

# Habitual Dietary Intake Is Associated with Stool Microbiota Composition in Monozygotic Twins<sup>1–3</sup>

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## Abstract

The impact of diet on the gut microbiota has usually been assessed by subjecting people to the same controlled diet and thereafter following the shifts in the microbiota. In the present study, we used habitual dietary intake, clinical data, quantitative polymerase chain reaction, and denaturing gradient gel electrophoresis (DGGE) to characterize the stool microbiota of Finnish monozygotic twins. The effect of diet on the numbers of bacteria was described through a hierarchical linear mixed model that included the twin individuals, stratified by body mass index, and their families as random effects. The abundance and diversity of the bacterial groups studied did not differ between normal-weight, overweight, and obese individuals with the techniques used. Intakes of energy, monounsaturated fatty acids, n3 polyunsaturated fatty acids (PUFAs), n6 PUFAs, and soluble fiber had significant associations with the stool bacterial numbers (e.g., increased energy intake was associated with reduced numbers of *Bacteroides* spp.). In addition, co-twins with identical energy intake had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. than did the co-twins with different intake. Moreover, the co-twins who ingested the same amounts of saturated fatty acids had very similar DGGE profiles of *Bacteroides* spp., whereas the co-twins with similar consumption of fiber had a very low bifidobacterial DGGE-profile similarity. In conclusion, our findings confirm that the diet plays an important role in the modulation of the stool microbiota, in particular *Bacteroides* spp. and bifidobacteria. J. Nutr. 143: 417–423, 2013.

## Introduction

The influence of the diet on the gastrointestinal tract microbiota composition, numbers, and activity has been discussed for several decades (1,2). Early culture-based studies comparing defined diets (e.g., Japanese versus Western) did not show major differences in the composition of the resident stool microbiota (3), whereas chemically defined diets produced diminished stool mass associated with compositional changes in the microbiota (4). Advances in molecular microbiologic techniques have expanded our knowledge on the gut microbial ecology (5), thus starting a new era of the study of the impact of diet and dietary changes on the resident microbiota. At the same time, the diet

itself has been changing worldwide as a result of alterations in lifestyle, agricultural practices, and population growth (6). Controlled diets, such as those having high protein and reduced carbohydrate content (7), or diets differing in nondigestible carbohydrate content (8) have been used to study the influence of the diet-induced changes in the microbiota. In addition, stool microbiota of individuals with different types of habitual diets [e.g., vegetarians or vegans versus omnivores (9,10)] or from geographically distinct areas (11,12) have been characterized. It has become evident that the diet has a dominant role on the stool microbiota and that the diet-driven changes in it occur within days to weeks (8).

Dietary and physical activity patterns contribute to weight-imbalance disorders. Recently, there has been increased interest in the potential relation between gut microbiota and the development of obesity. Studies on energy-restricted diets administered in overweight and obese individuals aiming to relate the amount of body weight or weight loss to specific microbial groups have reported contradictory results (13–15). On the other hand, other studies involving human volunteers have mainly characterized the microbiota in stools according to individual BMI, regardless of diet (16–18). Previous work on obesity has shown distinct between-subject variations in stool bacterial diversity (13,19,20). Mixed genetic backgrounds and differences in analysis methodologies used (21) may, however,

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<sup>3</sup> Supplemental Tables 1–3 and Supplemental Figures 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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explain some of the controversies found in these studies. Studies in monozygotic (MZ) weight-concordant twins have shown that the MZ twins had a more similar stool bacterial community structure than unrelated individuals, suggesting a role for host genetic factors (18,22). It will therefore be very difficult to distinguish whether the changes observed in the stool microbiota of obese people are due to obesity itself, the diet related to it, or the host genetic make-up when obese and lean individuals from different genetic pools are compared. In contrast, MZ twins discordant for BMI offer an excellent tool for studying changes in the gut microbiota of obese and normal-weight subjects perfectly matched for genotype. Our aim was to study whether there is a correlation between the diet and the numbers and/or diversity of the predominant bacterial groups in stools. We studied the *Eubacterium rectale* group, the *Clostridium leptum* group, and *Bacteroides* spp., which together account for 50–70% of the human stool microbiota (11). Lactobacilli and bifidobacteria, although comprising smaller proportions of the stool microbiota, were also included, because both of those groups have traditionally been regarded as beneficial to human health. The *Atopobium* group (i.e., Coriobacteriaceae) has received less attention in the diet-related studies, but because it has been shown with hybridization-based studies that the *Atopobium* group comprises 1–5% of the stool microbiota (11) and that the proportion of Actinobacteria in stool microbiota may presently be underestimated (21,23,24), we also included the *Atopobium* group in our study. In addition to studying the correlation between the predominant bacterial groups and diet, we also studied how the intrapair differences in nutritional intake of MZ twins, concordant or discordant for BMI, correlated with the intrapair differences of bacterial group cell numbers and similarities.

