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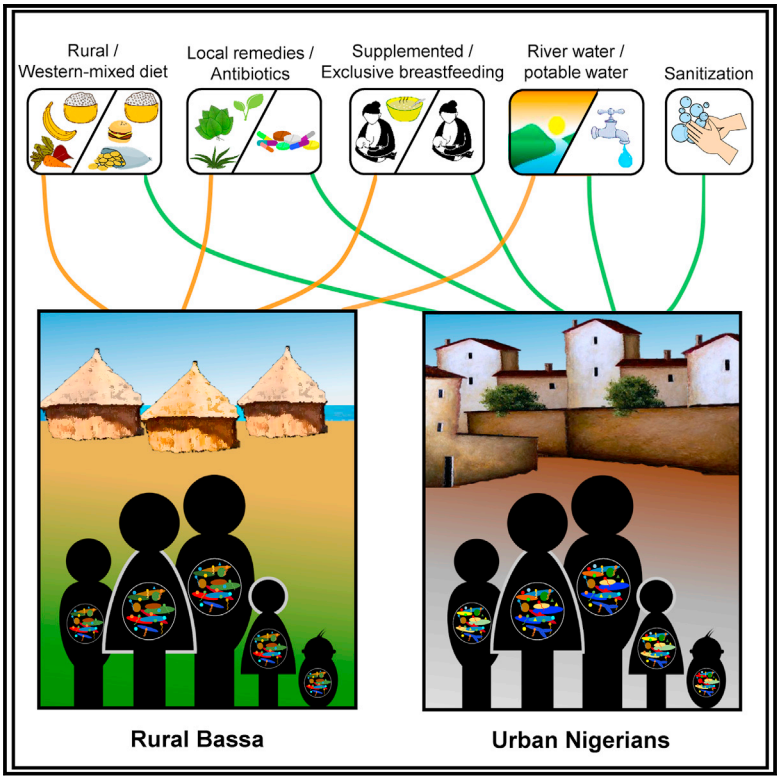
Infant and Adult Gut Microbiome and Metabolome in Rural Bassa and Urban Settlers from Nigeria

Graphical Abstract



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In Brief

Ayeni et al. characterize the fecal microbiome and metabolome of rural Bassa and urban individuals from Nigeria, including infants. Their findings stress the loss of ancient signatures along with urbanization and support distinct trajectories of development of the intestinal ecosystem in early life, depending on human subsistence.

Highlights

- Specific microbiome and metabolome traits are progressively lost with urbanization
- Bassa infants and adults have similar gut microbiomes and metabolomes
- Microbial dispersal nullifies the differences between infant and adult microbiomes
- The infant-type microbiome in urban Westerners may be a neotenic trait

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Infant and Adult Gut Microbiome and Metabolome in Rural Bassa and Urban Settlers from Nigeria

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SUMMARY

We assessed the subsistence-related variation of the human gut microbiome at a fine resolution for two of the main dimensions of microbiome variation, age and geography. For this, we investigated the fecal microbiome and metabolome in rural Bassa and urbanized individuals from Nigeria, including infants, and compared data with worldwide populations practicing varying subsistence. Our data highlight specific microbiome traits that are progressively lost with urbanization, such as the dominance of pristine fiber degraders and the low inter-individual variation. For the Bassa, this last feature is the result of their subsistence-related practices favoring microbial dispersal, such as their extensive environmental contact and the usage of untreated waters from the Usuma River. The high degree of microbial dispersal observed in the Bassa meta-community nullifies the differences between infant and adult intestinal ecosystems, suggesting that the infant-type microbiome in Western populations could be the result of microbiome-associated neotenic traits favored by urbanization.

INTRODUCTION

In recent years, we have witnessed a growing number of studies on the characterization of the human gut microbiome across the globe, in populations adhering to varying subsistence patterns, from more traditional to more urbanized (De Filippo et al., 2010; Yatsunenko et al., 2012; Schnorr et al., 2014; Martínez et al., 2015; Obregon-Tito et al., 2015; Rampelli et al., 2015; Gomez et al., 2016; Smits et al., 2017; Winglee et al., 2017). In addition to providing valuable information on the specific adaptations of the gut microbiota to diet and other lifestyle factors, such studies have evolutionary relevance, as they recall ways of life that accompanied our history, from the hunting and gathering of our Paleolithic ancestors, to small-scale agriculture and permanent settlements of the Neolithic, to the post-industrial Westernized lifestyle. This body of literature has consistently illustrated distinctive signatures of the urbanization process in intestinal microbial communities, including reduced diversity, loss of bacterial taxa with fibrolytic specializations, and the appearance of microorganisms as a potential adaptive response to the changes in diet, environment, use of antibiotics, and hygiene practices, brought on by the modern lifestyle.

However, most of the previous studies have focused on the taxonomic variation of the gut microbiota in adult populations living distinct lifestyles, thus leaving a number of unanswered questions, especially about potential subsistence-driven alterations in metabolic networks and the broad-scale application of this body of data toward informing about the infant gut microbiome alterations. Moreover, most studies comparing microbiomes from hunter-gatherers, rural agriculturalists, and urbanized communities have so far dealt with geographically and culturally distant populations, with obvious confounding factors, such as relatedness and local environment.

In an attempt to bridge these gaps, here we characterized the fecal microbiota and metabolome of two Nigerian communities, the Bassa rural agriculturalists and urban individuals from four state capitals (Ilorin, Abeokuta, Ado Ekiti, and Ibadan) and the Nigerian capital city (Abuja), which also include infants aged <3 years. The Bassa are an agrarian community with limited contact with other populations, who live on a hill about 500 m away from the Chibiri village in Kuje Area Council (Abuja), where they moved from Kogi State (a distance of 160 km) about 100 years ago (Figure 1). Their community comprises about 70–80 people who primarily eat what they grow on their farm, such as tubers, grains, fruit, and other small crops. The Bassa are thus a somewhat

