Early differences in fecal microbiota composition in children may predict overweight[1–3]

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ABSTRACT

Background: Experimental studies suggest that gut microbiota deviations predispose toward energy storage and obesity.

Objective: We wanted to establish whether early gut microbiota composition can guide weight development throughout early childhood.

Design: Overweight and obese children ($n = 25$) were selected from a prospective follow-up study at the age of 7 y and identified according to the International Obesity Task Force criteria. Normal-weight children ($n = 24$) were selected from the same cohort and matched for gestational age and body mass index at birth, mode of delivery, probiotic supplementation, duration of breastfeeding, use of antibiotics during infancy, and frequencies of atopic diseases and atopic sensitization. Early fecal microbiota composition was analyzed by fluorescent in situ hybridization (FISH) with microscopic and flow cytometry detection and by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The bifidobacterial numbers in fecal samples during infancy, as assessed by the FISH with flow cytometry, were higher in children remaining normal weight, [median: 2.19×10^9 cells/g (interquartile range: $1.10–5.28 \times 10^9$ cells/g)] than in children becoming overweight [1.20×10^9 cells/g ($0.48–1.59 \times 10^9$ cells/g); $P = 0.02$]. A similar tendency was found by FISH with microscopic detection and qRT-PCR. The microbiota aberrancy during infancy in children becoming overweight was also associated with a greater number of *Staphylococcus aureus* [0.64×10^6 cells/g ($0.33–1.00 \times 10^6$ cells/g)] than in children remaining normal weight [0.27×10^6 cells/g ($0.17–0.50 \times 10^6$ cells/g); $P = 0.013$].

Conclusion: Aberrant compositional development of the gut microbiota precedes overweight, offering new possibilities for preventive and therapeutic applications in weight management. *Am J Clin Nutr* 2008;87:534–8.

KEY WORDS Bifidobacteria, children, gut microbiota, infants, obesity, overweight, *Staphylococcus aureus*

INTRODUCTION

Obesity is viewed as one of the important public health problems of our times, and the velocity of propagation is highest in children (1). Children may find themselves in a vicious circle: obese children often become obese adults and maternal obesity overnourishes the fetus, thereby programming adult size and health with a heightened risk of obesity later in life (2). Recent scientific advances point to systemic low-grade inflammation and gut local microbiota as contributing factors for overnutrition (3, 4).

The gut microbiota enables hydrolysis of indigestible polysaccharides to easily absorbable monosaccharides and activation of lipoprotein lipase by direct action on the villous epithelium (4). Consequently, glucose is rapidly absorbed and fatty acids are excessively stored, which both processes boost weight gain. Indeed, increased numbers of *Bacteroides* in the gut microbiota were shown in an experimental animal model to predispose toward energy storage and obesity (4, 5). Gut microbiota alterations in humans during a critical maturational period to date were linked to the development of various inflammatory conditions such as allergy (6). An ambiguous relation between obesity and asthma was also suggested (7). We therefore have evaluated the gut microbiota in infants at high risk of allergy in relation to later weight gain.

SUBJECTS AND METHODS

Design

Subjects for the study were selected from a prospective follow-up on probiotics in allergic disease, as described in greater detail elsewhere (8). In brief, subjects were examined by one of the authors (MK) at birth and at the ages of 3, 6, 12, 18, and 24 mo and at 4 and 7 y. In addition, weight and height were assessed. Body mass index (BMI; in kg/m^2) at 7 y of age was calculated. Instead of adult BMI criteria, which underestimate the extent of adiposity in childhood (9), we established weight status by the International Obesity Task Force criteria for overweight and obesity (9). These criteria identify BMI values for each age associated with a predicted BMI of 25 or 30, respectively, at age 18 y. On the basis of the criteria, 25 overweight or

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[2] Supported by the Academy of Finland, Excellence Postdoctoral grant from Conselleria Empresa, Universidad y Ciencia de la Generalitat Valenciana, Spain (BPOSTDOC 06/016; MCC).

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Received March 26, 2007.

Accepted for publication October 8, 2007.

obese children were selected. Twenty-four normal-weight children were selected from the same cohort and matched for gestational age and BMI at birth, mode of delivery, probiotic intervention, duration of breastfeeding, use of antibiotics during infancy, and frequencies of atopic diseases and atopic sensitization at 7 y of age. Gut microbiota composition was analyzed at the ages of 6 and 12 mo.

