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Differences in Fecal Microbiota in Different European Study Populations in Relation to Age, Gender, and Country: a Cross-Sectional Study

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A cross-sectional study on intestinal microbiota composition was performed on 230 healthy subjects at four European locations in France, Germany, Italy, and Sweden. The study participants were assigned to two age groups: 20 to 50 years (mean age, 35 years; $n = 85$) and >60 years (mean age, 75 years; $n = 145$). A set of 14 group- and species-specific 16S rRNA-targeted oligonucleotide probes was applied to the analysis of fecal samples by fluorescence in situ hybridization coupled with flow cytometry. Marked country-age interactions were observed for the German and Italian study groups. These interactions were inverse for the predominant bacterial groups *Eubacterium rectale-Clostridium coccoides* and *Bacteroides-Prevotella*. Differences between European populations were observed for the *Bifidobacterium* group only. Proportions of bifidobacteria were two- to threefold higher in the Italian study population than in any other study group, and this effect was independent of age. Higher proportions of enterobacteria were found in all elderly volunteers independent of the location. Gender effects were observed for the *Bacteroides-Prevotella* group, with higher levels in males than in females. In summary, age-related differences in the microbiota makeup were detected but differed between the study populations from the four countries, each showing a characteristic colonization pattern.

The intestinal ecosystem is characterized by dynamic and reciprocal interactions between the host and its microbiota. Although the importance of the gut microbiota for human health has been increasingly recognized, the mechanisms underlying these interactions are too complex to be fully understood at present. Important functions of the microbiota include the processing of a wide range of dietary plant polysaccharides, provision of colonization resistance, shaping of the immune system, and regulation of host signaling pathways (23). Changes in the makeup of the intestinal microbial community may therefore influence host functions.

Asian studies revealed age-related changes in the composition of the human gut microbiota. Available data reported higher numbers of enterobacteria and lower numbers of anaerobic bacteria, including bifidobacteria in the elderly (29). However, the data are confined to the Asian population and might therefore be of limited relevance.

The mechanisms underlying the observed age-dependent differences in microbiota composition are unknown. Microbes have to acquire distinct and appropriate adaptation strategies and physiological traits to successfully occupy a niche and form a microbial population group that remains stable during adulthood. Progressive colonization seems to happen in old age where competitiveness and colonization resistance are often affected by medical treatment, in particular by antibiotics (3). Furthermore, host physiology may be compromised in aged populations owing to a reduced taste and smell perception. For example, achlorhydria, which is often observed in elderly subjects, may result in reduced colonization resistance and lead to bacterial overgrowth of the stomach and the small intestine. In addition, elderly tend to deviate from usual dietary habits, which in turn may influence bacterial colonization.

In the present study, we investigated the intestinal bacterial community structure in aged European populations and compared it to the bacterial community structure of healthy adults from the same geographical origin. A cross-sectional study was conducted to gain basic information on the fecal microbiota composition of different European study populations. Flow cytometry-based in situ hybridization was used to determine the fecal microbiota composition.

MATERIALS AND METHODS

Study design and subjects. A cross-sectional study was conducted. Healthy human subjects were recruited in four European locations: Stockholm, Potsdam, Paris, and Camerino (Italy). The study subjects included in the study were born and raised in the respective country, except for two French subjects born in former French territories and one born in the United Kingdom, who all came to France in their childhood. In each country they were allocated to one of two age groups: an adult group (aged 20 to 50 years) and an elderly group (>60 years of age) (Table 1). The following abbreviations were used: F, France; G, Germany; I, Italy; S, Sweden; A, adults; and E, elderly. Subjects with gastrointestinal disorders; hypercholesterolemia (cutoff, 5.2 mmol/liter); taking medication with antibiotics, sulfonamides, corticoid, or immunosuppressive agents within 3

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TABLE 1. Distribution of study group participants according to country, age, and sex