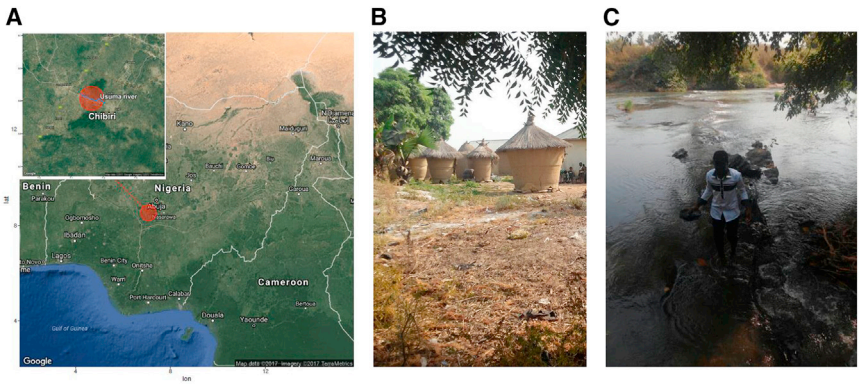

Figure 1. Geography and Lifestyle Patterns of the Bassa in Kuje Area Council, Abuja, Nigeria

(A) Location of Bassa land.

(B) Traditional Bassa village.

(C) The river Usuma, near the Bassa living place, serving as their main water source for drinking and cooking.

Photos by H.J.A.

isolated group, but nonetheless maintain a self-sufficient rural horticultural subsistence. Microbial communities in the Usuma River, which is a daily feature in Bassa life, both for nourishment and physical exposure, were characterized as well. The urban dwellers recruited in our study were randomly selected from different ethnic groups, including Hausas, Igbos, Yorubas and Ebira, as representative of people geographically close to the Bassa but who are embracing a Western lifestyle. Compositional microbiome data and metabolome profiles from these populations were interpreted across subsistence strategies and age, and integrated with available data from worldwide populations, with varying degrees of traditional or urban lifeways. By exploring, at a finer geographic and age resolution than previous efforts, the variation of the human gut ecosystem along the transition from rural to urbanized communities, this study led to uncovering specific adaptive gradients, at both structural and functional scale.

RESULTS

Dietary and Socio-economic Context

The Bassa diet mostly consists of foods derived from their farming activities, such as tubers (yams and cassava), grains (guinea corn, millet, and maize), fruit (banana and mango), and soup condiments (okra and melon). Grains are processed into a flour used to make the traditional stiff porridge "tuwo," and tubers are used to make solid foods (e.g., sokora). The Bassa regularly eat soups (e.g., ayoyo from *Corchorus* leaves and kuka from leaves of *Adansonia digitata*) and fish, given their proximity to the river Usuma. They rear goats and chickens that they sometimes sell to farmers who cross over to their land, but they rarely eat meat, except during festival periods. Breakfast usually consists of koko (or pap), made from the fermentation of maize, millet, and sorghum into a thick paste with hot water. The Usuma River serves as their main water source for drinking and cooking because they have no access to potable water. The river water is not heated or subjected to any form of treatment. The Bassa also bathe in the Usuma and cross it on foot, when it is not flooded, to get to neighboring communities and to the nearest school (attended by very few children). The Bassa breastfeed their infants up to 7–12 months, but they do not practice exclusive breastfeeding. As soon as the baby is able to handle other foods, they supplement breast milk with liquid and semi-liquid foods (e.g., koko, kunu, a popular drink consumed in Northern Nigeria, made from cereal fermentation, and dame, a paste obtained by dissolving in water 24-hour rested tuwo); then solid food is gradually introduced. Antibiotic use is infrequent, and they still rely on local remedies for health needs, favoring herbs to modern medicines, because there is no health care center nearby.

The diet of the urban dwellers was a mixture of traditional Nigerian foods and Westernized dietary items, with only 9 subjects (out of 30) declaring to follow a purely Nigerian diet. Breakfast was generally based on cereals, tea, bread, and pap, while lunch consisted of cooked rice, beans, pastries, and fruit, and dinner usually included foods made from common African tuber crops (e.g., iyan, eba, fufu, tuwo), eaten with spicy soup. In contrast to the Bassa, the urban infants were on average exclusively breastfed for the first 3 months of life (during the working mother national maternity leave) before the introduction of cereals, both traditional (e.g., koko or ogi) and more processed (e.g., flakes, oats, and commercial breakfast cereals). Enrolled urban Nigerians mostly (73%) belonged to the middle class, were educated, had a high level of hygiene and access to potable water, and made a moderate use of antibiotics. Only 5 infants out of the 12 enrolled (and none of the adults) came from the lower class, were exposed to low standards of hygiene, and were usually given natural remedies rather than antibiotics. See Table S1 for a short description of local ingredients and foods.

Characterization of the Bassa and Urban Nigerian Gut Microbiome

The gut microbiota structure from 18 Bassa (9 infants aged <3 years and 9 individuals aged 3–60 years, hereafter referred to as adults, according to previous evidence on the establishment of the typical adult-like microbiota profile; Yatsunenko et al., 2012) and 30 urban Nigerians (12 infants aged <3 years; 18 adults aged 5–75 years) was profiled through 16S rRNA gene sequencing of stool samples. A total of 3,346,376 high-quality reads (mean, 69,716; range, 8,480–133,340) were produced and analyzed.

According to common diversity indices, similar alpha diversity is seen regardless of community membership and age ($p > 0.05$, Wilcoxon rank-sum test) (Figures 2A and 2B; Figure S1). However, it should be noted that the observed number of operational taxonomic units (OTUs) in the microbiota of Bassa infants (mean, 758) is significantly higher compared with urban adults (651; $p = 0.03$) and tends to be greater than Bassa adults (636; $p = 0.08$) and urban infants (615; $p = 0.2$).