Gut microbiota analyses

Analysis of microbiota by in situ hybridization

The main groups of fecal bacteria were analyzed with the use of the fluorescent in situ hybridization (FISH) method as previously described (6). In brief, fecal samples were suspended in phosphate-buffered saline (PBS) and homogenized. Bacteria were fixed with 4% paraformaldehyde, washed with PBS, and stored in 50% ethanol-PBS at −20 °C until analyzed. Probes included Bac303 (5'-CCAATGTGGGGGACCTT) for the *Bacteroides-Prevotella* group, Bif164 (5'-CATCCGGCATTACCACCC) for the *Bifidobacterium* genus, CHis150 (5'-TTATGCGGTATTAAT CT(C/T)CCTTT) for the C*lostridium histolyticum* group, and Lab158 (5'-GGTATTAGCA(T/C)GTGTTTCCA) for the *Lactobacillus-Lactococcus-Enterococcus* group (6). Total bacterial counts were determined by staining with 4',6-diamino-2-phenylindole. The bacteria were washed and filtered on 0.22-μm polycarbonate filters. These were then mounted on slides and counted visually under an epifluorescence microscope (BX51; Olympus, Hamburg, Germany) with the use of Cy3-labeled probes and 4',6-diamino-2-phenylindole–specific filters. At least 15 random fields were counted on each slide, and the average count was used for analysis.

Analysis of microbiota by in situ hybridization combined with flow cytometry

Hybridization of samples was performed as described above for the FISH analysis. Probes included Bac303 for the *Bacteroides-Prevotella* group, Bif164 for the *Bifidobacterium* group; Cy3-labeled and total cells were enumerated with the use of an EUB338 (5'-GCTGCCTCCCGTAGGAGT)–fluorescein (FITC)–labeled probe. Flow cytometric analyses were performed with the use of a BD LSR II flow cytometer (Becton Dickinson and Co, Franklin Lakes, NJ) equipped with a 488-nm laser at 15 mW. This standard instrument is equipped with 2 light scatter detectors that measure forward and side scatter and 4 fluorescence detectors detecting appropriately filtered light at green (FL1, 525 nm), orange (FL3, 620 nm), and red (FL4, 675 nm) wavelengths. To avoid cell coincidence, the flow rate was kept at the lowest setting (data rate: 200–300 event/s). At least 30 000 events were recorded for each sample, and all experiments were conducted in duplicate. Data were stored as list-mode files and analyzed off line with the use of the BD FACSDIVA software version 4.1.1 (Becton Dickinson and Co).

Absolute bacterial cell counts were determined with the use of Flow-Count fluorospheres (Beckman Coulter, Berea, CA) following the manufacturer's instructions. To avoid loss of the signal intensity of hybridized cells, they were kept in the dark on ice at 4 °C until the flow cytometry assay. Results were expressed as the numbers of cells hybridizing with the specific group-Cy3 probe determined by total bacteria EUB 338-FITC hybridization.

Analysis of microbiota by quantitative real-time polymerase chain reaction

DNA extractions from pure cultures of the different microorganisms and fecal samples were extracted with the use of the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantitative polymerase chain reactions (qPCRs) were conducted as previously described (10, 11). For characterization of the fecal microbiota, PCR primers were designed to target different *Bifidobacterium* species or groups, including *Bifidobacterium* genus, *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve,* and *Bifidobacterium longum* group, according to Gueimonde (12, 13). The *Bacteroides fragilis* group was determined according to the method of Matsuki et al (11), and *Staphylococcus aureus* group was determined by conditions described by Fang and Hedin (14). These oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany). Briefly, PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Cheshire, United Kingdom). Each reaction mixture of 25 μL was composed of SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of each of the specific primers at a concentration of 0.25 μmol/L, and 1 μL of template DNA. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product.

Statistical analyses

Because of nonnormal distribution, microbial data are expressed as medians with interquartile ranges. To test for interaction between bacterial numbers at 6 and 12 mo of age, we calculated the rank of the ratio of the 12-mo to the 6-mo value of each bacterium studied. Thereafter, the ranks were compared between the groups by the Wilcoxon's rank-sum test. Because no statistically significant differences were observed between the groups, the mean number of each bacterium studied during infancy was obtained by dividing the sum of the bacterial numbers at 6 and 12 mo by 2. The Mann-Whitney *U* test was then applied in comparisons between normal-weight and overweight children. Dichotomous and continuous data on clinical characteristics were analyzed by chi-square tests and unpaired *t* tests, respectively. Wilcoxon's signed rank test was used to compare bacterial numbers between 6 and 12 mo of life in the whole study population. A P value < 0.05 was considered statistically significant. The SPSS 11.0.2 version for Mac OS X statistical software package (SPSS Inc, Chicago, IL) was used to analyze the data.

