Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa

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Gut microbial composition depends on different dietary habits just as health depends on microbial metabolism, but the association of microbiota with different diets in human populations has not yet been shown. In this work, we compared the fecal microbiota of European children (EU) and that of children from a rural African village of Burkina Faso (BF), where the diet, high in fiber content, is similar to that of early human settlements at the time of the birth of agriculture. By using high-throughput 16S rDNA sequencing and biochemical analyses, we found significant differences in gut microbiota between the two groups. BF children showed a significant enrichment in Bacteroidetes and depletion in Firmicutes ($P < 0.001$), with a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, completely lacking in the EU children. In addition, we found significantly more short-chain fatty acids ($P < 0.001$) in BF than in EU children. Also, *Enterobacteriaceae* (*Shigella* and *Escherichia)* were significantly underrepresented in BF than in EU children ($P < 0.05$). We hypothesize that gut microbiota coevolved with the polysaccharide-rich diet of BF individuals, allowing them to maximize energy intake from fibers while also protecting them from inflammations and noninfectious colonic diseases. This study investigates and compares human intestinal microbiota from children characterized by a modern western diet and a rural diet, indicating the importance of preserving this treasure of microbial diversity from ancient rural communities worldwide.

metagenomics | nutrigenomics | biodiversity | 454-pyrosequencing | short-chain fatty acids

The human gut "metagenome" is a complex consortium of trillions of microbes, whose collective genomes contain at least 100 times as many genes as our own eukaryote genome (1). This essential "organ," the microbiome, provides the host with enhanced metabolic capabilities, protection against pathogens, education of the immune system, and modulation of gastrointestinal (GI) development (2).

We do not yet completely understand how the different environments and wide range of diets that modern humans around the world experience has affected the microbial ecology of the human gut.

Contemporary human beings are genetically adapted to the environment in which their ancestors survived and which conditioned their genetic makeup. In mammals, both diet and phylogeny influence the increase in bacterial diversity from carnivore to omnivore to herbivore (3). Dietary habits are considered one of the main factors contributing to the diversity of human gut microbiota (2). Profound changes in diet and lifestyle conditions began with the so-called "Neolithic revolution" with the introduction of agriculture and animal husbandry ≈10,000 y ago (4). After that time, food resources became more abundant and constant, the concentration of large populations in limited areas created selective pressure that favored pathogens specialized in colonizing human hosts and probably produced the first wave of emerging human diseases (5). It has been hypothesized that bacteria specialized in human-associated niches, including our gut commensal flora, underwent intense transformation during the social and demographic changes that took place with the first Neolithic settlements (6).

Western developed countries successfully controlled infectious diseases during the second half of the last century, by improving sanitation and using antibiotics and vaccines. At the same time, a rise in new diseases such as allergic, autoimmune disorders, and inflammatory bowel disease (IBD) both in adults and in children has been observed (5), and it is hypothesized that improvements in hygiene together with decreased microbial exposure in childhood are considered responsible for this increase (7). The GI microflora plays a crucial role in the pathogenesis of IBD (8), and recent studies demonstrate that obesity is associated with imbalance in the normal gut microbiota (9, 10).

The aim of this study was to compare the gut microbiota of children aged 1–6 y living in a village of rural Africa in an environment that still resembles that of Neolithic subsistence farmers with the gut microbiota of western European children of the same age, eating the diet and living in an environment typical of the developed world. These two childhood populations provided an attractive model for assessing the impact of many environmental variables on the gut microbiota.

In our study, we address three general questions regarding the geography and evolution of the human microbiota: (*i*) how is bacterial diversity partitioned within and between the two populations studied; (*ii*) is there a possible correlation between bacterial diversity and diet; and (*iii*) what is the distribution of well-known bacterial pathogens in the two populations, given the different hygienic and geographic conditions?

Results and Discussion

Characterization of Dietary Habits of Children from the Boulpon Rural Village and from Florence, Italy. In this study, we characterized the fecal microbiota of 14 healthy children from the Mossi ethnic

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Data deposition: Data were submitted to the Sequence Read Archive (SRA) using ISA tools (ISAcreator and ISAconverter, http://isatab.sourceforge.net/index.html). The dataset is available at http://www.ebi.ac.uk/ena/data/view/ERP000133.