## Participants and Methods

**Participants and sample collection.** A total of 20 MZ twin pairs were recruited from a population-based longitudinal survey of 5 consecutive birth cohorts of twins (1975–1979) identified through the national population registry of Finland. The individuals were healthy based on their medical history and clinical examination, and their weight had been stable for at least 3 mo before the study. No medications other than contraceptives were used during the same period. Status of zygosity was confirmed by genotyping of 10 informative genetic markers (25). Weight, height, and body fat were measured as described previously (26). The participants were divided in categories according to their BMI (in kg/m<sup>2</sup>) as follows: normal weight (19 ≤ BMI < 25), overweight (25 ≤ BMI < 30), and obese (BMI ≥ 30) (Table 1). Nine twin pairs were concordant (BMI difference < 3 kg/m<sup>2</sup>) and 11 pairs were discordant for BMI (BMI difference ≥ 3 kg/m<sup>2</sup>); a BMI difference of 3 kg/m<sup>2</sup> represented the top 5% most discordant MZ twin pairs (25,27). Eleven of the twin pairs were female and 9 were male. The study protocols were approved by the ethics committee of the Hospital District of Helsinki and Uusimaa, Finland. Written informed consent was obtained from all participants. The participants collected the stool samples at home and stored them in their home freezer (−18°C) before taking the samples to the laboratory. The samples were stored in the laboratory at −70°C until analysis.

**Nutritional intake.** Dietary information was obtained from each individual based on a 3-d food diary (2 weekdays and 1 weekend day) that reflected the habitual dietary intake (Table 2). Although self-recorded estimates of food intake as food diaries may not provide accurate or unbiased estimates of a person's energy intake, the volunteers in our study were supervised by a specialist to ensure the best possible outcome. The food diaries were analyzed with the program DIET32 (Aivo Finland), which is based on a national database for food composition (28).

**TABLE 1** Characteristics of the participants according to BMI group<sup>1</sup>

	Normal weight	Overweight	Obese
Age, y	26 ± 3	29 ± 3	28 ± 4
Height, cm	169 ± 10	170 ± 8.9	177 ± 11
Weight, kg	66 ± 11	79 ± 10	102 ± 16
Waist, cm	78 ± 6	88 ± 7	102 ± 10
Hip, cm	95 ± 7	101 ± 4.9	113 ± 5
Body fat, kg	19.2 ± 7.3	26.7 ± 5.3	41.5 ± 8.1
BMI, kg/m <sup>2</sup>	22.9 ± 2.2	26.5 ± 1.2	32.4 ± 2.1

<sup>1</sup> Values are mean ± SD. BMI groups (in kg/m<sup>2</sup>): normal weight (19 ≤ BMI < 25), n = 11; overweight (25 ≤ BMI < 30), n = 18; obese (BMI ≥ 30); n = 11.

**DNA extraction and qPCR of the stool samples.** DNA extraction was performed from 0.2 g of stool sample as previously described (29). qPCR of “all” bacteria, *Bacteroides* spp., *E. rectale* group, *C. leptum* group, and *Atopobium* group in addition to bifidobacteria, was performed as previously described (21). qPCR of the *Lactobacillus* group was optimized and validated in the present study by using the primer pairs and bacteria listed (Supplemental Tables 1 and 2, respectively). qPCR amplifications of the *Lactobacillus* group were performed by using the High Resolution Melting Master Kit (Roche) with an adjustment of the MgCl<sub>2</sub> concentration of 3.0 mmol/L as follows: preincubation at 95°C for 10 min, an amplification step of 45 cycles of denaturing at 95°C for 15 s, primer annealing at 62°C for 20 s and elongation at 72°C for 25 s, a high-resolution melting step (95°C, 1 min; 40°C, 1 min; 65°C, 1 s; 95°C, 1 s), and cooling (40°C, 30 s).

