

ORIGINAL PAPER

Differences in the composition of intestinal *Bifidobacterium* species and the development of allergic diseases in infants in rural Japan

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Summary

Background *Bifidobacterium* is a dominant genus in the intestinal microbiota of infants and comprises many different species. A series of studies performed in northern Europe showed differences in the composition of *Bifidobacterium* species between allergic infants and healthy controls. Additional studies are needed to confirm this observation.

Objective To investigate the composition of fecal *Bifidobacterium* species in allergic infants and healthy controls in Japan, using molecular methods.

Methods Full-term born babies were followed up to 6 months of age at a local hospital in rural Japan. The presence or absence of allergy was determined based on allergic symptoms and skin prick tests. Fecal *Bifidobacterium* species in allergic infants ($n = 10$), and healthy controls ($n = 16$) were evaluated using nine *Bifidobacterium* species-specific or group-specific primers based on 16S rDNA sequences at 1, 3, and 6 months of age.

Results The number of the infants in whom no *Bifidobacterium* species could be found was four (15.4%) at 1 month, two (7.7%) at 3 months, and one (3.3%) at 6 months of age, all of whom were healthy controls. At 1 month of age, allergic infants had a higher prevalence of the *Bifidobacterium catenulatum* group than healthy controls (60.0% vs. 6.3%, $P < 0.01$).

At 6 months of age, allergic infants had a higher prevalence of *B. bifidum* than healthy controls (70.0% vs. 12.5%, $P < 0.01$). These differences were not related to feeding method.

Conclusions Our results in infants in rural Japan support the hypothesis that a compositional difference in intestinal *Bifidobacterium* species may be associated with the development of allergy in early infancy, although the responsible species might vary among countries or races.

Keywords allergy, *Bifidobacterium bifidum*, *Bifidobacterium catenulatum* group, infancy, polymerase chain reaction

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Introduction

It has been shown that microbial colonization influences the immune development of infants [1]. As *Bifidobacterium* is the predominant intestinal microbiota in the gastrointestinal tract of infants, it may play a role in the development of allergic diseases in infancy. Although the etiology of atopic dermatitis and food allergy remains unclear, a disturbance in the intestinal microbiota, especially of endogenous lactic acid bacteria such as *Bifidobacterium*, has been reported as one of the important events related to allergic diseases [2, 3].

The genus *Bifidobacterium* comprises many different species, and a series of studies performed in northern

Europe using a culture method showed a distinct difference in the pattern of *Bifidobacterium* flora between allergic infants and healthy controls. In their study, *Bifidobacterium bifidum* was found to colonize in most non-atopic healthy infants whereas *B. adolescentis* was found in most allergic infants [4, 5].

The composition of intestinal microbiota is influenced by genetic background and food custom [6, 7]. Therefore, we thought it necessary to analyse the relationship between *Bifidobacterium* species and the development of allergy in countries outside northern Europe. Here we report the results of a prospective study on intestinal microbiota in which infants in rural Japan were followed from birth to 6 months of age. Nine major species of

Bifidobacterium in allergic infants and controls who remained non-allergic at 6 months of age were analysed in fecal samples collected at 1, 3, and 6 months of age by a recently developed, sensitive PCR technique.

Materials and methods

Subjects

This study was conducted at a local hospital in Omigawa town, located in a rural region of Chiba prefecture in Japan. The mothers of full-term babies born from May to December, 2001, entered the study with written informed consent, and were asked to bring fresh feces from their babies in a diaper at 1, 3, and 6 months of age. At every visit, a detailed history of the feeding method was obtained, and the babies underwent a physical examination performed by a pediatric allergist. Babies with non-allergic diseases, who had been administered antibiotics, or who had eaten yogurt or foods containing live bacteria within 7 days before the collection of feces were excluded from the study. Atopic eczema was defined as flexural, itching eczema, as characterized by Hanifin and Rajka [8]. Recurrent wheezing was defined as reversible bronchial obstruction occurring at least three times and verified at least once by a doctor. At 6 months of age, skin prick tests (SPTs) were performed on the volar aspects of the forearms with standardized extracts (Torii Pharmaceutical Co. Ltd, Tokyo, Japan) from egg white, cow's milk, wheat, soy bean, rice, house dust mite (HDM), and house dust. A result was regarded as positive if the diameter of the weal was more than 3 mm [9]. Infants with any allergic diseases or one or more positive results in the SPTs were considered allergic; infants without a history of allergic symptoms and without a positive SPT result were considered non-allergic.