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Fig. 1. Life in a rural village of Burkina Faso. (*A*) Village of Boulpon. (*B*) Traditional Mossi dwelling. (*C*) Map of Burkina Faso (modified from the United States CIA's World Factbook, 34). (*D*) Millet and sorghum (basic components of Mossi diet) grain and flour in typical bowls. (*E*) Millet and sorghum is ground into flour on a grinding stone to produce a thick porridge called Tô.

group (BF) living in the small village of Boulpon in Burkina Faso (Fig. 1) and compared it with that of 15 healthy European children (EU) living in the urban area of Florence, Italy (Table S1). The BF children from Boulpon village were selected as representative consumers of a traditional rural African diet. The diet of BF children is low in fat and animal protein and rich in starch, fiber, and plant polysaccharides, and predominantly vegetarian (Table S2). All food resources are completely produced locally, cultivated and harvested nearby the village by women. The BF diet consists mainly of cereals (millet grain, sorghum), legumes (black-eyed peas, called Niébé), and vegetables, so the content of carbohydrate, fiber and nonanimal protein is very high. Millet and sorghum are ground into flour on a flat stone and made into thick porridge called millet-based Tô, dipped into a sauce made of local vegetables (Néré) and herbs. Although the intake of animal protein is very low, sometimes they eat a small amount of meat (chicken) and termites that we verified to be occasionally part of the BF children's diet in the rainy season.

Children are breast-fed up to the age of 2 y as a complement to a mixed diet. The average amount of fiber in BF diet is 10.0 g/d (2.26%) in 1- to 2-y-old children and 14.2 g/d (3.19%) in 2- to 6-y-old children (Table S2). To represent a Western population (EU), we selected children of the same age who are generally concordant for growth, socially homogeneous and eating the diet and living in an environment typical of the developed world. EU children were breast-fed for up to 1 y of age. They were eating a typical western diet high in animal protein, sugar, starch, and fat and low in fiber. The fiber average content in EU diet is 5.6 g/d (0.67%) in 1- to 2-y-old children and 8.4 g/d (0.9%) in 2- to 6-y-old children (Table S3). The amount of calories (average) consumed varies considerably in the two populations (BF children: 1–2 y old, 672.2 kcal/d; 2–6 y old, 996 kcal/d; EU children: 1–2 y old, 1,068.7 kcal/d; 2–6 y old, 1,512.7 kcal/d; Tables S2 and S3). The isolation of the BF village where the children whom we investigated live, in comparison with the urbanized world, suggests that their diet very likely resembles that of the Neolithic African rural populations following the agriculture revolution.

Dominance of the Bacteroidetes in Gut Microbiota of Burkina Faso Compared with European Children. To characterize the bacterial lineages present in the fecal microbiotas of these 29 children, we performed multiplex pyrosequencing of the V5 and V6 hypervariable regions of 16S rRNA gene with a 454 FLX instrument (Roche). We generated a dataset consisting of 438,219 filtered high-quality, classifiable 16S rRNA gene sequences with a mean average (± SD) of 15,111 ± 3,774 sequences per sample (Table S4). More than 94.2% of the sequences in all of the BF and EU samples were found to belong to the four most populated bacterial phyla, namely Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, in agreement with previous studies describing such phyla as those contributing to the majority of human gut microbiota (2, 11). Relevant differences were found in the proportions of four phyla: Actinobacteria and Bacteroidetes were more represented in BF than in EU children's microbiota (10.1% versus 6.7% and 57.7% versus 22.4%, respectively), whereas Firmicutes and Proteobacteria were more abundant in EU than in BF children (63.7% versus 27.3% and 6.7% versus

0.8%, respectively). The differential distribution of Firmicutes and Bacteroidetes delineates profound differences between the two groups (Fig. S1).