Standard curves were obtained from genomic DNA templates isolated from pure cultures listed below. The extracted DNA was quantified by using NanoDrop 2000c equipment (Thermo Scientific). For each microorganism of interest, the number of cells present in the volume loaded to the qPCR reaction was calculated on the basis of the genome size and the respective 16S ribosomal RNA copy number per cell, identified through the National Center for Biotechnology Information genome database (30). A series of six 10-fold dilutions were performed per bacterial group qPCR by using the following type strains: *Anaerostipes caccae* VTT E-052773T (universal), *Bacteroides thetaiotaomicron* VTT E-022249 (*Bacteroides* spp.), *Roseburia intestinalis* VTT E-052785T (*E. rectale* group), *Anaerotruncus colibominis* VTT E-062942T (*C. leptum* group), *Bifidobacterium longum* VTT E-96664T (bifidobacteria), *Atopobium parvulum* E-052774T (*Atopobium* group), and *Lactobacillus casei* VTT E-58225T (*Lactobacillus* group). All qPCR reactions were performed by using

**TABLE 2** Composition of the daily dietary intake of participants obtained from 3-d food diaries according to BMI group<sup>1</sup>

	Normal weight	Overweight	Obese
Energy, MJ	8.0 ± 1.7	8.4 ± 2.2	9.8 ± 2.0
Protein, g	85 ± 29	86 ± 31	81 ± 31
Total fat, g	77 ± 29	75 ± 26	85 ± 22
SFA, g	30 ± 12	28 ± 9.9	32 ± 8.6
MUFA, g	23 ± 8.9	19 ± 6.9	23 ± 6.9
PUFA, g	10 ± 4.0	10 ± 5.1	13 ± 5.2
n3 PUFA, g	1.8 ± 0.7	1.5 ± 0.7	1.6 ± 0.6
n6 PUFA, g	7.9 ± 3.2	8.6 ± 4.5	11 ± 4.4
Carbohydrates, g	200 ± 50	219 ± 58	255 ± 51
Sugars, g	86 ± 29	88 ± 36	129 ± 26
Starch, g	107 ± 39	106 ± 39	103 ± 40
Total fiber, g	21 ± 14	16 ± 6.1	17 ± 5.5
Soluble fiber, g	4.8 ± 3.6	3.8 ± 1.4	4.0 ± 1.5
Insoluble fiber, g	16 ± 12	11 ± 4.1	13 ± 3.9

<sup>1</sup> Values are mean ± SD. BMI groups (in kg/m<sup>2</sup>): normal weight (19 ≤ BMI < 25), n = 11; overweight (25 ≤ BMI < 30), n = 18; obese (BMI ≥ 30); n = 11.

the LightCycler 480 System (Roche), and the results were analyzed with the LightCycler 480 software version 1.5 (Roche).

**Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene fragments.** Denaturing gradient gel electrophoresis (DGGE) analysis of the predominant bacteria, *E. rectale* group, *C. leptum* group, *Bacteroides* spp., bifidobacteria, and *Lactobacillus* group, were performed as previously described (21,31). DGGE profiles were analyzed with BioNumerics software version 5.1 (Applied Maths BVBA). Clustering was performed with the Pearson correlation and the unweighted-pair group method by using an optimization of 0.5% and a position tolerance of 1.0%. Bands with a total surface area of  $\geq 1\%$  were included in the similarity analysis as previously described (31).

**Statistical analysis.** The study participants were considered both individually and as twin pairs. A linear mixed model was applied to study the effect of BMI and dietary intake of the individuals in the numbers of bacteria obtained by qPCR. The logarithm-transformed number of cells was modeled through linear relationships with the dietary quantitative variable where intercepts and slopes were assumed to depend on the combination of BMI and the bacterial group. The dietary variables considered were energy intake (kcal/d) and the following macronutrients (g/d): protein, SFA, MUFA, n3 PUFA, n6 PUFA, insoluble fiber, and soluble noncellulosic polysaccharides. These variables were considered as fixed effects in the model. Twin individuals and their families were treated as random effects to reflect the hierarchical structure of the data and accounting for interpair (between families) and intrapair (between co-twins) variation in the data. In addition, the residual variation was assumed to differ across the bacterial groups, and a general unstructured residual correlation assumption, which allows any correlation pattern, was included to reflect arbitrary dependencies between bacterial groups. The model assumptions were assessed for the initial model as described above. Subsequently, stepwise backward elimination of nonsignificant effects was performed by using likelihood ratio tests. For the resulting simplified model, pairwise comparisons of estimated mean intercepts and slopes between BMI groups within each bacterial group were performed with appropriate adjustment of *P* values for multiple testing.