Of 26 infants followed until 6 months of age, 10 were allergic and 16 were without allergy and defined as healthy controls. The characteristics of allergic infants and healthy controls, including maternal age, gestational age, body weight at birth, sex, mode of delivery, and atopic heredity in at least one second-degree or closer relative in the family are shown in Table 1. There were no significant differences between allergic infants and healthy controls except in terms of atopic heredity (100% vs. 37.5%, respectively, $P < 0.01$). There was no significant difference in the numbers of babies fed by a particular method after 1 month of age between allergic infants and healthy controls: that is, the distribution of breastfeeding, mixed feeding, and formula feeding was 3:2:5 for allergic infants and 4:5:7 for healthy controls. Of 10 allergic infants, seven had atopic eczema by 6 months of age, and two had recurrent wheezing. As for the SPTs, three allergic infants had more than two positive reactions (cow's milk, 3; egg white, 2; wheat, 1; HDM, 1). Four had one positive reaction (egg white, 2;

Table 1. Characteristics of infants in the study ($n = 26$)

	Allergic infants ($n = 10$)	Healthy controls ($n = 16$)
Maternal age (years)	27.1 $\pm$ 4.5	26.3 $\pm$ 4.2
Gestational age (weeks $\pm$ SD)	39.3 $\pm$ 1.1	39.7 $\pm$ 1.3
Body weight (g $\pm$ SD)	2964 $\pm$ 357	2952 $\pm$ 312
Sex (male : female)	4 : 6	6 : 10
Mode of delivery (vaginal : cesarean)	9 : 1	14 : 2
Atopic heredity* (%)	100	31.3

* $P < 0.01$ for comparison between groups.

cow's milk, 1; HDM, 1), and the remaining three had no positive reaction despite distinct allergic manifestations (all three had atopic eczema and two infants had recurrent wheezing).

Definition of type of feeding

Babies were administered water containing 10% glucose at about 6 h after delivery, and were fed formula milk until receiving breast milk from their mothers in the maternity ward of the hospital until discharge. Consequently, all babies were fed breast milk and formula before 5 days of age. 'Breastfed' infants in this study are defined as those who had occasional formula milk but were exclusively breastfed after 1 month of age. 'Formula-fed' infants are those who had occasional breast milk but were exclusively formula-fed after 1 month of age. 'Mixed-fed' infants are those who were fed breast milk and formula throughout the study period.

Fecal storage and processing for polymerase chain reaction analysis of *Bifidobacterium* species

Fecal samples were frozen at -20°C until analysis. Samples of 100 mg were washed three times by suspension in 1.0 mL of phosphate buffered saline and centrifugation at 14 000 g to reduce the level of PCR inhibitors. The fecal pellets were resuspended in 450 μL of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 μL of 10% sodium dodecyl sulphate. Then, 300 mg of glass beads (diameter, 0.1 mm) and 500 μL of buffer-saturated phenol were added to the suspension, and the samples were mixed vigorously for 30 s using a FastPrep FP 120 (Bio101 Inc, Vista, CA, USA) at a power level of 5.0. After centrifugation at 14 000 g for 5 min, the supernatants were collected, and extracted with phenol-chloroform. DNA was obtained by isopropanol precipitation, and suspended in 100 μL of TE Buffer.