Age group (yr)	Mean age in yr (range) (*n*)									
	France		Germany		Italy		Sweden		Total	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Adults (20–50)	30 (25–43) (10)	35 (26–45) (12)	39 (29–46) (8)	36 (26–43) (15)	30 (25–38) (11)	31 (27–39) (19)	33 (26–49) (6)	34 (24–44) (14)	33 (25–49) (35)	34 (24–45) (50)
Elderly (>60)	79 (71–87) (10)	80 (70–90) (17)	70 (65–81) (14)	70 (65–84) (24)	79 (64–100) (16)	80 (68–94) (24)	72 (61–88) (23)	73 (65–87) (17)	74 (61–100) (63)	76 (65–94) (82)

months prior to the study; type 2 diabetes treated with oral pharmaceuticals; extreme types of constipation (less than two stools per week); and on extreme diets (vegan diet, total fasting, extremely high consumption of alcohol [>50 g/day]) were excluded from the study. Prescreened subjects underwent a medical examination before start of the study. The degree of independence based on AGGIR (Autonomie Gérontologique-Groupes Iso-Resources) rank (44) was assessed for the elderly study group. Nutritional habits were assessed with a 3-day food record.

The study was approved by the local Ethics Committee. Written informed consent was obtained from all participants. A total of 230 individuals took part in the study.

Fecal collection and fixation. Freshly voided fecal samples were collected in plastic boxes, kept at 4°C, and processed within 3 h after defecation. For bacterial enumeration by flow cytometry-based fluorescence in situ hybridization, 0.5 g of feces (wet weight) was subsequently suspended in 4.5 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 9 mM Na_2HPO_4, 1 mM NaH_2PO_4 [pH 7.4]) and thoroughly vortexed for 3 min after the addition of glass beads (3 mm in diameter). A portion (200 μl) of the resulting suspension was mixed with 600 μl of 4% paraformaldehyde solution and kept at 4°C for 3 to 4 h. Fixed fecal suspensions were stored at −80°C until analysis.

The samples from France and Italy were treated according to the above protocol, while a centrifugation step at 300 × *g* for 1 min at 4°C was applied to the samples collected in Germany and Sweden to remove debris. The latter samples were stored at −20°C. For the five bacterial probes: Bif 164, Erec 482, Rbro 730, Fprau 645, and Bac 303 (listed in Table 2), 10 human fecal samples were carefully analyzed to find out whether the application of the two protocols to the same samples leads to different results. The correlation between the two methods was high, with coefficients of correlation as follows: 0.951 for Erec 482, 0.980 for Fprau 645, 0.949 for Bac 303, 0.916 for Rbro 730, and 0.872 for Bif 164. A tendency to overestimate the values was observed for Bac 303 (7.2% ± 6.3%) and to underestimate was observed for Fprau 645 (2.7% ± 2.09%) with the additional centrifugation step. However, it is very unlikely that this had any impact on the results presented here because differences between age groups and countries do not show any systematic effect of different protocols.

Fluorescence in situ hybridization analyses. (i) Probe panel. The 16S rRNA-targeted oligonucleotide probes used in the present study are listed in Table 2. The EUB 338 probe, which targets all bacteria (1), was used as a positive control probe. The NON EUB 338 probe (45) was applied as a negative control probe to recognize unspecific binding of the fluorochrome. These oligonucleotide probes were covalently linked at their 5′ end either to fluorescein isothiocyanate (FITC) or to indodicarbocyanine (Cy5; Interactiva). The group- and species-specific probes were covalently linked with Cy5 at their 5′ ends. Unlabeled competitor oligonucleotides were used for improved in situ accessibility and specificity as described previously (40).