The intra-twin pair difference in dietary intake (same nutrients mentioned above) measured between co-twins of the same family, BMI, and body fat were related with the intrapair difference of number of cells per bacterial group, the intrapair difference in diversity, and bacterial profile similarities between co-twins. General linear models were fitted for each individual bacterial group. Slopes of the regression lines were assumed to vary according to the concordance/discordance status of the twin pair for BMI. Backward stepwise elimination of nonsignificant effects was performed by using likelihood ratio tests.

The comparison of the group-specific DGGE profiles between the co-twins was performed by calculating a similarity percentage. The intrapair similarities were divided into intervals for each bacterial group analyzed and correlated with the intrapair difference in dietary intake of the macronutrients mentioned previously. Similarity groups were labeled as “very low” (0–25% similarity), “low” (26–50% similarity), “high” (51–80% similarity), and “very high” (81–100% similarity). In the case of bifidobacteria, no similarity values were obtained above 80%. Mean differences between groups were evaluated by ANOVA.

The statistical environment R (32) was used for statistical analysis, in particular the R extension packages “nlme” and “multcomp” (33,34). *P* values  $< 0.05$  were considered to be significant.

## Results

**Association of nutritional intake with numbers of stool bacteria as studied with qPCR.** The numbers of bacteria within the different bacterial groups, as measured by qPCR, did not differ between BMI groups (Supplemental Fig. 1; Supplemental Table 3). The association of the nutritional intake (all

dietary components together) with the numbers of the different stool bacteria across the studied population was described through a hierarchical linear mixed model (Fig. 1). Intakes of energy, MUFA, n3 PUFA, n6 PUFA, and soluble fiber affected the numbers of the bacterial groups studied ( $P < 0.01$ ). Individuals with high energy intake had significantly lower numbers of *Bacteroides* spp. ( $P = 0.007$ ) and slightly higher numbers of bifidobacteria ( $P = 0.02$ ) than did individuals with lower energy intake (Fig. 1A). The greater MUFA consumption was associated with lower bifidobacterial numbers ( $P = 0.0005$ ) (Fig. 1B). Moreover, the increased ingestion of n3 PUFA had a significant association with higher numbers of bacteria within the *Lactobacillus* group ( $P = 0.02$ ) (Fig. 1C). In contrast, greater n6 PUFA consumption was negatively correlated with the numbers of bifidobacteria ( $P = 0.003$ ) (Fig. 1D). Soluble fiber intake had a positive association with the *Bacteroides* spp. numbers ( $P = 0.009$ ) (Fig. 1E).