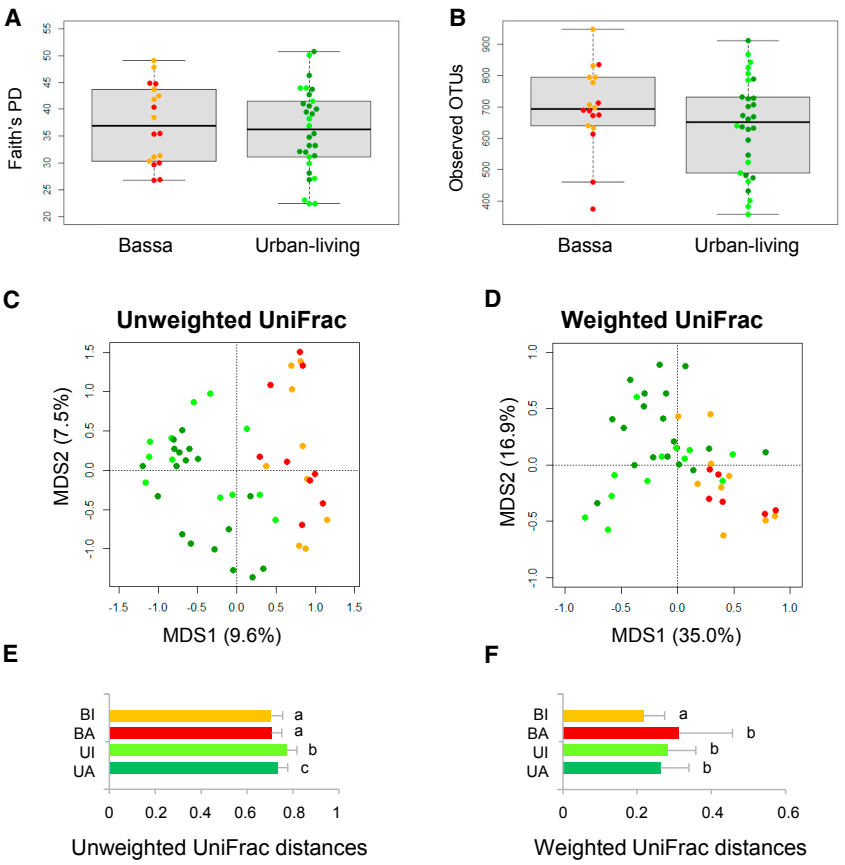

Figure 2. Diversity of the Gut Microbiome of Bassa and Urban Nigerians

(A and B) Alpha diversity computed with Faith's PD (A) and observed OTUs (B) metrics. Subject groups are identified with colored dots within boxes (orange, Bassa infants; red, Bassa adults; green, urban infants; olive green, urban adults). Bassa infants show a higher number of OTUs compared with urban adults ($p = 0.03$, Wilcoxon rank-sum test).

(C–F) Beta diversity, based on unweighted (C and E) and weighted (D and F) UniFrac distances, and PCoA plots. A significant separation between Bassa and urbanized Nigerians was found ($p < 0.05$, adonis). Different letters in the bar plots (means ± SD) indicate significant differences ($p < 0.05$, Kruskal-Wallis test). Same color code as in (A).

BA, Bassa adults; BI, Bassa infants; UA, urban adults; UI, urban infants. See also Figure S1.

UniFrac (weighted and unweighted) and Bray-Curtis distance ordination show separation between Bassa and urban individuals (Figures 2C and 2D; Figure S1; Table S2). No clustering is observed within the populations between infants and adults, except for the unweighted UniFrac analysis, which shows barely separable clusters by age group among urban individuals (adonis: $p = 0.03$, $R^2 = 0.03$; ANOSIM: $p = 0.02$, $R = 0.14$). Regardless of the metrics used, lower interpersonal variation is observed in the microbiota structure of Bassa compared with urban individuals ($p \leq 0.001$, Wilcoxon rank-sum test). With regard to age, greater inter-individual variation is found among urban infants compared with urban adults, according to unweighted UniFrac metrics ($p = 3 \times 10^{-7}$) (Figure 2E). The opposite is true for the Bassa based on weighted UniFrac distances, with a lower inter-individual variation among infants than among adults ($p = 0.009$), as well as compared with urban groups ($p \leq 3 \times 10^{-4}$) (Figure 2F). The analysis based on Bray-Curtis dissimilarities confirms the lower beta diversity for Bassa infants compared with urban groups (Figure S1).

At phylum level, Firmicutes and Bacteroidetes dominate the gut microbiota of rural and urban populations (Figure 3A), but with different ratios (Firmicutes-to-Bacteroidetes ratio: Bassa versus urban, 1.37 versus 2.85; $p = 3 \times 10^{-4}$). Moreover, the Bassa microbial communities are comparatively enriched in Spirochaetes (relative abundance, Bassa versus urban, 1.54% versus 0.18%) and Fusobacteria (0.94% versus 0.01%), while depleted in Actinobacteria (1.13% versus 3.87%) ($p \leq 0.005$). The main discriminant genera are members of the *Lachnospiraceae* and *Ruminococcaceae* families, such as *Blautia*, *Coprococcus*, *Lachnospira*, *Faecalibacterium*, and *Oscillospira*, which are less abundant in the microbiota of Bassa compared with urban individuals ($p \leq 0.03$) (Figure 3B). The Bassa are also depleted in other common commensal inhabitants of the Western gut, including *Bacteroides* and *Bifidobacterium* ($p \leq 4 \times 10^{-6}$). As expected based on previous reports for non-Western populations (De Filippo et al., 2010; Yatsunenko et al., 2012; Schnorr et al., 2014; Martínez et al., 2015; Obregon-Tito et al., 2015; Gomez et al., 2016; Smits et al., 2017), the Bassa microbiome is enriched in *Prevotella* and other Bacteroidales members (including [*Prevotella*] and an unknown S24-7 genus), as well as in *Bulleidia*, [*Eubacterium*], *Cetobacterium*, *Succinivibrio*, and unclassified *Peptostreptococcaceae*. Furthermore, in accordance with available data on traditional populations (Mancabelli et al., 2017), *Phascolarctobacterium* and *Treponema* are more abundant in the microbial ecosystem of Bassa compared with the urban community ($p \leq 0.03$). Notably, *Ruminobacter* and *Butyrivibrio* are exclusively present in the Bassa microbiota, whereas *Megamonas* is only detected in urban individuals (Figure 3C). The relative abundance of several taxa increase with age within the urban cohort, including *Oscillospira*, *Clostridium*, *Odoribacter*, and unknown genera in the Clostridiales order and in the families *Christensenellaceae*, *Rikenellaceae*, and [*Barnesiellaceae*] ($p < 0.05$, Spearman's correlation test). Otherwise, no differences are found between the two Bassa age groups at the various taxonomic levels. However, the exact age of Bassa is uncertain, which prevented us from assessing the precise contribution of age to microbial variation. Likewise, sex was not recorded for participating