(ii) In situ hybridization. Depending on the cell density, 200 μl to 400 μl of fecal suspension was diluted with PBS to a final volume of 1 ml. Before hybridization, cells were pelleted at 8,000 × *g* for 3 min and resuspended in 1 ml of PBS. After a wash with Tris-EDTA buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA), the pellets were resuspended in Tris-EDTA buffer containing 1 mg of lysozyme (Serva, Heidelberg, Germany) ml^{-1} and incubated for 10 min at room temperature. Cells were subsequently washed in PBS and equilibrated in the hybridization solution (900 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.01% sodium dodecyl sulfate, 30% formamide). Hybridization was performed in 96-well microtiter plates overnight at 35°C in 50 μl of hybridization solution containing 4 μl of probe (50 ng μl^{-1}) resulting in a final concentration of 4 ng μl^{-1}. Competitor oligonucleotides were added as required. After hybridization, 150 μl of hybridization solution was added in each well to the hybridization mix, and the cells were pelleted at 2,254 × *g* for 15 min at room temperature. Nonspecifically binding probes were removed by incubation at 37°C for 20 min in 200 μl of washing solution (65 mM NaCl, 20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 0.01% sodium dodecyl sulfate). Cells were finally pelleted and resuspended in 200 μl of PBS. Aliquots of 100 μl were added to 200 μl of FACS Flow (Becton Dickinson) for data acquisition by flow cytometry.

(iii) Flow cytometry. Data acquisition was performed with a FACS Calibur flow cytometer (Becton Dickinson) as described previously (36), equipped with an air-cooled argon ion laser providing 15 mW at 488 nm combined with a 635-nm red-diode laser. All of the parameters were collected as logarithmic signals. The 488-nm laser was used to measure the forward-angle light scatter (in the 488-nm band-pass filter), the side-angle light scatter (in the 488-nm band pass), and the green fluorescence intensity conferred by FITC-labeled probes (FL1, in the 530-nm band-pass filter). The red-diode laser was used to detect the red fluorescence conferred by Cy5-labeled probes (FL4 in a 660-nm band-pass filter). The acquisition threshold was set in the side-scatter channel. The rate of

TABLE 2. Panel of group- and species-specific 16S rRNA-targeted oligonucleotide probes

Target	Probe	Sequence (5′ to 3′)[c]	OPD code[a]	Reference
Domain *Bacteria*	EUB 338	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18	1
	NON 338	ACATCCTACGGGAGGC	NA[b]	45
Bacteroides-Prevotella	Bac 303	CCAATGTGGGGGACCTT	S-*-Bacto-0303-a-A-17	28
Bacteroides putredinis	Bputre 698	GTTCTGTATGATCTCTAAGC	S-S-Bputr-0698-a-A-20	37
Bacteroides vulgatus	Bvulg 1017	AGATGCCTTGCGGCTTACGGC	S-S-Bvulg-1017-a-A-21	37
Enterobacteria	Enter 1432	CTTTTGCAACCCACT	S-*-Ent-1432-a-A-15	41
Lactobacillus-Streptococcus group	Lab 158	GGTATTAGCAYCTGTTTCCA	S-G-Lab-0158-a-A-20	14
Bifidobacterium group	Bif 164	CATCCGGCATTACCACCC	S-G-Bif-0164-a-A-18	25
Atopobium cluster	Ato 291	GGTCGGTCTCTCAACCC	S-*-Ato-0291-a-A-17	16
Clostridium coccoides-Eubacterium rectale cluster	Erec 482	GCTTCTTAGTCARGTACCG	S-*-Erec-0482-a-A-19	12
Faecalibacterium prausnitzii cluster	Fprau 645	CCTCTGCACTACTCAAGAAAAAC	S-*-Fprau-0645-a-A-23	43
Ruminococcus group	Rbro 730	TAAAGCCCAGYAGGCCGC	S-*-Rbro-0730-a-A-18	15
	cpRbro1[d]	TAAAGCCCAGYAAGCCGC		26
	cpRbro2[d]	GGTGCCCAGYAGGCCGC		26
Clostridium viride group	Cvir 1414	GGGTGTTCCCGRCTCTCA	S-*-Cvir-1414-a-A-18	26
	cpCvir1[d]	GGGTGTTDCCGRCTCTCA		26
	cpCvir2[d]	GGGTGTTCCCGRCTTTCA		26
	cpCvir3[d]	GGGTCCCCCCGRCTCTCA		26
Eubacterium cylindroides subgroup	Ecyl 387	CGCGGCATTGCTCGTTCA	S-*-Ecyl-0387-a-A-18	15
Streptococcus-Lactococcus group	Strc 493	GTTAGCCGTCCCTTTCTGG	S-*-Strc-0493-a-A-19	12
	cpStrc[d]	TTTAGCCGTCCCTTTCTGG		26
Clostridium ramosum assemblage	Cra 757	CCACGCTTTCGKGAMTGA	S-S-Cra-0757-a-A-18	26
	cpCra[d]	CCACGCTTTCGKGAMTCA		26