Statistical analysis using a parametric test (ANOVA) indicates that Firmicutes ($P = 7.89 \times 10^{-5}$) and Bacteroidetes ($P = 1.19 \times 10^{-6}$) significantly differentiate the BF from the EU children. This result is strengthened by the nonparametric Kruskal–Wallis test, which again indicated significant discriminating factors in Firmicutes ($P = 3.38 \times 10^{-5}$), Bacteroidetes ($P = 4.80 \times 10^{-4}$), Actinobacteria ($P = 8.82 \times 10^{-3}$), and Spirochaetes ($P = 1.11 \times 10^{-5}$) phyla. Firmicutes are twice as abundant in the EU children as evidenced by the different ratio between Firmicutes and Bacteroidetes (F/B ratio ± SD, 2.8 ± 0.06 in EU and 0.47 ± 0.05 in BF), suggesting a dramatically different bacterial colonization of the human gut in the two populations. Interestingly, *Prevotella*, *Xylanibacter* (Bacteroidetes) and *Treponema* (Spirochaetes) are present exclusively in BF children microbiota (Figs. 2 *A* and *B*, Fig. S2, and Table S5). We can hypothesize that among the environmental factors separating the two populations (diet, sanitation, hygiene, geography, and climate) the presence of these three genera could be a consequence of high fiber intake, maximizing metabolic energy extraction from ingested plant polysaccharides.

Diet plays a central role in shaping the microbiota, as demonstrated by the fact that bacterial species associated with a high-fat, high-sugar diet promote obesity in gnotobiotic mice (12). In such a model, indigenous bacteria maintain energy homeostasis by influencing metabolic processes. The ratio of Firmicutes to Bacteroidetes differs in obese and lean humans, and this proportion decreases with weight loss on low-calorie diet (9). It is therefore reasonable to surmise that the increase in the F/B ratio in EU children, probably driven by their high-calorie diet, might predispose them to future obesity. This F/B ratio may also be considered a useful obesity biomarker.

16S rRNA Gene Surveys Reveal Hierarchical Separation of the Two Pediatric Populations. We further assessed differences in the total bacterial community at the single sample level by clustering the EU and BF samples according to their bacterial genera as found by the RDP classifier (Ribosomal Database Project v. 2.1).