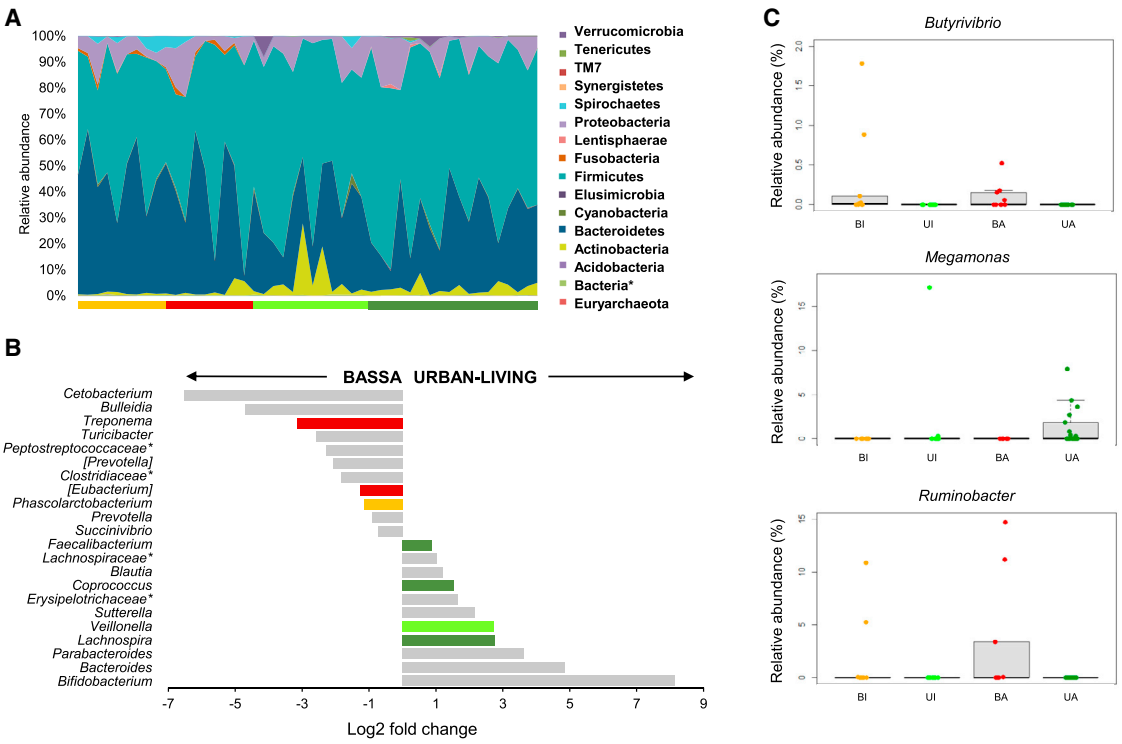

Figure 3. Gut Microbiome Profile of Bassa and Urban Nigerians

(A) Relative abundances of phylum-level taxa. Bars below the area chart are colored by tribe and age (orange, Bassa infants; red, Bassa adults; green, urban infants; olive green, urban adults).

(B) Log2 fold changes of the main discriminant genera between Bassa and urban Nigerians ($p < 0.05$, Wilcoxon rank-sum test). Genera with $\geq$0.5% of mean relative abundance in at least one population were considered. When the difference was significant only between adults or infants, the bar was colored according to the population with the highest relative abundance of that genus (same color code as in A). Asterisk (*) indicates unclassified OTU reported at higher taxonomic level.

(C) Boxplots showing the relative abundance distribution of genera that were uniquely detected in the gut microbiota of Bassa (*Butyrivibrio* and *Ruminobacter*) or urban individuals (*Megamonas*).

Bassa individuals, making us blind to possible sex-related differences.

The intestinal microbiota structure of Bassa and urban settlers was further analyzed through the determination of co-abundance groups (CAGs) (Claesson et al., 2012). Four CAGs were identified based on pairwise Kendall correlation of taxa from the genus-level summarized taxa table, and each one was named according to the most abundant taxon (Figure 4; Figure S2). Interestingly, the *Faecalibacterium* CAG (yellow), showing the co-abundance of several Western-like short-chain fatty acid (SCFA) producers (including the butyrate-producing *Faecalibacterium* and *Coprococcus*, and the acetate-producing *Blautia, Bacteroides*, *Parabacteroides*, *Bifidobacterium*, and *Lachnospira*) (Flint et al., 2015), is absent in the Bassa microbiota, regardless of age, suggesting a different ecological layout supporting SCFA production in the intestinal ecosystem of study populations. On the other hand, the *Prevotella* CAG (light blue) is far more represented in Bassa rather than urban individuals and shows the Bassa-specific co-abundance of bacteria with xylan- and/or starch-degrading and succinogenic capabilities, such as *Prevotella* and *Succinivibrio* (Hippe et al., 1999; Flint et al., 2008), and *Phascolarctobacterium*, an asaccharolytic succinate-utilizing and propionate-producing bacterium (Watanabe et al., 2012), suggesting the establishment of cross-feeding mechanisms leading to propionate production. With regard to the transition from infants to adults, population-specific inter- and intra-CAG rearrangements are observed. In particular, for the urban community, changes in the proportions of common Western SCFA producers and other commensals accompany the shift from the infant to the adult group, along with an increasing representation of the *Roseburia* CAG (dark blue). Alternatively, the *Roseburia* CAG decreases in representation among the Bassa from infants to adults. Furthermore, in the transition to Bassa adults, a shuffling of the *Dialister* CAG (pink) is observed, along with increased proportions of *Turicibacter*, *Streptococcus*, and [*Eubacterium*] in the *Prevotella* CAG.