[a] OPD code, oligonucleotide probe database code.
[b] NA, not applicable.
[c] Sequence in IUPAC code: R = G/A, Y = T/C, M = A/C, K = G/T, S = G/C, W = A/T, H = A/C/T, B = G/T/C, V = G/C/A, D = G/A/T, N = G/A/T/C.
[d] Competitor oligonucleotide probe.

events in the flow was generally below 3,000 events s^{-1}. A total of 100,000 events was stored in list mode files. Subsequent analyses were conducted by using the CellQuest Software (Becton Dickinson, Erembodegem-Aalst, Belgium).

As previously described (36), cells belonging to a bacterial group or species were enumerated by combining a group or species-specific probe labeled with Cy5 with the EUB 338 FITC probe. An FL1 histogram was used to determine the number of bacteria hybridizing with the EUB 338 FITC probe. A region R1 was delineated in this histogram defining all of the events considered as bacterial cells. Region R1 was used to gate all density plots. For each Cy5-labeled specific probe, a rectangular region (R2, . . .) was delineated within the density plot to encompass doubly labeled bacteria. These regions allowed to position histogram markers (M2, . . .), which defined the sections, for which the area under the curve was integrated and the bacterial cell numbers were counted. These steps allowed estimation of the proportion of bacterial group or species targeted by the Cy5-labeled probe as a proportion of total bacteria (EUB 338 FITC-labeled cells). This proportion was corrected by subtracting the background fluorescence obtained with the negative control probe NON 338. The results are expressed as cells hybridizing with the group- or species-Cy5 probe as a proportion of total bacteria hybridizing with the EUB 338-FITC *Bacteria* domain probe. A potential limitation of the method lies in the detection limit which does not permit the reliable detection of bacterial target groups whose relative proportion is below 0.5%.

Statistical analyses. All statistical analyses were performed by using the software package SPSS, version 11.5 for Windows (SPSS, Inc., Chicago, IL). Bacterial data were logarithmically transformed. The normality of data was checked by using the Kolmogorov-Smirnov test. To assess mean differences for country or country-age interactions, a univariate analysis of variance, followed by a Tukey's post hoc multiple comparison test ($P < 0.05$), was used. All values are given as geometric means and standard errors. Parameter estimates were adjusted for country, age, and gender. Prevalence is defined as the relative frequency of bacterial groups which were present ≥1%.

RESULTS

Age and country related effects in dominant groups of bacteria. The analyses of fecal samples collected from subjects of the four European study groups indicated age-related structural differences in the bacterial community (Table 3). The age effects were found to differ from country to country for the following phylogenetic groups: *Eubacterium rectale-Clostridium coccoides* ($P = 0.003$), *Bacteroides-Prevotella* ($P < 0.001$), *Faecalibacterium prausnitzii* ($P = 0.002$), and *Atopobium* ($P = 0.050$). These country-age interactions were mainly based on effects observed in the German and Italian study groups, while the bacterial community structure of the French and Swedish study populations was not at all affected by age.

In all study populations, *Eubacterium rectale-Clostridium coccoides*, *Bacteroides-Prevotella*, and *Faecalibacterium prausnitzii* were the most dominant phylogenetic groups. Bacteria belonging to the *Eubacterium rectale-Clostridium coccoides* cluster were not only found in each fecal sample but they were also numerically dominant in all study groups with the exception of the Swedish adults. For the *Eubacterium rectale-Clostridium coccoides* cluster, country-age interactions were inverse in German and Italian population groups, with higher levels in group GE compared to group GA and lower levels in group IE compared to group IA. Similar country-age interactions were also observed for the *Bacteroides-Prevotella* group. Both phylogenetic groups correlated with each other (Pearson correlation coefficient = 0.499, $P < 0.01$). GA group members had a 2- to 3.5-fold lower proportion of *Bacteroides-Prevotella* than members of groups FA, IA, and SA. In contrast, group IE members showed lower proportions of these bacteria than those of groups FE, GE, and SE. *Bacteroides-Prevotella* was detected in all fecal samples of groups FA, IA, and SA but in only 90% of the fecal samples of group GA and in ca. 94% of the fecal samples of all elderly.