RESULTS

The clinical characteristics of the subjects are presented in **Table 1**. Of the 25 overweight children, 7 were obese at the age of 7 y. The median (interquartile range) BMI at 7 y was 19.74 (18.91–20.77) in the overweight children and 15.53 (14.79–16.20) in the controls ($P < 0.0001$). The respective BMI values during infancy were 18.17 (17.36–19.24) and 16.96 (16.00–17.36) at 6 mo ($P < 0.0001$) and 18.20 (17.26–18.92) and 16.73 (16.00–17.34) at 12 mo ($P < 0.0001$).

TABLE 1
Clinical characteristics of the study subjects[1]

Characteristics	Normal-weight children ($n = 24$)	Overweight children ($n = 25$)
Gestational age at birth (wk)[1]	39.8 ± 1.06[2]	39.0 ± 1.51
BMI at birth (kg/m^2)[1]	13.71 ± 0.95	14.46 ± 1.61
BMI at 7 y of age (kg/m^2)	15.56 ± 0.85	20.16 ± 1.50
Vaginal delivery (*n*/total [%])	19/24 [79]	22/25 [88]
Probiotic supplementation (*n*/total [%])	11/24 [46]	11/25 [44]
Breastfeeding (mo)[1]		
Exclusive	2.7 ± 2.0	2.5 ± 1.6
Total	7.7 ± 3.9	6.3 ± 4.0
Use of antibiotics (*n*/total [%])		
By 6 mo	3/24 [13]	10/25 [40]
Between 7th and 12th mo	10/24 [42]	13/25 [52]
Eczema (*n*/total [%])	10/24 [42]	9/25 [36]
Allergic rhinitis (*n*/total [%])	4/24 [17]	2/25 [8]
Asthma (*n*/total [%])	3/24 [13]	4/25 [16]
Atopic sensitization (*n*/total [%])	7/24 [29]	6/22 [27]

[1] None of the clinical characteristics were significantly different between normal-weight and overweight children ($P > 0.05$ in all comparisons by chi-square test or unpaired *t* test).
[2] $\bar{x}$ ± SD (all such values).

The bifidobacterial numbers in fecal samples during infancy, as assessed by FISH with flow cytometry, were higher in children remaining at normal-weight, 2.19 × 10^9 cells/g (1.10–5.28 × 10^9 cells/g; $n = 13$) than were children becoming overweight [1.20 × 10^9 cells/g (0.48–1.59 × 10^9 cells/g; $n = 19$; $P = 0.02$]. Similar tendency in bifidobacterial numbers was also found by FISH with microscopic detection and qRT-PCR, as shown in **Table 2** and **Table 3**, respectively. Normal-weight development was further linked with lower numbers of fecal *S. aureus* (Table 3). Bifidobacterial numbers, as assessed by FISH with microscopic detection, were lower at 12 mo than at 6 mo in the whole study population, probably reflecting the effect of decreasing exposure to breast milk ($P < 0.0001$; data not shown). All the other bacterial numbers were not different at these time points (data not shown).

DISCUSSION

We show here for the first time in the human situation that differences in the intestinal microbiota may precede overweight development. The genus *Bifidobacterium*, affecting both the quantity and quality of the microbiota during the first year of life, was shown to be higher in number in children who remained normal weight at 7 y than in children developing overweight. This finding was confirmed by FISH analysis with cytometry detection, an improved method of accurate bacterial counting (15, 16). Similar tendency was also found by 2 other methods: traditional FISH analysis, a reliable method for major microbiota characterization also in human studies (6), together with qPCR analysis, a real-time assessment of quantitative and qualitative composition of bifidobacterial microbiota (13). In addition, fecal numbers of *S. aureus*, as assessed by real-time qPCR, were lower in children remaining normal weight than in children developing overweight. Thus, these findings unequivocally imply that high numbers of bifidobacteria and low numbers of *S. aureus* in infancy may provide protection against overweight and obesity development.