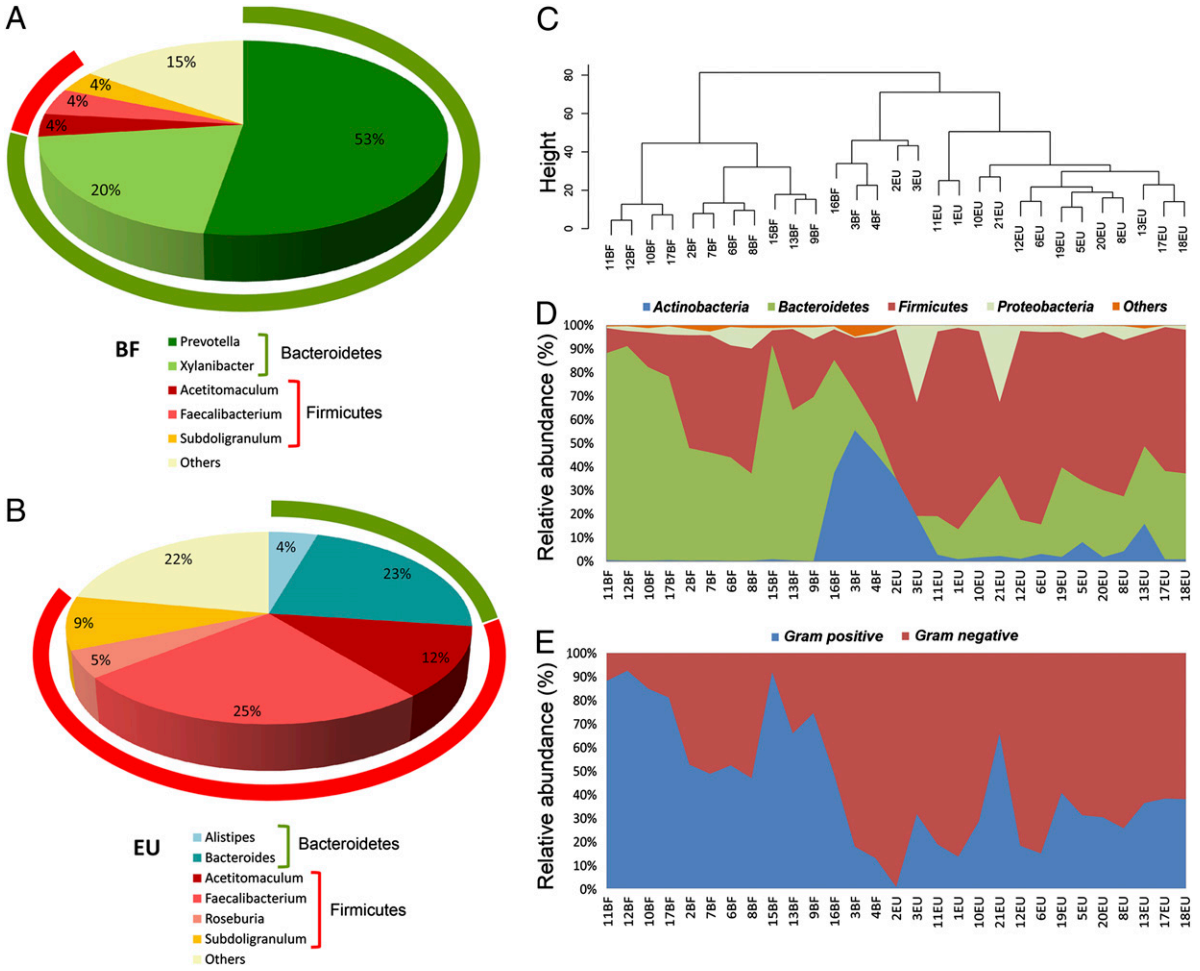

Fig. 2. 16S rRNA gene surveys reveal a clear separation of two children populations investigated. (*A* and *B*) Pie charts of median values of bacterial genera present in fecal samples of BF and EU children (>3%) found by RDP classifier v. 2.1. Rings represent corresponding phylum (Bacteroidetes in green and Firmicutes in red) for each of the most frequently represented genera. (*C*) Dendrogram obtained with complete linkage hierarchical clustering of the samples from BF and EU populations based on their genera. The subcluster located in the middle of the tree contains samples taken from the three youngest (1–2 y old) children of the BF group (16BF, 3BF, and 4BF) and two 1-y-old children of the EU group (2EU and 3EU). (*D*) Relative abundances (percentage of sequences) of the four most abundant bacterial phyla in each individual among the BF and EU children. Blue area in middle shows abundance of Actinobacteria, mainly represented by *Bifidobacterium* genus, in the five youngest EU and BF children. (*E*) Relative abundance (percentage of sequences) of Gram-negative and Gram-positive bacteria in each individual. Different distributions of Gram-negative and Gram-positive in the BF and EU populations reflect differences in the two most represented phyla, Bacteroidetes and Firmicutes.

Complete linkage hierarchical clustering produced a net separation of BF and EU populations (Fig. 2*C*). It is noteworthy that the subcluster that joins the two major clusters (located in the middle of the tree) contains samples taken from the five youngest EU and BF children (1–2 y old). This can be explained by the fact that BF children are breast-fed up to the age of 2 y and resemble the younger EU children who were breast fed-up to 1 y of age. It is also noteworthy that only in these three BF subjects we observed abundant Actinobacteria, mainly represented by *Bifidobacterium* genus (Fig. 2*D*), that were found in all EU subjects (Table S5) and that is known to be strictly related to breast-feeding in infants (13). This result provides a clear indication of the dominant role of diet over the variables mentioned above in shaping the microbial composition of the gut. Studies on the genetic variability between Mossi and Europeans showed polymorphisms in the major histocompatibility complex (MHC) genes (14), but no significant differences in the expression of key genes regulating immune function such as *TGFβ, TGFβR, CTLA4,* and *FOXP3,* suggesting a functional similarity (15). Also, the two populations are different for many other variables such as sanitation, hygiene, geography, and climate. Yet, if any of these variables had prevailed over diet, these five children would have fallen into two main clusters instead of creating a third, significantly separated, cluster.

In western populations, the human intestinal microbiota undergoes maturation from birth to adulthood and aging, with particular emphasis on the F/B ratio, that evolves during different life stages (16). Our results can be explained by the fact that, as soon as breast-feeding is substituted by solid foods, the differences in microbiota between the two populations increase, reflecting the dietary and environmental separation that results in a differentiation between the F/B ratio. As a consequence of the different F/B ratio in the two populations, we found Gram-negative bacteria (mainly Bacteroidetes) more abundant (58.5%) than Gram-positive bacteria (37.4%) in the BF population, whereas Gram-positive (mainly Firmicutes) were more abundant than Gram-negative bacteria (70.4% versus 29.1% respectively) in the EU population (Fig. 2*E*).