The SA group had the highest proportion of *Faecalibacterium prausnitzii*. Even though this proportion was somewhat smaller in the SE than in the SA group, it was still higher than in any other of the E groups. Significantly higher levels of *Faecalibacterium prausnitzii* were found in group IA than in IE.

TABLE 3. Proportions of phylogenetic groups in feces of adults and elderly of four European study groups

Probe	Geometric mean (SE range) (%)[a]								Country-age interaction term (p value)
	France		Germany		Italy		Sweden		
	20–50 yr (n = 22)	>60 yr (n = 26)	20–50 yr (n = 22)	>60 yr (n = 37)	20–50 yr (n = 20)	>60 yr (n = 39)	20–50 yr (n = 20)	>60 yr (n = 39)	
Erec 482	20.6 (17.9–23.2)	21.1 (18.7–23.8)	14.5 (12.7–16.5)	23.5 (21.3–26.0)	25.9 (22.6–29.7)	17.2 (15.6–18.9)	16.9 (14.8–19.4)	18.6 (16.8–20.5)	0.003
Ecyl 87	0.4 (0.3–0.5)	0.6 (0.5–0.7)	0.3 (0.3–0.4)	0.4 (0.4–0.5)	0.3 (0.2–0.3)	0.3 (0.3–0.4)	0.3 (0.2–0.3)	0.3 (0.3–0.4)	0.290
Cra 757	0.2 (0.2–0.2)	0.2 (0.2–0.2)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.2 (0.1–0.2)	0.2 (0.1–0.2)	0.2 (0.1–0.2)	0.1 (0.1–0.2)	0.534
Fprau 645	6.0 (4.8–7.6)	7.7 (6.2–9.5)	5.0 (4.0–6.4)	8.5 (7.1–10.3)	13.4 (10.5–17.1)	4.9 (4.1–5.9)	20.4 (15.9–26.1)	12.8 (10.7–15.2)	0.002
Rbro 730	2.8 (2.3–3.5)	3.7 (3.0–4.6)	1.8 (1.5–2.2)	2.4 (2.0–2.9)	2.9 (2.3–3.5)	3.8 (3.2–4.6)	4.6 (3.7–5.7)	6.1 (5.1–7.4)	0.703
Cvir 1414	1.5 (1.3–1.8)	1.6 (1.4–1.9)	0.6 (0.5–0.7)	0.6 (0.5–0.7)	0.9 (0.7–1.0)	0.9 (0.8–1.1)	1.2 (1.0–1.5)	1.3 (1.1–1.5)	0.182
Bac 303	9.3 (7.6–11.4)	8.0 (6.6–9.6)	3.9 (3.2–4.8)	8.4 (7.2–9.8)	13.6 (11.0–16.8)	5.3 (4.6–6.2)	13.5 (10.9–16.7)	11.1 (9.5–12.9)	<0.001
Bvulg 1017[b]	0.8 (0.6–1.1)	0.7 (0.5–0.9)	0.2 (0.2–0.3)	0.4 (0.3–0.6)	1.3 (0.9–1.8)	0.4 (0.3–0.5)	0.8 (0.6–1.1)	1.2 (0.9–1.5)	0.016
Bputre 698	0.3 (0.2–0.3)	0.2 (0.2–0.2)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.8 (0.6–0.9)	0.2 (0.2–0.3)	0.1 (0.1–0.2)	0.2 (0.2–0.2)	<0.001
Ato 291	1.6 (0.3–2.2)	1.0 (0.8–1.3)	1.6 (1.2–2.1)	3.8 (3.1–4.7)	0.8 (0.6–1.1)	1.2 (0.9–1.4)	3.8 (2.8–5.0)	6.3 (5.2–7.8)	0.050
Bif 164	1.4 (1.1–1.7)	1.1 (0.9–1.4)	1.5 (1.2–1.8)	1.2 (1.0–1.5)	4.3 (3.6–5.3)	3.5 (3.0–4.2)	2.2 (1.8–2.6)	1.7 (1.5–2.1)	0.195
Lab 158	0.3 (0.2–0.3)	0.5 (0.4–0.6)	0.2 (0.2–0.3)	0.5 (0.5–0.6)	0.4 (0.3–0.5)	0.4 (0.4–0.5)	0.8 (0.7–1.0)	0.9 (0.8–1.0)	0.050
Strc 493	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.5 (0.4–0.6)	0.8 (0.6–1.0)	0.3 (0.2–0.4)	1.9 (1.4–2.5)	1.1 (0.9–1.3)	0.014
Enter 1432	0.1 (0.1–0.2)	0.2 (0.2–0.2)	0.1 (0.1–0.1)	0.2 (0.2–0.2)	0.1 (0.1–0.2)	0.2 (0.2–0.3)	0.1 (0.1–0.2)	0.2 (0.2–0.3)	0.853
Sum	58.3 (53.1–63.9)	58.4 (53.6–63.6)	37.6 (34.2–41.2)	59.8 (55.7–64.3)	81.9 (74.4–90.2)	55.8 (52.1–59.8)	72.9 (66.1–80.3)	72.2 (67.3–77.3)	<0.001