### Association of nutritional intake with diversity and quantification of stool microbial groups within the twin pairs.

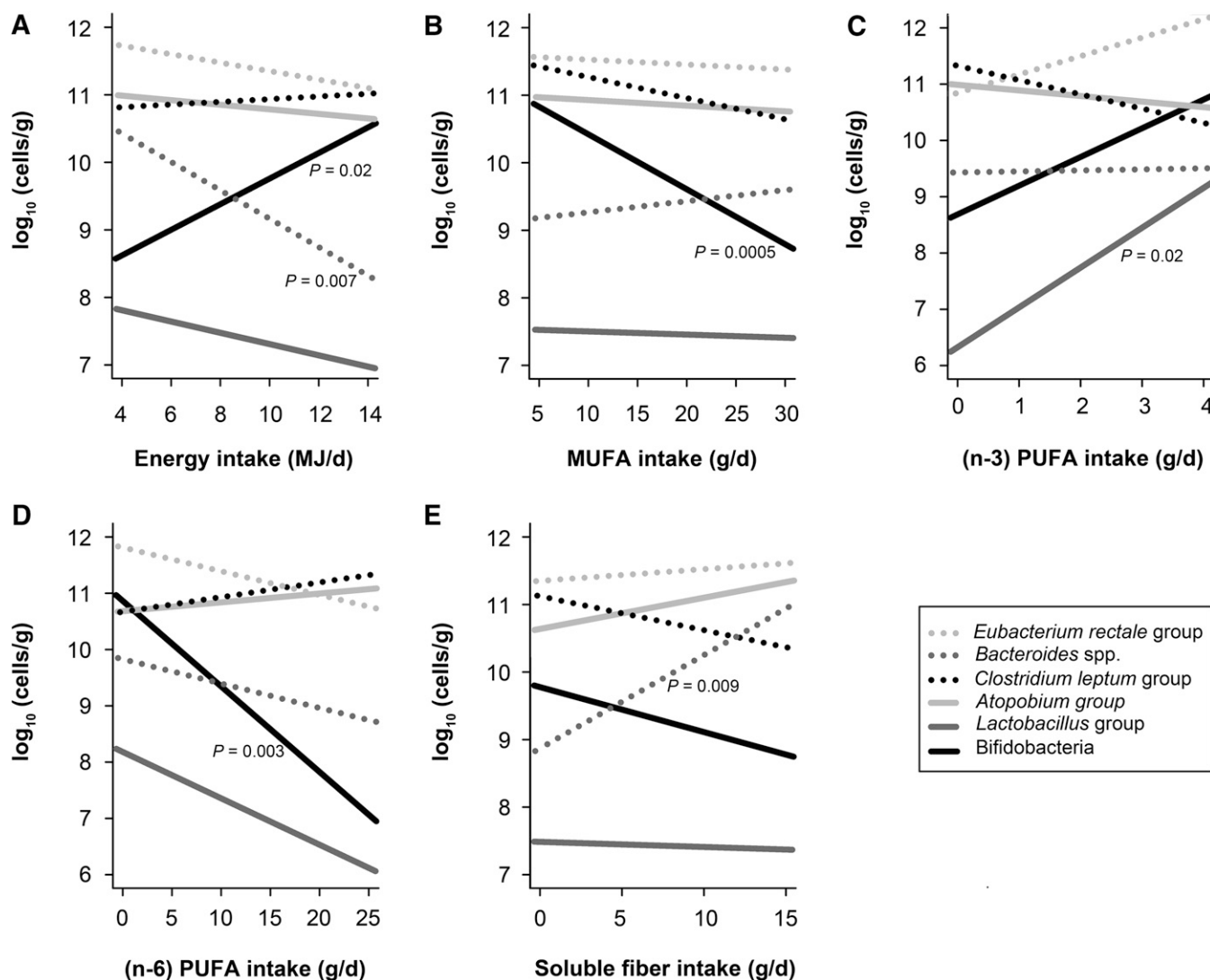
The diversity of the studied bacterial groups, defined as the number of the bands obtained by different group-specific PCR-DGGE, did not differ between BMI groups (Supplemental Fig. 2). However, in co-twins with identical energy intake, the diversity of *Bacteroides* spp. as measured with group-specific DGGE was more similar than in co-twins with different intakes of energy ( $P = 0.02$ ,  $R^2 = 0.3$ ; data not shown). In addition, the co-twins with identical energy intake had more similar numbers of *Bacteroides* spp. than did the co-twins with different intake ( $P = 0.03$ ,  $R^2 = 0.3$ ; data not shown). No significant differences were obtained in the comparison of the numbers of bacteria within the other bacterial groups and diet. Moreover, no significant association was found between the intrapair difference in diversity or numbers of cells per gram of stool samples of the studied bacterial groups and concordance of co-twins for BMI, intrapair difference in BMI, or body fat.

### Intra-twin pair similarities of DGGE microbiota profiles.

The similarities (in %) of the bacterial group-specific DGGE profiles were calculated between co-twins. In addition, the difference in the amount of nutrients ingested was calculated within each twin pair and afterwards correlated with the DGGE similarities. Co-twins with the same SFA intake had very similar *Bacteroides* spp. profiles (80–100% similarity), which was significantly different from the twin pairs with distinct SFA intake (0–25% similarity;  $P = 0.003$ ) (Fig. 2A). The group of co-twins who daily consumed the same amount of fiber had very low bifidobacterial similarity (0–25%), which was significantly different from the group with high similarity (56–80%) ( $P = 0.008$ ) (Fig. 2B). For the other bacterial groups studied by using DGGE, no significant associations were obtained regarding dietary intake. No relation was found between the intrapair DGGE-profile similarities and the co-twin concordance for BMI, intrapair difference in BMI, or body fat.