Detection of *Bifidobacterium* species by polymerase chain reaction using species-specific or group-specific primers

The presence of *Bifidobacterium* species was determined using nine *Bifidobacterium* species-specific or group-

specific primers based on the 16S rDNA sequences as reported by Matsuki et al. [10]. Briefly, PCR mixtures (50 µL) were composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 mM deoxynucleoside triphosphate, 0.25 µM species-specific or group-specific primer, template DNA, and 1.25 U of *E_x*Taq DNA polymerase (TaKaRa, Ohtsu, Japan). PCR was carried out with a GeneAmp[®] PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using an amplification program consisting of one cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, and, finally, one cycle of 72 °C for 5 min. The amplification products were subjected to gel electrophoresis in 2.5% agarose gels, and visualized by ethidium bromide staining.

Statistical evaluation

Data were analysed using computer software in the statistical package for social sciences (SPSS standard version 11.0; SPSS Inc, Cary, NC, USA). $P < 0.05$ was considered significant. Maternal age, gestational age, and body weight at birth were compared between allergic infants and healthy controls using the Student's *t*-test. Proportion of sex, mode of delivery, atopic heredity, distribution of feeding method, and frequencies of *Bifidobacterium* species between groups were evaluated using Fisher's exact test.

Ethical considerations

This study was approved by the ethical committee of Omigawa General Hospital and performed in accordance with the Declaration of Helsinki.

Results

Frequencies of *Bifidobacterium* species in allergic infants and healthy controls

Figure 1 shows the frequencies of nine fecal *Bifidobacterium* species in 10 allergic infants and 16 healthy controls.

At 1 month of age, the most frequently detected species belonged to the *B. adolescentis* group in healthy controls (43.8%), whereas it was the *B. catenulatum* group in allergic infants in whom the frequency was significantly higher than in healthy controls (60.0% vs. 6.3%, respectively, $P < 0.01$).

At 3 months of age, the most frequently detected species remained those of the *B. adolescentis* group in healthy controls (68.8%), whereas *B. breve* was the most frequently detected species in allergic infants (70.0%). The frequency of *B. catenulatum* group species in allergic infants decreased dramatically and they became less frequent than in healthy controls (20.0% vs. 31.3%, respectively). Allergic infants had a tendency to have a

higher frequency of *B. bifidum* than healthy controls (50.0% vs. 12.5%, respectively, $P = 0.069$).

At 6 months of age, *B. adolescentis* group species, *B. breve*, and *B. longum* biotype *longum* were found with high frequencies in both groups. In contrast, *B. bifidum* was found with high frequency only in allergic infants, with a significant difference as compared with healthy controls (70.0% vs. 12.5%, respectively, $P < 0.01$).

The number of infants in whom no *Bifidobacterium* species could be found was four (15.4%) at 1 month, two (7.7%) at 3 months, and one (3.3%) at 6 months of age; all of them were healthy controls. Deletion of these data did not affect the significance of the overall findings (data not shown).

Effect of feeding method on the distribution of *Bifidobacterium* species at 6 months of age

As the feeding method may influence the colonization of *Bifidobacterium* species, we compared the distribution of *Bifidobacterium* species based on feeding method. As shown in Fig. 2, there were no differences in the distribution of *Bifidobacterium* species due to feeding method at 1, 3, or 6 months of age.

Discussion

In the present study, the composition of fecal *Bifidobacterium* was found to differ between allergic infants and healthy controls. Allergic infants had a higher prevalence of *B. catenulatum* group species at 1 month of age and *B. bifidum* at 6 months of age than healthy controls. There was no difference in the prevalence of *B. adolescentis* between allergic infants and controls. Feeding method was not related to the differences in the composition of fecal *Bifidobacterium*. The finding of a difference in *Bifidobacterium* species between allergic subjects and non-allergic controls is consistent with the report of Ouwehand from Finland [4, 5]. However, the dominant species in allergic and non-allergic subjects in the present study differs somewhat from those in their data, which showed *B. bifidum* to colonize most non-atopic healthy infants whereas *B. adolescentis* colonized most allergic infants. *B. catenulatum* is generally categorized as an adult-type *Bifidobacterium* species; thus the findings in the two studies are basically the same in terms of the presence of an adult-type *Bifidobacterium* species in allergic subjects. However, the rate of detection of *B. bifidum* is opposite between the Finnish study and our present study.