[a] A univariate analyses of variance was used for multiple comparisons. Estimated marginal means were adjusted for country, age, and gender. Results are expressed as geometric means and standard errors.
[b] The overlapping probe was excluded from the summation.

The volunteers of groups FA and FE did not show marked differences in the *Faecalibacterium prausnitzii* cluster. An age-related effect similar to the one observed for the *Eubacterium rectale-Clostridium coccoides* cluster and the *Bacteroides-Prevotella* group were not detectable within the German study group for *Faecalibacterium prausnitzii*. GE group members showed slightly but not significantly higher levels compared to GA group members. Nevertheless, a high correlation between these three dominant bacterial groups was shown. *Eubacterium rectale-Clostridium coccoides* correlated with *Faecalibacterium prausnitzii* (Pearson correlation coefficient = 0.555, $P < 0.01$) and *Faecalibacterium prausnitzii* with *Bacteroides-Prevotella* (Pearson correlation coefficient = 0.628, $P < 0.01$). Organisms of the *Faecalibacterium prausnitzii* cluster were detected in the feces of all Swedish volunteers and all members of the IA study group and in at least 80% of the remaining volunteers.

Age effects which were based on country-age interactions were also detected for the *Atopobium* cluster. The proportion of members of the *Atopobium* cluster in group GE was 2.4-fold higher than in group GA, indicating significant age-dependent differences in this bacterial population. Elevated *Atopobium* levels were also observed for group SE which exhibited the highest proportion of this cluster. In contrast, there were no age effects in the French and the Italian study groups. *Atopobium* was detected in all group SE volunteers and also detectable in almost all samples of group GE but found in only 50% of the participants of groups FE and IE.

For the *Bifidobacterium* group a country effect ($P < 0.001$) was observed. Approximately two- to threefold-higher proportions of bifidobacteria were detected in the Italian study participants (groups IA and IE) than in the other three groups (Table 3). The prevalence of bifidobacteria was 85% in groups IA, IE, and SA but only 46% in FE. Age effects were not significant, but bifidobacteria tended to be lower in groups FE, GE, IE, and SE than in the corresponding adults (FA, GA, IA, and SA).

Age and country related effects in minor groups of bacteria. Each of the following groups: *Lactobacillus-Enterococcus*, *Streptococcus-Lactococcus*, enterobacteria, and *Eubacterium cylindroides* on average made up less than 1% of total bacteria. Independent of the study groups, enterobacteria were 1.7-fold higher ($P < 0.001$) in the elderly compared to the adults. Enterobacteria were found to be present in 10% of the elderly (GE, FE, IE, and SE), but in only 5% of the subjects from group SA and in no subject from groups GA, FA, and IA. This result indicates a clear age effect.