We also examined the microbial community in water samples from the Usuma River, which is a daily feature, both in nourishment and physical exposure, in Bassa life. Consistent with previous findings on other African rivers (Jordaan and Bezuidenhout, 2016; Vezzulli et al., 2017), the dominant phyla in the Usuma are Proteobacteria (relative abundance, 37.13%) and Firmicutes (15.11%), with Acidobacteria (13.54%), Chloroflexi (5.26%), Bacteroidetes (5.04%), Planctomycetes (4.86%), and

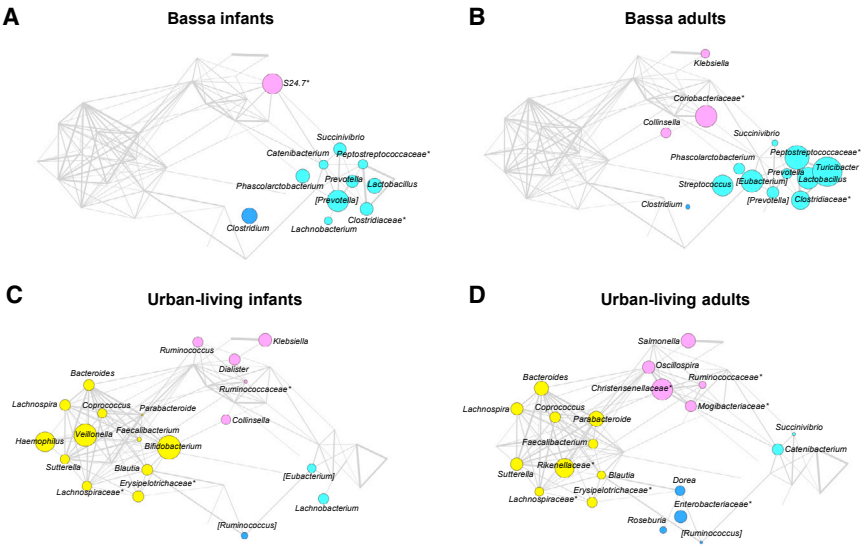

Figure 4. Distinct Bacterial Co-abundance Groups Define Each Population

(A–D) Wiggum plots indicate pattern of variation of the four identified CAGs in Bassa (top) and urban Nigerians (bottom), both in infants (A and C) and in adults (B and D). CAGs were named according to the most abundant genera, as follows: *Faecalibacterium* (yellow), *Dialister* (pink), *Roseburia* (dark blue), and *Prevotella* (light blue). Each node represents a genus, and its dimension is proportional to the over-abundance relative to background. Connections between nodes indicate positive and significant Kendall correlations between genera ($p < 0.05$). Line thickness is proportional to correlation strength. Only genera with $\geq$0.1% relative abundance in at least 30% of subjects were considered. Asterisk (*) indicates unclassified OTU reported at higher taxonomic level.
See also Figure S2.

Actinobacteria (4.51%) as other major groups (Figure 5A). Within Proteobacteria, the Betaproteobacteria members (mainly *Comamonadaceae* and *Oxalobacteraceae* families, and unknown taxa in the SC-I-84 and Ellin6067 orders) dominate the ecosystem, followed by Alphaproteobacteria (mainly *Sphingomonadaceae*, *Rhodospirillaceae*, and *Hyphomicrobiaceae*), Gammaproteobacteria (with *Coxiellaceae* as the most abundant family), and Deltaproteobacteria (with an unknown Myxococcales family as the most represented) members (Figure 5B). Interestingly, among the most abundant (>1%) genera, we could recognize typical commensals of the human gut microbiota belonging to the Clostridiales order and specifically to the *Lachnospiraceae* and *Ruminococcaceae* families, such as *Oscillospira* and *Blautia* (cumulative relative abundance, 10.62%) (Figure 5C). It should be pointed out that during the wet season, when sampling took place, the rain waters wash down the surrounding hill-lands into the river, including the bush and scrub land near the Bassa settlement where Bassa usually defecate. Therefore, the high presence of human-associated commensal microorganisms in river waters could be the consequence of fecal pollution from anthropogenic sources, as well as other wild fauna. These observations are supported by recent work demonstrating that *Ruminococcaceae* and especially *Lachnospiraceae* bacteria could serve as human fecal signatures, as an alternative to traditional indicators based on enterococci and enterobacteria (Newton et al., 2013; McLellan and Eren, 2014).

Characterization of the Bassa and Urban Nigerian Fecal Metabolome

Gas chromatograph-mass spectrometry (GC-MS) analysis of SCFAs revealed a different profile for Bassa (3 infants and 8 adults) compared with urban individuals (8 infants and 15 adults). Bassa are significantly enriched in propionate (relative abundance, Bassa versus urban settlers, 21.86% versus 12.53%) and proportionally depleted in acetate (65.22% versus 78.07%) ($p \leq 0.005$, Wilcoxon rank-sum test). These signatures are shared by both infant and adult groups within the urban population, while the Bassa exhibit varying SCFA profiles depending on the age group. Specifically, compared with adults, Bassa infants show a greater abundance of acetate and valerate ($p = 0.04$) (Figures 6A).

Principal component analysis (PCA) of the relative abundance profiles of 185 key metabolites from core metabolic pathways, as detected through a semi-untargeted metabolomics approach (Table S3), shows separation between Bassa (3 infants, 6 adults) and urban populations (7 infants, 14 adults), which is particularly evident along PC2 (adonis: $p = 0.04$, $R^2 = 0.11$; ANOSIM: $p = 0.03$, $R = 0.21$) (Figure 6B). Procrustes analysis of Euclidean distances between metabolomes and weighted or unweighted UniFrac distances highlights significant association between the microbiota taxonomic and metabolic profiles across the entire cohort (for weighted UniFrac: m12 squared = 0.78, correlation value = 0.47; for unweighted UniFrac: m12 squared = 0.55, correlation value = 0.67; $p \leq 0.002$, PROTEST) (Figure S3). As expected, PC2 coordinate-related genera are among those previously identified as discriminatory for the Bassa from the above taxonomic findings. In particular, *Prevotella*, [*Prevotella*], and *Succinivibrio* are positively correlated to the axis, whereas *Bifidobacterium*, *Bacteroides*, *Blautia*, and unclassified *Lachnospiraceae* and *Erysipelotrichaceae* correlate with negative values of PC2 coordinates ($p \leq 0.007$, Kendall tau correlation test) (Figure S4). According to a Random Forests analysis (Breiman, 2001), seven metabolites are highly discriminatory between Bassa and urban individuals, of which five are over-represented in the former (histamine, alanine, lysoPC a C16:1, lysoPC a C18:1, and C10:2) and two in the latter (serotonin and tyrosine).