## Discussion

The main environmental factors that affect the gut microbiota composition in generally healthy adults are diet and medication. The impact of the diet on the stool microbiota has usually been assessed by subjecting a group of individuals to the same controlled diet and consequently following the shifts in the microbiota. In the present study, the effect of habitual dietary intake in the stool microbiota of a population of Finnish MZ

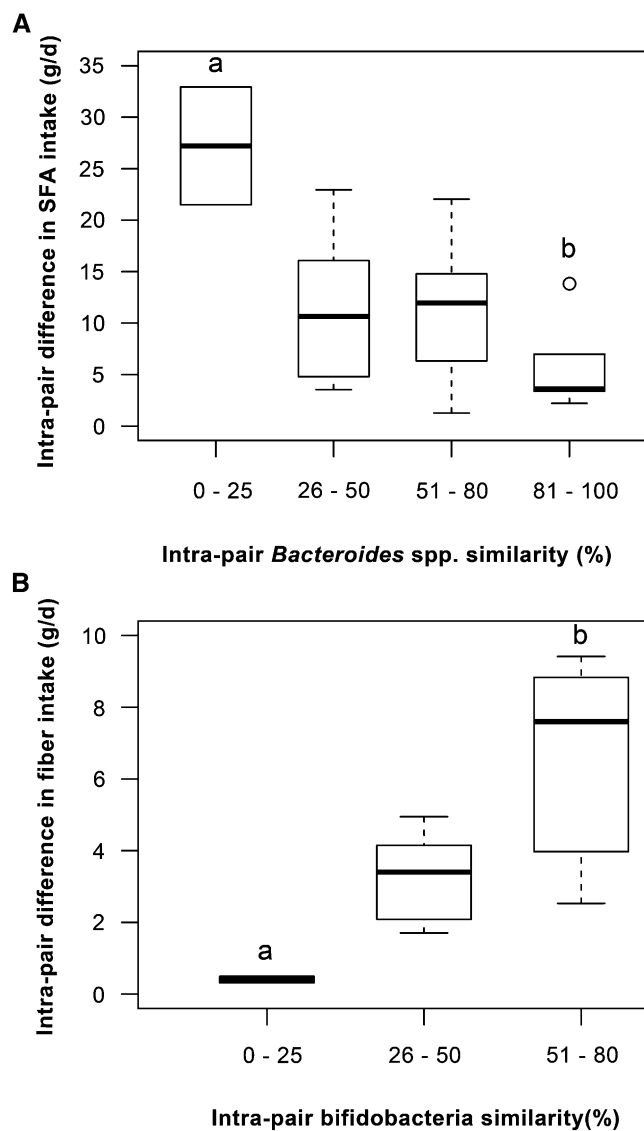


**FIGURE 1** Association between the dietary intake of monozygotic twins and the number of cells of the stool bacterial groups, as measured by using group-specific qPCR;  $n = 40$ . Data are logarithm-transformed numbers of cells in the bacterial groups per gram of wet stool versus energy intake (A), MUFA intake (B), n3 PUFA intake (C), n6 PUFA intake (D), and soluble fiber intake (E).

twins was assessed through a hierarchical linear mixed model accounting for interpair and intrapair variations. No significant differences in the cell numbers of stool bacteria within the bacterial groups studied were observed between different BMI groups. In a longitudinal study in obese and lean twins, Turnbaugh et al. (18) reported a higher proportion of bacteria of the phylum Actinobacteria and lower proportion of the phylum Bacteroidetes in obese twins compared with lean twins, whereas no significant differences in the members of the phylum Firmicutes were observed. Although no difference in *Bacteroides* spp. numbers was detected in our study between BMI categories, the abundance of this bacterial group significantly decreased when the total energy intake increased. In addition, the co-twins of our study with similar daily energetic intake had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. as compared with the twin pairs with different energy intakes. In accordance with our results, previous studies have also found a correlation between a low proportion of *Bacteroides/Prevotella* and high energy intake, rather than obesity (35). Moreover, according to Hildebrandt et al. (36), administration of a high-fat diet to both wild-type and *RELM $\beta$*  knockout mice, resistant to fat-induced obesity, increased the relative proportions of the

phyla Proteobacteria, Firmicutes, and Actinobacteria in the feces, whereas the levels of Bacteroidetes decreased in both mice. This result indicated that the fat content in the diet itself rather than the obese state of the host induced the changes in microbiota composition. Although BMI is a validated measure of nutritional status, other physiologic, metabolic, and genetic factors, in addition to inadequate physical activity, are behind the etiology of the weight-balance disorders. Therefore, studies on the relationship between the gut microbiota and the host's health should not rely solely on BMI values but should also consider other variables such as diet composition.