One explanation for this difference involves genetic and environmental differences between Finland and Japan, as the implantation and development of *Bifidobacterium* in the neonate are greatly influenced by prematurity, method of birth, early feeding regime, endogenous substrate availability from breast milk or formula and the environment [11]. There is a clear

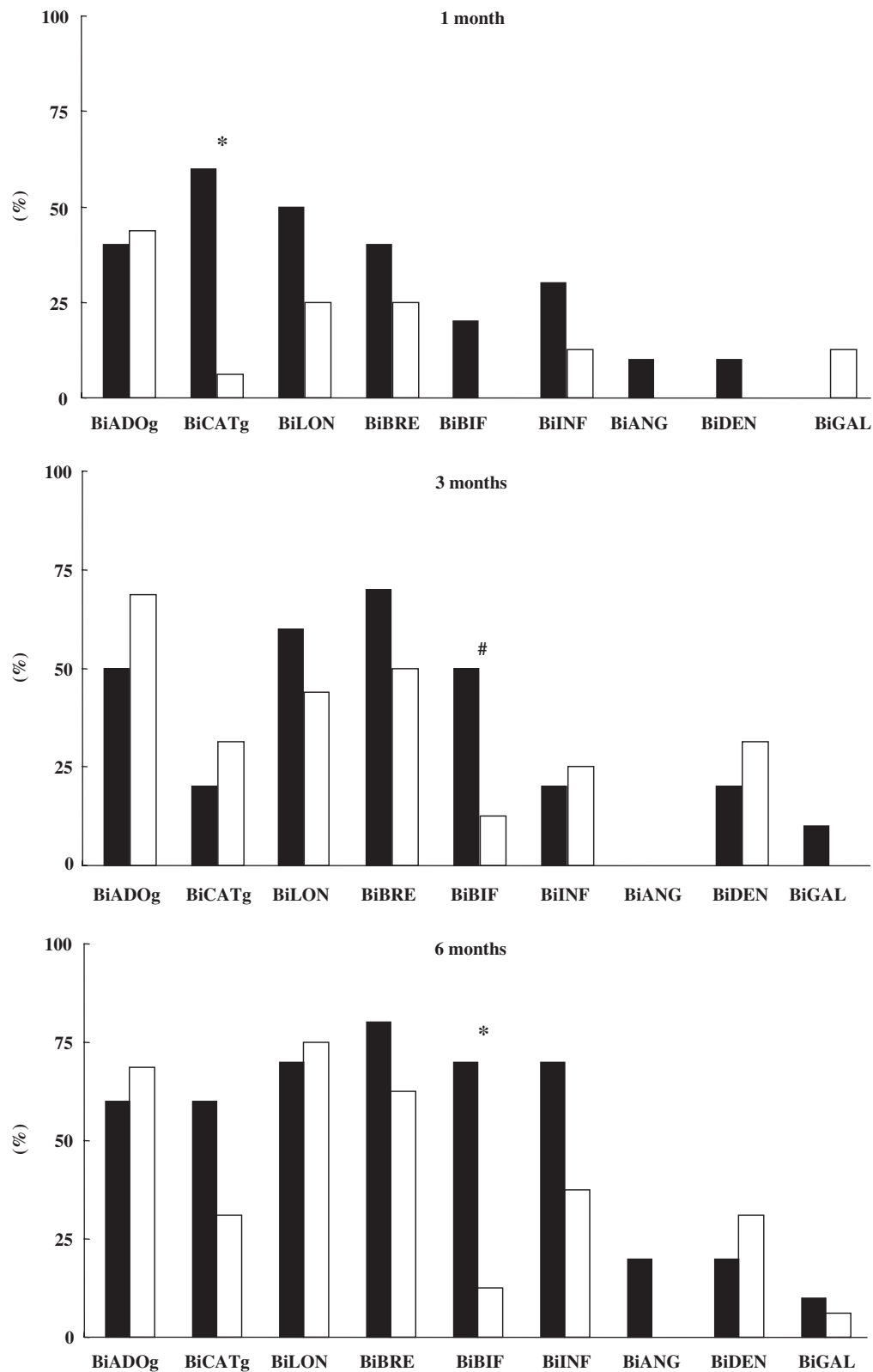


Fig. 1. Frequencies of *Bifidobacterium* species in allergic infants ($n = 10$, black bars) and healthy controls ($n = 16$, white bars) at 1, 3, and 6 months of age. Primers BiADOg, BiCATg, BiLON, BiBRE, BiBIF, BiINF, BiANG, BiDEN, and BiGAL are specific for the *B. adolescentis* group, the *B. catenulatum* group, *B. longum* biotype *longum*, *B. breve*, *B. bifidum*, *B. longum* biotype *infantis*, *B. angulatum*, *B. dentium*, and *B. gallicum*, respectively. * $P < 0.01$, # $P < 0.1$.