Age-related significant differences in the proportion of the *Lactobacillus-Enterococcus* group were country specific ($P = 0.050$). No age-dependent differences with respect to this bacterial group were observed for the Italian and Swedish study groups. However, such differences were observed for the French and German study groups, with the elderly showing higher levels. The Swedish study subjects (SA and SE) had the highest levels of the *Lactobacillus-Enterococcus* group. The prevalence of this bacterial group was ca. 50 and 40% in groups SA and SE, respectively.

The Swedish adult subjects also harbored the highest proportion of members of the *Streptococcus-Lactococcus* group. Age-related effects were detected in the Italian and the Swedish study groups, with significantly lower levels in the Italian elderly (IE). A

similar trend was observed for the Swedish elderly (SE), but the differences were not statistically significant.

Gender-specific effects. Gender-specific differences ($P = 0.036$) in the *Bacteroides-Prevotella* group (Bac303) were observed in the total study population with higher levels in males than in females. However, no gender-related differences were observed for the two species-specific probes targeting *Bacteroides vulgatus* and *Bacteroides putredinis*. *Bacteroides vulgatus* was found to be a dominant component of the *Bacteroides* species detected with the Bac303 probe, accounting for up to 10% of the bacteria detected with this probe. No gender effect was detectable for any of the other microbial groups.

Coverage. With the set of probes used in the present study, it was possible to detect 72% of total cells (detected with the probe EUB338) in the feces of the Swedish study subjects (groups SA and SE). In contrast, the coverage for groups FA, FE, GE, and IE reached ca. 60%. The largest difference in coverage was observed between groups GA (38%) and IA (82%).

DISCUSSION

In light of the importance of the gut microbiota to health, age-dependent changes in its composition could be of major significance. Earlier studies, which were mainly conducted on Asian populations, indicated a decrease in bifidobacteria and an increase in lactobacilli and clostridia in elderly compared to adult subjects. The underlying data were solely based on the use of classical microbiological methods (9–11, 29–31). In the meantime, molecular culture-independent methods have become available and have been applied to elderly Asian study populations (17–19). The data collected in our study make it feasible to compare European and Asian populations with respect to their age-dependent microbiota composition.

Clone libraries from three adults and one elderly Japanese revealed striking differences in the phylogenetic groups investigated (18, 19). Members of the *Clostridium* rRNA subcluster XIVa (*Clostridium coccoides*) tended to be lower in elderly than in younger Japanese subjects (18, 19, 29). This result was discussed in relation to the findings that *Ruminococcus obeum* and related phylotypes, which comprised ca. 2.5% of total bacteria and 16% of the *Eubacterium rectale-Clostridium coccoides* cluster in Dutch adults (47), were apparently absent from the samples of elderly subjects (19, 21, 47). This might be one reason for the observation that the *Eubacterium rectale-Clostridium coccoides* cluster makes up a smaller proportion of the fecal microbiota of aged compared to adult populations. We detected age-related differences in this cluster in both the German and the Italian subjects, but these differences were inverse.

Butyrate-producing bacteria are widely distributed across several *Clostridium* clusters, including clusters I, IV, XIVa, XV, and XVI (35), many of which belong to the *Clostridium coccoides-Eubacterium rectale* cluster (*Clostridium* cluster XIVa) and to the *Faecalibacterium prausnitzii* cluster (*Clostridium* cluster IV) (2, 22, 43). Significant changes within these clusters could therefore influence functional properties, such as butyrate production. We were unable to confirm results from an earlier study, in which numbers of the *Eubacterium rectale-Clostridium coccoides* cluster were outnumbered by bifidobacteria in elderly subjects (20).