Bifidobacteria, especially *B. breve*, *B. infantis*, and *B. longum*, typify the gut microbiota composition of the healthy breastfed infant (17). Recent meta-analyses have concluded that having been breastfed is associated with a 13–22% reduced likelihood of overweight or obesity in childhood and that the duration of breastfeeding is inversely associated with the risk of overweight

TABLE 2
Bacterial counts in fecal samples analyzed by fluorescent in situ hybridization during infancy

	Normal-weight children	Overweight children	Mann-Whitney *U* test
Bifidobacteria (×10^9 cells/g)			
6 mo	2.47 (1.52–4.21) [16][1]	1.46 (0.35–2.35) [23]	
12 mo	0.70 (0.39–1.69) [19]	0.20 (0.14–0.88) [22]	
During infancy[2]	1.62 (1.01–3.73) [14]	1.05 (0.61–1.38) [20]	0.08
Bacteroides (×10^8 cells/g)			
6 mo	0.62 (0.12–3.05) [16]	0.83 (0.24–2.28) [23]	
12 mo	0.43 (0.13–2.50) [19]	0.91 (0.39–4.45) [22]	
During infancy[2]	0.96 (0.24–4.0) [14]	1.26 (0.49–2.77) [20]	0.44
Lactobacilli (×10^8 cells/g)			
6 mo	3.26 (0.57–8.25) [16]	1.14 (0.60–2.54) [23]	
12 mo	1.20 (0.64–2.30) [19]	1.13 (0.26–2.76) [22]	
During infancy[2]	2.46 (1.24–4.59) [14]	1.43 (0.82–2.23) [20]	0.11
Clostridia (×10^8 cells/g)			
6 mo	0.91 (0.38–3.63) [16]	0.73 (0.24–1.78) [23]	
12 mo	1.48 (0.44–5.87) [19]	0.45 (0.065–2.78) [22]	
During infancy[2]	2.18 (1.18–3.74) [14]	1.49 (0.39–2.45) [20]	0.15
Total cell count (×10^9 cells/g)			
6 mo	5.47 (3.43–10.10) [16]	3.52 (2.26–5.09) [23]	
12 mo	3.44 (2.07–10.35) [19]	3.22 (1.96–5.03) [22]	
During infancy[2]	4.44 (2.68–9.39) [14]	3.54 (2.27–5.11) [20]	0.29

[1] Median; interquartile range in parentheses; *n* in brackets (all such values).
[2] The mean bacterial number was obtained by dividing the sum of the bacterial numbers at 6 and 12 mo by 2.

TABLE 3
Bacterial counts in fecal samples analyzed by quantitative real-time polymerase chain reaction during infancy[1]

	Normal-weight children	Overweight children	Mann-Whitney *U* test
Bifidobacterium genus ($\times 10^9$ cells/g)			
6 mo	10.50 (4.76–17.71) [17][2]	1.58 (0.93–7.60) [21–22]	
12 mo	5.79 (1.85–14.83) [18]	1.52 (0.16–8.13) [20–21]	
During infancy[3]	8.23 (2.67–11.6) [8–13]	4.91 (1.53–9.60) [16–18]	0.20
Bifidobacterium longum ($\times 10^8$ cells/g)			
6 mo	56.2 (0.6–102.0) [17]	4.8 (0.016–32.01) [21–22]	
12 mo	2.27 (0.15–6.83) [18]	1.08 (0.02–2.62) [20–21]	
During infancy[3]	4.05 (0.17–7.20) [8–13]	1.57 (0.24–5.69) [16–18]	0.42
Bifidobacterium breve ($\times 10^6$ cells/g)			
6 mo	3.32 (ND–268.50) [17]	0.11 (ND–52.50) [21–22]	
12 mo	9.28 (0.038–54.60) [18]	ND (ND–9.58) [20–21]	
During infancy[3]	25.52 (12.22–290.5) [8–13]	0.53 (ND–198.5) [16–18]	0.59
Bifidobacterium bifidum ($\times 10^6$ cells/g)			
6 mo	0.21 (ND–312.65) [17]	ND (ND–1.64) [21–22]	
12 mo	0.044 (ND–222.30) [18]	0.044 (ND–294.0) [20–21]	
During infancy[3]	8.04 (ND–230.5) [8–13]	50.7 (0.03–287.3) [16–18]	0.49
Bifidobacterium adolescentis ($\times 10^6$ cells/g)			
6 mo	ND (ND–139.5) [17]	ND (ND–0.15) [21–22]	
12 mo	ND (ND–5.08) [18]	ND (ND–ND) [20–21]	
During infancy[3]	0.05 (ND–445.5) [8–13]	ND (ND–33.7) [16–18]	0.55
Bacteroides fragilis ($\times 10^6$ cells/g)			
6 mo	2.69 (0.11–74.45) [17]	14.33 (3.01–95.79) [21–22]	
12 mo	12.59 (0.16–64.64) [18]	9.31 (2.37–53.45) [20–21]	
During infancy[3]	33.3 (2.50–191.9) [8–13]	22.9 (7.47–78.3) [16–18]	0.88
Staphylococcus aureus ($\times 10^6$ cells/g)			
6 mo	0.28 (0.097–0.58) [17]	1.00 (0.40–1.14) [21–22]	
12 mo	0.13 (0.085–0.26) [18]	0.27 (0.15–1.00) [20–21]	
During infancy[3]	0.27 (0.17–0.50) [8–13]	0.64 (0.33–1.00) [16–18]	0.013