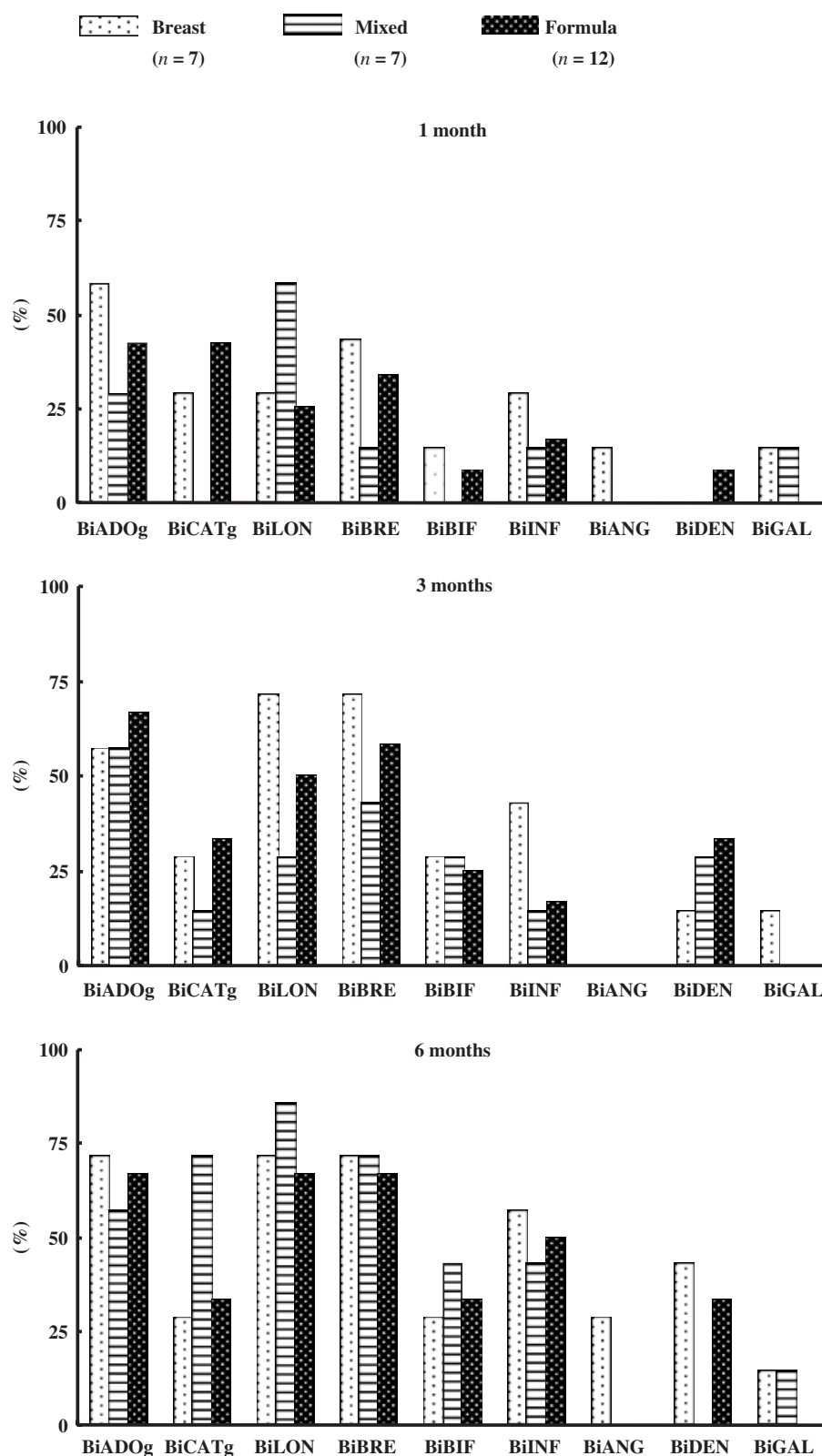


Fig. 2. Frequencies of *Bifidobacterium* species at 1, 3, and 6 months of age among babies fed by three different feeding methods. Open bars with black dots: breastfeeding (n = 7); striped bars (n = 7): mixed-feeding; closed bars with white dots: formula-feeding (n = 12). There was no difference of the distribution of *Bifidobacterium* species according to feeding method at any age. Primers BiADOG, BiCATg, BiLON, BiBRE, BiBIF, BiINF, BiANG, BiDEN, and BiGAL are specific for the *B. adolescentis* group, the *B. catenulatum* group, *B. longum* biotype *longum*, *B. breve*, *B. bifidum*, *B. longum* biotype *infantis*, *B. angulatum*, *B. dentium*, and *B. gallicum*, respectively.

difference in the composition of *Bifidobacterium* species other than members of the *B. bifidum* and *B. adolescentis* groups between infants in the report by Ouwehand and those in the present study. Ouwehand found very low frequencies of *B. breve* and *B. longum* biotype *longum* in both allergic and healthy infants (2–7 months of age). In contrast, in the present study, we found *B. longum* biotype *longum* and *B. breve* in more than 50% of infants irrespective of the presence or absence of allergy. Matsuki et al. [12] also showed that 37% of 1-month-old Japanese infants were colonized with *B. longum* biotype *longum* and 70% with *B. breve*. Bjorksten et al. [3] followed babies in Sweden and Estonia until 1 year of age, and compared fecal microbiota between 18 allergic and 26 feeding-matched controls. In their study, the prevalence of *Bifidobacterium* at 6 months of age was 28% in allergic infants and 42% in controls. In contrast, 100% of the infants were colonized with *Bifidobacterium* irrespective of allergic status in the present study (data not shown). Nambu