We also observed age-related differences in the *Bacteroides* group, which exhibited lower levels in the Italian elderly subjects. Similar age-related differences were reported for Dutch subjects (20) and subjects from the United Kingdom (46), although the latter study was based on classical microbiology. In the latter study, a reduced number of bacteroides was accompanied by reduced species diversity (46). Another study reported a reduced abundance of bacteroides in elderly hospitalized patients, in particular in patients receiving antibiotics compared to healthy elderly (24). Interestingly, the age-dependent differences in bacteroides observed in our study was country specific. The observed differences may have consequences because members of the *Bacteroides* group play an important role in the hydrolysis and fermentation of exogenous fiber and endogenous mucins, as well as the conversion of bile acids and the production of toxins (27, 39). Marked reductions in this groups may have an impact on the complex cross-feeding network among bacterial species in the colon. The most abundant and frequently cultivated species include *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides distasonis*, *Bacteroides fragilis*, and *Bacteroides ovatus* (7, 10, 28). Our study also indicates a positive correlation between *Bacteroides* and the *Eubacterium rectale-Clostridium coccoides* cluster. Moreover, this is the first time that a gender effect has been observed for the *Bacteroides-Prevotella* group.

Both 16S rRNA gene clone libraries and culture-dependent enumerations indicate that *Faecalibacterium prausnitzii* is one of the most abundant species in the human intestine (22, 32, 42, 43). This finding is also confirmed by our study in which we used a probe targeting the *Faecalibacterium prausnitzii* cluster, which comprises the species *Faecalibacterium prausnitzii* and closely related species (8, 33, 43). We also observed country- and age-specific differences for this bacterial cluster. The observed age effects are in accordance with data from a study conducted on Dutch elderly subjects (20). In contrast, *Faecalibacterium prausnitzii* and relatives, which require strictly anaerobic conditions, were hardly detectable in a study which used plating for the enumeration of these organisms (4).

It has generally been assumed that the fecal microbiota of elderly subjects is more diverse than that of adults (5). An inspection of 16S rRNA clone libraries revealed that many phylotypes correspond to undescribed species (42). Approximately 75% of the sequences in 16S rRNA libraries derived from fecal samples of adults were novel phylotypes (17, 21, 42). Most of the new phylotypes obtained from healthy Asian adults were affiliated with the *Clostridium* rRNA subcluster XIVa (*Clostridium coccoides*). Potentially, new species were also affiliated with the *Clostridium* rRNA cluster IX (*Sporomusa*), which were particularly abundant in the elderly (18, 19). The low coverage observed in our study, especially in the German adults, might indicate the presence of phylotypes that were not detectable with the set of probes used.

Diet-related differences are well documented. The gut microbiota of vegetarians differs from that of individuals on a Western diet in that the former is dominated by clones belonging to the *Clostridium* rRNA subcluster XIVa (also called *Clostridium coccoides*) and the *Clostridium* rRNA cluster XVIII (also called *Clostridium ramosum*) (10, 17). Interestingly, the detection of *Faecalibacterium prausnitzii* in a strict vegetarian failed (17). In our cross-sectional study we detected the highest levels of the *Faecalibacterium prausnitzii* cluster in the Swedish study groups, a

population whose dietary habits are characterized by a high consumption of fish and meat. Furthermore, higher numbers of bifidobacteria but significantly lower levels of anaerobic bacteria and lecithinase-negative clostridia were detected in rural elderly compared to urban elderly in Japan (4). The latter have a lower intake of dietary fiber. Multivariate analyses led us to conclude that the country effects observed in our study with respect to the phylogenetic groups may be due to differences in dietary habits. It has been demonstrated that the consumption of fructooligosaccharides leads to an increase in fecal bifidobacteria (concept of prebiotics) (6, 13, 34, 38).

Conclusion. To our knowledge, this is the first molecular cross-sectional study that focused on age-related and geography related differences in the fecal microbiota composition across Europe. A unique feature of microbiota composition in European adults and elderly could not be identified. However, the geographic affiliation was associated with differences in microbiota composition. Marked country-age interactions were observed for the German and Italian study groups. Host specificities or diet-related differences may explain variations observed in the microbiota composition. It should be kept in mind that the data presented were obtained for subjects who live in a specific geographical region which do not necessarily reflect the situation in the whole country. To better define lifestyle- or diet-related effects, a more detailed structural and functional analysis of intestinal microbiota is required. A discrimination at species or even strain level might be of interest for some bacterial groups to detect subtle variations in dominant groups.

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