Along PC1, which accounted for nearly all of the variation (81.4%), the infant groups segregate from the respective adult groups, with the metabolomics profiles of the latter located at positive values of the axis ($p \leq 0.04$, Wilcoxon rank-sum test). Microbial taxa that positively correlate to PC1 (i.e., to the intra-group segregation by metabolites) include *Oscillospira* and unclassified *Christensenellaceae* ($p \leq 3 \times 10^{-4}$, Kendall tau

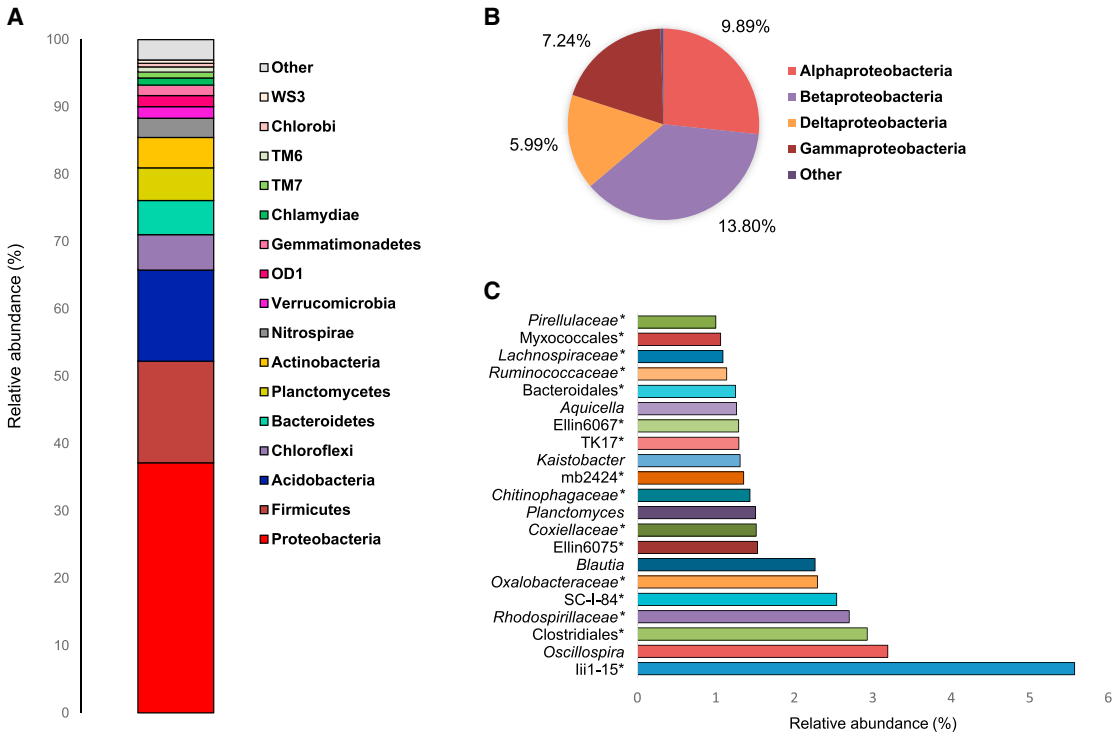

Figure 5. The Microbiota of the Usuma River

(A) Relative abundance of the major phyla.

(B) Distribution of Proteobacteria classes.

(C) Top 21 most abundant genera, with relative abundance >1%.

Asterisk (*) indicates unclassified OTU reported at higher taxonomic level.

correlation test) (Figure 6B; Figure S4). Regardless of population, adult individuals are characterized by a greater presence of amino acids (infants versus adults: Bassa, 54.75% versus 83.03%; urban, 73.67% versus 85.82%; $p \leq 0.04$, Wilcoxon rank-sum test) (Figure S5). When looking at individual metabolites, alanine is the main discriminant amino acid of Bassa adults, while phenylalanine, tyrosine, and branched-chain amino acids (BCAAs; leucine, isoleucine, valine) are far more represented in urban adults. With respect to infants, hexoses are largely dominant in the Bassa, while taurine is greatly overrepresented in the urban infant metabolomes (Figure 6B). Compared with infants, urban adults also have different proportions of glycerophospholipids (infants versus adults, 1.84% versus 1.67%), sphingolipids (0.25% versus 0.22%), biogenic amines (5.34% versus 2.53%), and hexoses (18.36% versus 9.28%) ($p \leq 0.04$). Aside from the previously described amino acid category, the Bassa adult metabolome significantly differs from that of infants by a slightly diminished abundance of acylcarnitines (infants versus adults, 0.88% versus 0.35%; $p = 0.02$) (Figure S5).

The pattern of metabolome variation between populations was further explored through a CAG analysis, leading to the identification of three CAGs of correlated metabolites (Figure 6C; Figure S6). According to our findings, the alanine CAG (brownish), including most of the amino acids, is exclusively present in the fecal metabolome of adults, but far more represented in urban compared with rural individuals. Conversely, the hexoses CAG (ivory), also including several glycerophospholipids, some acylcarnitines, along with most of the biogenic amines, is more represented in the infant metabolome, but far more in rural than urban. The threonine CAG (purple) is variously represented in all study groups except in Bassa infants. It is worth noting the Bassa-specific age-independent co-abundance of asparagine, histamine, and acetylornithine, with the first being widely distributed in the plant kingdom, the second derived by enzymatic decarboxylation of histidine, and the last available in several "rural-like" foods used by Bassa (http://www.hmdb.ca/; http://foodb.ca/). At the same time, it is interesting to note the absence of histamine (with the abundance of histidine), as well as that of acetylornithine and ornithine (with the abundance of putrescine, a product of ornithine decarboxylation), in the CAGs of urban infants, suggesting the establishment of alternate co-metabolisms in early life. Contrarily to Bassa, no overlap between infant and adult CAGs is observed for the urban population, consistent with the Western-type age-related differentiation of microbiota profiles.