Microbial Richness and Biodiversity. We then compared the microbial richness, estimated by the Chao1 index, and the biodiversity, assessed by a nonparametric Shannon index for the two BF and EU groups. In our calculations we took into account different OTU distance unit cutoffs, namely 0.03, 0.05, and 0.10 (Fig. S3). Using the nonparametric Kruskal–Wallis test for comparisons, we found significant differences ($P < 0.01$) in both richness and biodiversity between BF and EU samples at the Operational Taxonomic Unit (OTU) cutoff 0.10, with a higher microbial richness and biodiversity in BF samples than in EU samples (Table S6).

Exposure to the large variety of environmental microbes associated with a high-fiber diet could increase the potentially beneficial bacterial genomes, enriching the microbiome. Reduction in microbial richness is possibly one of the undesirable effects of globalization and of eating generic, nutrient-rich, uncontaminated foods. Both in the Western world and in developing countries diets rich in fat, protein, and sugar, together with reduced intake of unabsorbable fibers, are associated with a rapid increase in the incidence of noninfectious intestinal diseases. The potential protective effects of the diet on bowel disorders was first described by Burkitt (17) who, working in Africa in the 1960s, noticed the remarkable absence of noninfectious colonic diseases in Africans consuming a traditional diet rich in fiber.

***Xylanibacter, Prevotella, Butyrivibrio, and Treponema* Genera May Enhance the Ability to Extract Calories from Indigestible Polysaccharides in BF Children.** Whole grains are concentrated sources of dietary fiber, resistant starch, and oligosaccharides, as well as carbohydrates that escape digestion in the small intestine and are fermented in the gut, producing short-chain fatty acids (SCFAs). *Xylanibacter*, *Prevotella, Butyrivibrio,* and *Treponema* are exclusive to the BF children (Fig. S2) and indicate the presence of a bacterial community using xylane, xylose, and carboxymethylcellulose to produce high levels of SCFAs (18) whose protective role against gut inflammation has been well proven (19). These bacteria can ferment both xylan and cellulose through carbohydrate-active enzymes such as xylanase, carboxymethylcellulase, and endoglucanase (http://www.cazy.org).

Other SCFA-producing bacteria, such as *Bacteroides* and *Faecalibacterium* species, particularly *F. prausnitzii* (Table S5), found in both populations, could generally indicate the importance of maintaining a microflora with potentially anti-inflammatory capability (20).

To associate the presence of SCFA-producing bacterial communities with the effective increase in the concentration of SCFAs in fecal samples, we determined the levels of acetic, propionic, butyric, and valeric acids using solid phase microextraction–gas chromatography–mass spectrometry (SPME-GC-MS) analysis. It is