Naturally occurring fats are mixtures of SFA, MUFA, and PUFA with one predominating type in most foods. Therefore, we also analyzed how the intake of different types of fats correlated with the stool microbiota composition. The high intake of MUFA was associated with lower numbers of bifidobacteria and slightly higher numbers of *Bacteroides* spp. In addition, the co-twins of our study who ingested identical levels of SFA had very similar *Bacteroides* spp. DGGE profiles (80–100%), suggesting that the intake of SFA affects the diversity of *Bacteroides* spp. by targeting specific strains within the same group. In a recent metagenomic study in healthy volunteers, the



**FIGURE 2** Association between the intra-twin pair difference in dietary intake and the intra-twin pair similarities as measured with bacterial group-specific denaturing gradient gel electrophoresis. *Bacteroides* spp. similarity versus intrapair difference in SFA intake (g),  $n = 5-7$  twin pairs/group (A); bifidobacteria similarity versus intrapair difference in insoluble fiber intake,  $n = 4-7$  twin pairs/group (B). Boxes show the medians (black line inside the box) and IQRs, and whiskers represent either the maximum and minimum values or 1.5 times the IQR of the data in case outliers are present. Circles outside the boxes represent outliers. Groups labeled with different letters differ,  $P < 0.05$ .

*Bacteroides* enterotype was found to be highly associated with the consumption of fat, in particular with mono- and saturated fat (37). These observations suggest that the consumption of fat and animal-derived products, typically present in the Western diet, are associated with increased *Bacteroides* spp. prevalence in the human gut microbiota. There are only a few studies in which the correlations between the types of dietary fat and the stool bacterial composition have been investigated. In our study, n3 PUFA intake resulted in a significant positive association with *Lactobacillus* group abundance. The increase in stool *Lactobacillus* group bacterial numbers after n3 PUFA intake was also previously reported in a mouse study (38). In addition, in a human study by Santacruz et al. (15), the numbers of lactobacilli remained at the same level, even though the ingested amount of total PUFA was greatly reduced. The increase in n3 PUFA has

been reported to be effective in supporting epithelial barrier integrity by improving trans-epithelial resistance and by reducing IL-4-mediated permeability (39), and several lactobacilli have been shown to enhance the function of the intestinal barrier (40,41). Higher n6 PUFA intake was associated with decreased numbers of bifidobacteria in our study. This result is in agreement with previous studies, which reported that high n6 PUFA intakes decrease certain immune functions, such as antigen presentation, adhesion molecule expression, proinflammatory cytokines, and T-helper (Th) 1 and Th2 responses (42). Furthermore, genomic DNA of some bifidobacterial strains has been shown to stimulate the production of Th1 and proinflammatory cytokines, IFN- $\gamma$ , and TNF- $\alpha$  (43). Overall, our results indicate an association between dietary fat types and their distinct effect on the fecal microbiota. As a consequence, it seems that balanced diet with regard to fat consumption is critical not only for the host's health but also for the gut microbiota.

Diet-derived carbohydrates are one of the main fermentative substrates of dietary origin in the colon and include plant cell wall polysaccharides (nonstarch polysaccharides), oligosaccharides, and resistant starch. These polymers arrive in various states of solubility, chain length, and association with other molecules (44). In the human colon, the microbiota metabolizes these indigestible fibers to short-chain fatty acids, composed mainly of acetic, propionic, and butyric acids, which have been implicated to have both local and systemic beneficial biological effects in the human body. Butyrate, for example, is the preferred fuel of colonocytes and also plays a major role in the regulation of cell proliferation and differentiation (45,46). In our study, the numbers of *Bacteroides* spp., *C. leptum* group bacteria, and *E. rectale* group bacteria were increased in individuals who consumed higher quantities of soluble fiber. Bacteria within the *Bacteroides* spp. have been recognized as versatile members of the dominant microbiota, carrying a vast array of polysaccharide hydrolyzing enzymes. Moreover, *Bacteroides* spp. bacteria have been associated with the use of soluble fibers (44). It has been reported in several studies that the high consumption of fiber is associated with an increased proportion of butyrate-producing bacteria (47,48). Because most of the butyrate-producing bacteria belong to the *C. leptum* and *E. rectale* groups, our results are in agreement with previous studies (49,50). In conclusion, our findings confirm that diet plays an important role in the modulation of the intestinal microbiota, in particular *Bacteroides* spp. and bifidobacteria.

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