Taxonomic and Metabolic-Level Comparison with Worldwide Data from Populations Adhering to Different Subsistence Strategies

To further explore the variation of the gut microbiome across populations with different geographic origin and lifeway, the genus-level abundance profiles of the Bassa and urbanite

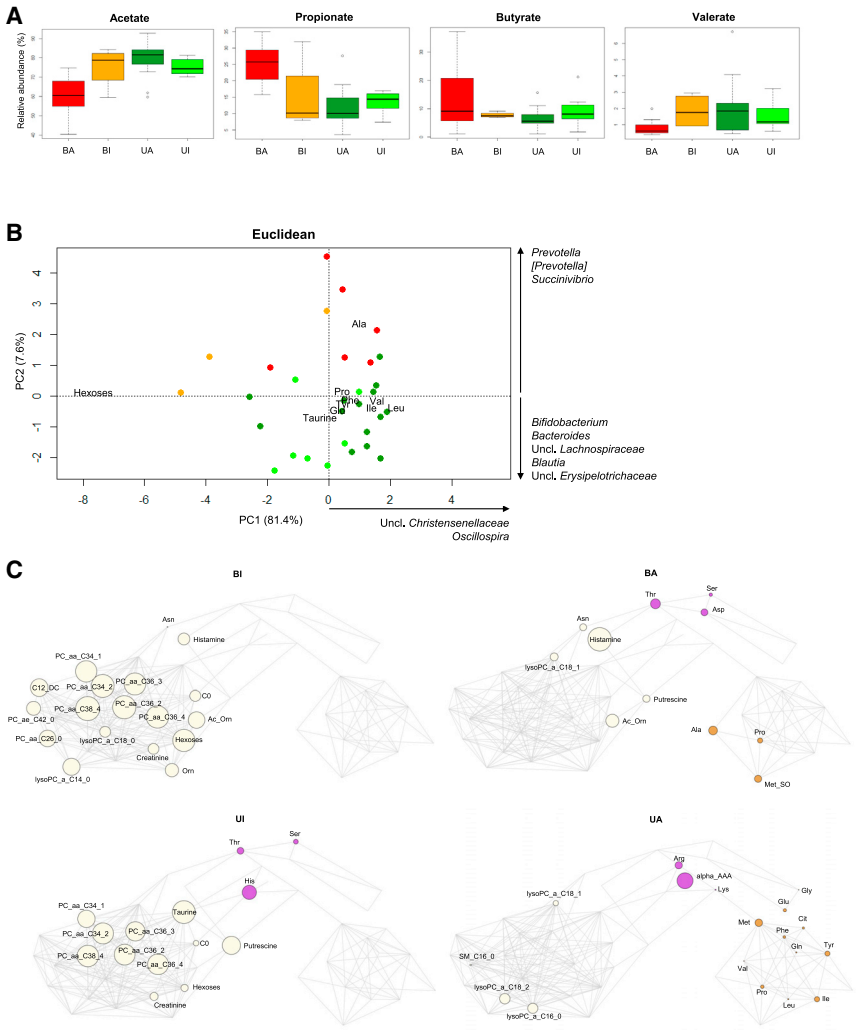

Figure 6. Fecal Metabolome of Bassa and Urban Nigerians

(A) Boxplots showing the relative abundance distribution for short-chain fatty acids. Acetate and propionate levels are different between study groups ($p \leq 0.004$, Kruskal-Wallis test). Valerate is enriched in Bassa infants compared with adults ($p = 0.04$, Wilcoxon rank-sum test).

(B) PCA of Euclidean distances between the metabolic profiles of the study populations, assessed using a semi-untargeted metabolomics approach (Turroni et al., 2016). $p = 0.04$, adonis. The main discriminant metabolites are mapped on the plot. Genera of the gut microbiota significantly correlated to PC1 and PC2 ($p < 0.05$, Kendall tau correlation test) are displayed at the bottom and on the right, respectively. BA, Bassa adults (red); BI, Bassa infants (orange); UA, urban adults (olive green); UI, urban infants (green).

(C) Wiggum plots indicate pattern of variation of the three identified metabolic co-abundance groups (CAGs) in Bassa (top) and urban Nigerians (bottom), both in infants (left) and in adults (right). Each node represents a metabolite, and its dimension is proportional to the over-abundance relative to background. Connections between nodes indicate positive and significant Kendall correlations between metabolites ($p < 0.05$). Only metabolites with $\geq 0.1\%$ relative abundance in at least two subjects were considered.

BA, Bassa adults; BI, Bassa infants; UA, urban adults; UI, urban infants. See also Figures S3–S6.

Nigerians were compared with those from two previous works, dealing with a hunter-gatherer community (Hadza from Tanzania), rural agricultural communities from Malawi and Amazonas State of Venezuela, and urban-industrialized societies (from Italy and the USA) (Yatsunenko et al., 2012; Schnorr et al., 2014). As expected, the Bray-Curtis distances between the Bassa and the other traditionally living rural populations are lower than those between Bassa and urban groups (Figure 7A). Alternatively, the Bray-Curtis distances between urban Nigerians and the other groups are overall similar. A source tracking analysis using the previously published datasets as source populations confirmed that rural sources account for the vast majority (mean, 81%) of the Bassa microbiome, with the hunter-gatherer source making a substantial contribution (up to 59%) in eight individuals (Figures 7B and 7C). Compared with adults, Bassa infants have a greater contribution from rural (84% versus adults, 78%) as well as hunter-gatherer (15% versus 11%) sources, with a corresponding decrease in unknown sources (0.8% versus 11%). Supporting the inference that the urban Nigerian population represents a middle ground between a rural-traditional and fully urban-industrial lifestyle, the Nigerian urban profiles generally have a higher contribution from rural (mean, 57%) than urban sources (32%) (Figures 7B and 7C). It is worth noting that the rural source contributions decrease from infants (66%) to adults (50%), parallel to the increase in the urban source proportion (infants, 29%; adults, 34%). The hunter-gatherer source contributes 12%–88% in six urban individuals (five adults and one infant).