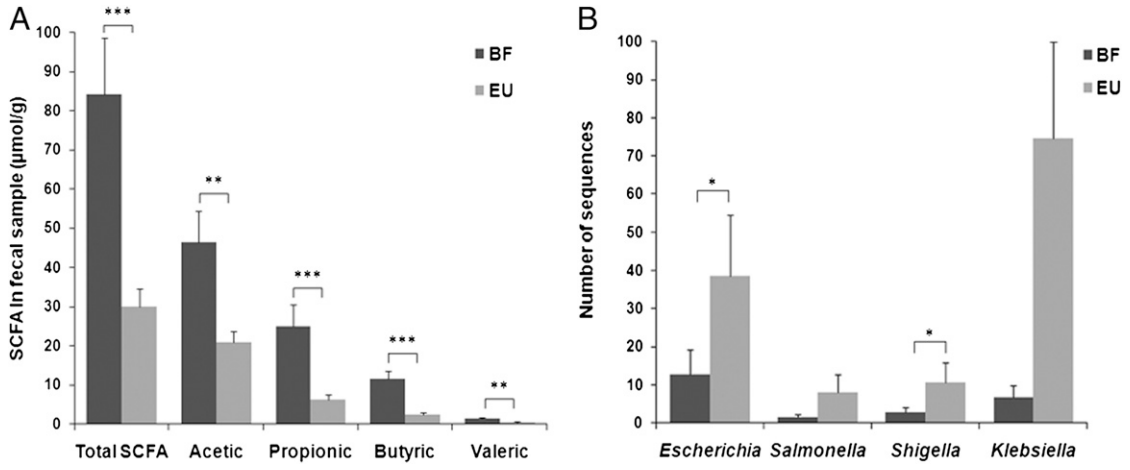

Fig. 3. SCFA-producing bacteria could help to prevent establishment of some potentially pathogenic intestinal bacteria. (*A*) Quantification of SCFAs in fecal samples from BF and EU populations by SPME-GC-MS. (*B*) Number of sequences relative to principal *Enterobacteriaceae* genera, in BF and EU children microbiota. Mean values (±SEM) are plotted. Asterisks indicate significant differences (one-tailed Student *t* test of all data points: $*P < 0.05$; $**P \leq 0.01$; $***P \leq 0.001$).

noteworthy that in BF children we found a significantly higher amount of total SCFAs compared with EU children (one-tailed Student t test, $P = 4.5 \times 10^{-4}$; Fig. 3*A*). In particular, propionic and butyric acids are nearly four times more abundant in BF than in EU fecal samples (one-tailed Student t test, $P = 1.3 \times 10^{-3}$ and $P = 1.6 \times 10^{-4}$, respectively), whereas acetic and valeric acids were comparable in both groups (one-tailed Student's t test, respectively $P = 2.0 \times 10^{-3}$ and $P = 2.4 \times 10^{-3}$ (Fig. 3*A* and Table S7). Normal colonic epithelia derive 60–70% of their energy supply from SCFAs, particularly butyrate (21). Propionate is largely taken up by the liver and is a good precursor for gluconeogenesis, liponeogenesis, and protein synthesis (22). Acetate enters the peripheral circulation to be metabolized by peripheral tissues and is a substrate for cholesterol synthesis (23). Previous analyses on the physiological significance of SCFA (24) showed how SCFA are rapidly absorbed from the colon. Therefore, an abundance of SCFA in the feces indicates production of SCFA from microflora at levels far above the absorption rate. Our results allow us to hypothesize that a diet rich in plant polysaccharides and low in sugar and fat could select SCFA-producing bacteria.

Altogether, our results indicate a correlation between polysaccharide-degrading microbiota and the calories that the host can extract from his/her diet, potentially influencing the survival and fitness of the host. We can hypothesize that microbiota coevolved with the diet of BF individuals, allowing them to maximize the energy intake from indigestible components, such as plant polysaccharides, by producing high levels of SCFAs that supply the host with an additional amount of energy. Given that enhanced ability to obtain energy-rich food is considered to be one factor that has driven human evolution. Substantial microbiota adaptation has probably accompanied the dietary changes that have occurred throughout human history. In fact it is well known that changes in food production agricultural and preparation have profoundly influenced the intestinal microflora.

Our results suggest that diet has a dominant role over other possible variables such as ethnicity, sanitation, hygiene, geography, and climate, in shaping the gut microbiota. We can hypothesize that the reduction in richness we observe in EU compared with BF children, could indicate how the consumption of sugar, animal fat, and calorie-dense foods in industrialized countries is rapidly limiting the adaptive potential of the microbiota. This microbial simplification harbors the risk of depriving our microbial gene pool of potentially useful environmental gene reservoirs that allow adaptation to peculiar diets, as we observed in BF population and as recently shown by diet-induced horizontal gene transfer in Japanese individuals consuming algae in their diet (25).

Gut microbial richness could have several health-related effects. The SCFA-producing bacteria that are abundant in the BF children's gut possibly help to prevent the establishment of some potentially pathogenic intestinal microbes (26) causing diarrhea, as seen by the fact that *Enterobacteriaceae*, such as *Shigella* and *Escherichia*, were significantly underrepresented in BF than in EU children ($P < 0.05$, one-tailed t test; Fig. 3*B*). Increased gut microbial diversity and reduced quantities of potentially pathogenic strains in BF would agree with the "old friend" hypothesis, indicating a role of microbiota in protecting children from pathogens as well as from gastrointestinal diseases (27).

