characteristic of infants before weaning who later develop allergy, and this trend might be a prospective marker for allergy crisis in infancy.

Recently, an *in vitro* study²⁴ using peripheral blood mononuclear cells from Japanese cedar pollinosis subjects indicated that strains of the *Bacteroides fragilis* group induced significantly more helper T cell (T_H) type 2 cytokine (IL-6) but fewer T_H1 -type cytokines (IFN- γ and IL-12) than those of *Bifidobacteria*. Also, it is known that *B. fragilis* LPS shows proinflammatory effects *via* the Toll-like receptor (TLR) 4 pathway, as enterobacterial LPS does, but that its potency is 100–1000-fold lower.²⁵ On the contrary, it has been reported that *Bacteroides thetaiotaomicron* attenuated inflammation by regulating intracellular signaling downstream of TLR signaling and NF- κ B activation.²⁶ With only this information, we cannot speculate the mode of action of bacteroides-mediated allergy induction in infancy. However, taking into account the present data showing the possibility that a high population of bacteroides in early infancy correlates with later allergy development, it is worthwhile to address the mode of action *in vivo*, *e.g.*,

using gnotobiotic mouse model in addition to *in vitro* experiments.

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References

- 1) Mitsuoka, T., Human microbiota research: present and future. *J. Intestinal Microbiol.*, **19**, 179–192 (2005).
- 2) Favier, C. F., Vaughan, E. E., de Vos, W. M., and Akkermans, A. D. L., Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.*, **68**, 219–226 (2002).
- 3) Songjinda, P., Nakayama, J., Kuroki, Y., Tanaka, S., Fukuda, S., Kiyohara, C., Yamamoto, T., Izuchi, K., Shirakawa, T., and Sonomoto, K., Molecular monitoring of the developmental bacterial community in the gastrointestinal tract of Japanese infants. *Biosci. Biotechnol. Biochem.*, **69**, 638–641 (2005).
- 4) Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, P. A., and Stobberingh, E. E., Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, **118**, 511–521 (2006).
- 5) Bailey, M., Haverson, K., Inman, C., Harris, C., Jones, P., Corfield, G., Miller, B., and Sokes, C., The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. *Proc. Nutr. Soc.*, **64**, 451–457 (2005).
- 6) von Mutius, E., The rising trends in asthma and allergic disease. *Clin. Exp. Allergy*, **28** (Suppl. 5), 45–49 (1998).
- 7) Saeki, H., Iizuka, H., Mori, Y., Akasaka, T., Takagi, H., Kitajima, Y., Tezuka, T., Tanaka, T., Hide, M., Yamamoto, S., Hirose, Y., Kodama, H., Urabe, K., Furuse, M., Kasaki, F., Torii, H., Nakamura, K., Morita, E., Tsuemi, Y., and Tamaki, K., Prevalence of atopic dermatitis in Japanese elementary schoolchildren. *Br. J. Dermatol.*, **152**, 110–114 (2005).
- 8) Eder, W., Ege, M. J., and von Mutius, E., The asthma epidemic. *N. Eng. J. Med.*, **23**, 2226–2235 (2006).
- 9) Noverr, M. C., and Huffnagle, G. B., The 'microflora hypothesis' of allergic diseases. *Clin. Exp. Allergy*, **35**, 1511–1520 (2005).
- 10) Sepp, E., Julge, K., Vasar, M., Naaber, P., Björkstén, B., and Mikelsaar, M., Intestinal microflora of Estonian and Swedish infants. *Acta Paediatr.*, **86**, 956–961 (1997).
- 11) Riikjäär, M. A., Julge, K., Vasar, M., Bråbäck, L., Knutsson, A., and Björkstén, B., The prevalence of atopic sensitization and respiratory symptoms among Estonian schoolchildren. *Clin. Exp. Allergy*, **25**, 1198–1204 (1995).
- 12) Björkstén, B., Naaber, P., Sepp, E., and Mikelsaar, M., The intestinal microflora in allergic Estonian and

- Swedish 2-year-old children. *Clin. Exp. Allergy*, **29**, 342–346 (1999).
- 13) Björkstén, B., Sepp, B., Julge, K., Voor, T., and Mikelsaar, M., Allergy development and the intestinal microflora during the first year of life. *J. Allergy Clin. Immunol.*, **108**, 516–520 (2001).
- 14) Asher, M. I., Keil, U., Anderson, H. R., Beasley, R., Martinez, F., Mitchell, E. A., Pearce, N., Sibbald, B., Stewart, A. W., Strachan, D., Weiland, S. K., and Williams, H. C., International study of asthma and allergies in childhood (ISAAC): rationale and methods. *Eur. Respir. J.*, **8**, 483–491 (1995).
- 15) Rinttilä, T., Kassinen, A., Malinen, E., Krogus, L., and Palva, A., Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in fecal samples by real-time PCR. *J. Appl. Microbiol.*, **97**, 1166–1177 (2004).
- 16) Bartosch, S., Fite, A., Macfarlane, G. T., and McMurdo, M. E., Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.*, **70**, 3575–3581 (2004).
- 17) Malinen, E., Rinttilä, T., Kajander, K., Matto, J., Kassinen, A., Krogus, L., Saarela, M., Korpela, R., and Palva, A., Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am. J. Gastroenterol.*, **100**, 373–382 (2005).
- 18) Kalliomäki, M., Kirjavainen, P., Eerola, E., Kero, P., Salminen, S., and Isolauri, E., Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J. Allergy Clin. Immunol.*, **107**, 129–134 (2001).
- 19) Lapa e Silva, J. R., da Silva, P., Lefort, J., and Vargaftig, B. B., Endotoxins, asthma, and allergic immune responses. *Toxicology*, **152**, 31–35 (2000).
- 20) Lodinova-Zadnikova, R., Cukrowska, B., and Tlaskalova-Hogenova, H., Oral administration of probiotic *Escherichia coli* after birth reduces frequency of allergies and repeated infections later in life (after 10 and 20 years). *Allergy Immunol.*, **131**, 209–211 (2003).
- 21) Renz, H., and Herz, U., The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *Eur. Respir. J.*, **19**, 158–171 (2002).
- 22) Kirjavainen, P. V., Apostolou, E., Arvola, T., Salminen, S. J., Gibson, G. R., and Isolauri, E., Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunol. Med. Microbiol.*, **32**, 1–7 (2001).
- 23) Kirjavainen, P. V., Arvola, T., Salminen, S. J., and Isolauri, E., Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? *Gut*, **51**, 51–55 (2002).
- 24) Odamaki, T., Xiao, J.-Z., Iwabuchi, N., Sakamoto, M., Takahashi, N., Kondo, S., Iwatsuki, K., Kokubo, S., Togashi, H., Enomoto, T., and Benno, Y., Fluctuation of fecal microbiota in individuals with Japanese cedar pollinosis during the pollen season and influence of probiotic intake. *J. Investig. Allergol. Clin. Immunol.*, **17**, 92–100 (2007).
- 25) Mancuso, G., Midiri, A., Biondo, C., Beninati, C., Gambuzza, M., Macri, D., Bellantoni, A., Weintraub, A., Espevik, T., and Teti, G., *Bacteroides fragilis*-derived lipopolysaccharide produces cell activation and lethal toxicity via Toll-like receptor 4. *Infect. Immun.*, **73**, 5620–5627 (2005).
- 26) Kelly, D., Campbell, J. I., King, T. P., Grant, G., Jansson, E. A., Coutts, A. G. P., Pettersson, S., and Conway, S., Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR- γ and RelA. *Nat. Immunol.*, **5**, 104–112 (2004).