et al. [13] studied intestinal microbiota in 4-month-old Japanese infants, and also found *Bifidobacterium* in all infants studied. Young et al. [14] reported a significant species difference in *Bifidobacterium* among residents of Ghana, New Zealand and the United Kingdom. These results suggest significant differences in the prevalence of *Bifidobacterium* species among countries and races.

Another possibility for the different detection rate of *Bifidobacterium* species is the methods used. Ouwehand detected *Bifidobacterium* species by culture methods while we used PCR-based detection of *Bifidobacterium* species. The detection limit of the PCR method by Matsuki et al. [15] is 10^6 – 10^7 cells/g wet feces, while that of the classic culture method is 10^9 – 10^{10} cells/g wet feces. This suggests that PCR is more sensitive than the culture method in the detection of *Bifidobacterium*. This might be a cause for the different detection rates of *Bifidobacterium* species between our study and that of Ouwehand.

The present work is the first to determine differences in *Bifidobacterium* species between allergic and healthy infants in Japan. When intestinal microbiota at 6 months of age were examined by a conventional culture method, there were no statistically significant differences in the numbers or prevalence of major flora, including *Bifidobacterium*, between allergic and non-allergic infants (data not shown). Thus, the results indicate some correlation between species differences in intestinal *Bifidobacterium* and allergic diseases in infants, although our data are in contrast with the report from Finland. The biological mechanisms for this difference in the development of allergic diseases in infancy remain to be determined.

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