[1] ND, not detected.
[2] Median; interquartile range in parentheses; *n* in brackets (all such values).
[3] The mean bacterial number was obtained by dividing the sum of the bacterial numbers at 6 and 12 mo by 2.

(1, 18, 19). We suggest that bifidobacteria constitute an internal link between breastfeeding and weight development. First, human milk contains bifidobacteria and also oligosaccharides that favor their thriving in the infant gut (20, 21). Genomic studies have shown convincingly that bifidobacteria present in the gut of breastfed infants, eg, *B. longum*, are specially equipped to use breast milk oligosaccharides as nutrients. *B. longum* is also adapted to the conditions in the lower human gut, where energy harvest from slowly absorbable carbohydrates takes place (22). These bacteria dominate the gut microbiota, representing 60–90% of the total microbiota in healthy infants during breastfeeding, and they influence the total metabolic activity of the gut microbiota by the mucosal cross-talk between microbes and the host. Moreover, breast milk also contains soluble pattern recognition receptors that recognize microbial constituents in the gut and thereby regulate activation of the innate immune system in such a way that immunopathology is dampened (23–25). Breast-milk-derived soluble receptors are also vital in linking fatty acids with the innate immune system, further strengthening the beneficial host microbe cross-talk for inflammation control (26). In view of the recent demonstration that adiposity is characterized by low-grade inflammation, the control of inflammatory pathways may provide new potential approaches for future therapy.

Infants receive their first microbial inoculation at the time of delivery. These inoculated bacteria reflect the microbiota of the mother's vagina and gastrointestinal tract. This inoculation is further reinforced during breastfeeding by breast-milk-derived galactooligosaccharides, bacteria in breast milk and the breast's skin. Thus, the part of the maternal microbiota is transferred to the infant, influencing further intestinal microbiota development. Recent studies suggest that especially skin-derived bacteria may have an important contribution in early colonization of the gut. Unlike a few decades ago (27–29), coagulase-negative staphylococci are now recognized as characteristic first-colonizers of the newborn gut, regardless of the mode of delivery (30). Improved hygiene was suggested to be a cause for this early colonization of the gut by traditional skin bacteria (30, 31). Moreover, a majority of the infants are colonized by *S. aureus* during the first months of life by parental skin (32). Such a colonization is long term in nature, and many strains produce toxins that can act as superantigens, carrying proinflammatory potential (31, 33). Indeed, a recent study found a strong specific association between early intestinal colonization with *S. aureus* and an increase in circulating soluble CD14, a marker of systemic inflammation (34). Hence, we speculate that *S. aureus* may indeed act as a trigger of low-grade inflammation (34), contributing to the development of obesity.

Taken together, a physically active lifestyle and avoidance of excessive energy intake lay the foundation for the prevention of obesity. Our results, however, indicate that changes in the gut microbiota may be linked not only to the development of allergy but also to other chronic inflammatory conditions common in the Western world, among them obesity, thus extending the concept of the hygiene hypothesis. Exploring and understanding these

mechanisms of action thoroughly may offer a new resort in the fight against the alarming pandemic of obesity.

We thank Tuija Poussa, MSc, for statistical consultation and Robert MacGilleon, MSc, for grammatical review of the manuscript.

The author's responsibilities were as follows—SS, MK, and EI: made study concept and design; MK and MCC: acquired data and made statistical analyses; all authors participated in analysis and interpretation of data and in preparation of the manuscript. None of the authors had a personal or financial conflict of interest.

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