The lessons learned from the BF children's microbiota prove the importance of sampling and preserving microbial biodiversity from regions where the effects of globalization on diet are less profound. The worldwide diversity of the microbiome from ancient communities, where gastrointestinal infections can make the difference between life and death, represents a goldmine for studies aimed at elucidating the role of gut microbiota on the subtle balance between health and disease and for the development of novel probiotics.

Materials and Methods

Population Enrollment, Fecal Sample Collection, and DNA Extraction. We enrolled 15 healthy children (nine male and six female) living in the rural village of Boulpon district of Nanoro, Boulkiemde province, Burkina Faso, and 15 healthy children (nine male and six female) living in the urban area of Florence, Italy. All children were 1–6 y of age, had not taken antibiotics or probiotics in the 6 mo before the sampling dates, and had not been hospitalized in the previous 6 mo (Table S1). A detailed medical and lifestyle report was obtained from EU children's parents, and a 3-d dietary questionnaire and an in-depth interview on BF children's diet was obtained directly from their mothers.

Despite the high incidence of infectious disease, including malaria and malnutrition in the area, all children were apparently healthy at the time of sample collection. Upper midarm measurement excluded both severe and moderate malnutrition. As representative of a healthy Western population (EU), we selected children of the same age who were generally concordant for growth, socially homogeneous, and eating the diet and living in an environment typical of the developed world. Fecal samples were collected by physicians and preserved in RNAlater (Qiagen) at −80 °C until extraction of genomic DNA (28) (details in *SI Materials and Methods*).

Sequencing of 16S rRNA Gene Amplicons. For each sample, we amplified 16S rRNA genes using a primer set specific for V5 and V6 hypervariable 16S RNA region. The forward primer contained the sequence of the Titanium A adaptor and a barcode sequence. Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche) following Titanium chemistry (details in *SI Materials and Methods*). Data were submitted to the Sequence Read Archive (SRA) using ISA tools (ISAcreator and ISAconverter, http://isatab.sourceforge.net/index.html); the dataset is available at http://www.ebi.ac.uk/ena/data/view/ERP000133.

Taxonomic Assignment to 16S Reads. RDP classifier (v 2.1) software was used (29) to classify the sequences according to the taxonomy proposed by Garrity et al. (30), maintained at the Ribosomal Database Project (RDP 10 database, Update 18). RDP classifier also emits, for each taxonomic rank, a confidence estimate (CE) based on a bootstrapping procedure, allowing to append the notation of "_uncertain" to assignments with CE lower than a defined cutoff , usually 50% (Table S4). Bacterial species were assigned using a speed-optimized procedure based on BLAST and on the creation of genus specific subsamples of the RDP 10 database (details in *SI Materials and Methods*).

Quantifying and Comparing Diversity Between BF and EU Populations. Differences between populations have been analyzed using parametric (ANOVA) and nonparametric (Kruskal-Wallis test) statistical methods. Even if, in principle, multivariate ANOVA would be more appropriate to catch the whole information available from such dataset, some of the assumptions (e.g., normality of residuals) were not met, as tested with Shapiro–Wilk W test and the energy E test. We then preferred to use univariate methods such as ANOVA and the nonparametric, rank-driven Kruskall-Wallis test, which performs well in the absence of distributional assumptions (details in *SI Materials and Methods*).

Complete Linkage Hierarchical Clustering. The clustering of EU and BF samples was performed on genera obtained from RDP Classifier by means of a complete linkage hierarchical clustering technique using the R package hclust (details in *SI Materials and Methods*).

Richness and Diversity Index. To obtain the matrix containing pairwise sequence distances, all reads were first aligned with muscle v3.7 (31) and converted to Phylip format for downstream calculations. Richness and biodiversity indices were obtained with the Mothur software package (32). For richness estimation, related to the number of observed operational taxonomic units (OTUs), we used the Chao1 index. Biodiversity that depends how uniformly the sequences are spread into the different observed OTUs, was instead estimated with the nonparametric Shannon formula (33). Both indexes were evaluated at a different distance unit cutoff, to test different selectivity in the definition of OTUs.

Determination of SCFAs in Fecal Samples. For determination of SCFAs we used 250 mg frozen fecal samples. Concentrations of SCFAs were determined in a 1:25 dilution of 500 μL supernatant. SPME-GC-MS determinations were performed using a Varian Saturn 2000 GC-MS instrument with 8200 CX SPME autosampler (details in *SI Materials and Methods*).

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