With regard to metabolite data, the Bassa and urban Nigerians cluster in distinct groups alongside either rural (hunter-gatherer) or urban individuals for which metabolomics data produced through the same analytical method are available (Turroni et al., 2016). Additionally, these cross-study populations cluster into distinct groups according to subsistence strategy and age (adonis: $p = 1 \times 10^{-5}$, $R^2 = 0.56$; ANOSIM: $p = 1 \times 10^{-4}$, $R = 0.30$) (Figure 7D). In particular, the Bassa population separates from all the others, whereas urban Nigerians overlap with urban Italians and are similarly enriched in phenylalanine and tyrosine. Despite this, Nigerian urban individuals are still distinguishable from Italians by higher proportions of glycerophospholipids (Nigerians versus Italians, 1.73% versus 0.60%), sphingolipids (0.23% versus 0.07%), and acylcarnitines (0.50% versus 0.26%) ($p \leq 0.02$, Wilcoxon rank-sum test). The same signatures emerge from the comparison between Bassa and

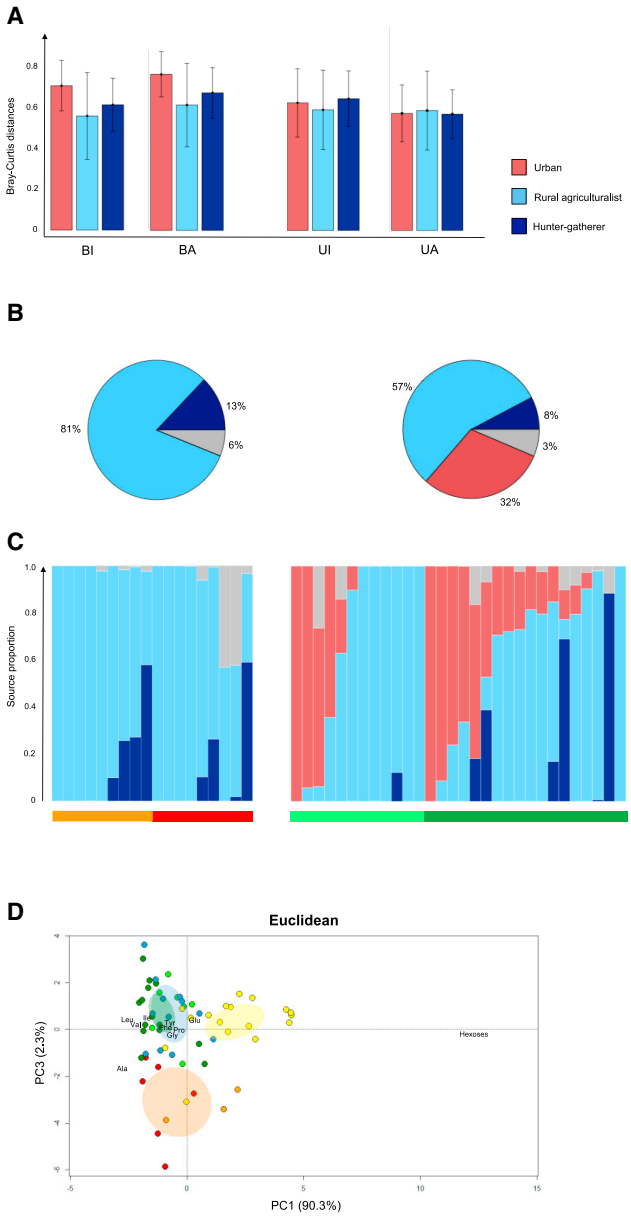

Figure 7. Taxonomic and Metabolic-Level Comparison with Worldwide Data from Populations Adhering to Different Subsistence Strategies

(A) Bray-Curtis distances (means ± SD) between genus-level microbiota profiles. Publicly available sequences from Hadza hunter-gatherers and urban Italian adults (Schnorr et al., 2014), rural agriculturalists from Malawi and Amazonas State of Venezuela, and urban US adults (Yatsunenko et al., 2012) were used.

(B and C) Bayesian source-tracking analysis. Source contributions, estimated using SourceTracker (Knights et al., 2011), are shown averaged within Bassa or urban Nigerians (B), and for individual samples (C). Bars below the histograms are colored by population and age (orange, Bassa infants; red, Bassa adults; green, urban infants; olive green, urban adults). Hunter-gatherer, rural agricultural, urban, and unknown sources are colored in dark blue, light blue, brick red, and gray, respectively (as in Obregon-Tito et al., 2015).

(D) PCA of Euclidean distances between the metabolic profiles of the study populations (same color code as the horizontal bars in C), as well as Hadza (yellow) and urban Italians (blue) (Turroni et al., 2016), as assessed using a semi-untargeted metabolomics approach. Ellipses include 99% confidence area based on the SE of the weighted average of sample coordinates and are colored by population (salmon, Bassa; green, urbanite Nigerians; yellow, Hadza; light blue, Italians). $p = 1 \times 10^{-5}$, adonis. The main discriminant metabolites are mapped on the plot.

See also Figure S5.

Italians (Figure S5). On the other hand, differences between the traditional subsistence groups, the Bassa and the Hadza, include enrichment in biogenic amines (Bassa versus Hadza, 2.75% versus 2.50%) and amino acids (73.61% versus 45.12%) for Bassa, and significant enrichment in hexoses (21.13% versus 50.54%) for Hadza ($p \leq 0.005$). The same differences are seen by comparing the Hadza with Bassa adults, but not with infants, whose metabolic profiles are overall more similar to those of the Hadza, with hexoses as main discriminant metabolites (Figure 7D; Figure S5).

DISCUSSION

In this study, we demonstrate that two human communities living in a geographically proximate region in Nigeria follow a predictive pattern of dissimilarity in taxonomic and metabolic traits of the gut microbiome that mirror the traditional and/or rural versus urban and/or industrialized subsistence dichotomy. Importantly, these results have allowed us to witness specific traits that indicate a progressive adaption of the intestinal microbial ecosystem toward urbanization.

Consistent with prior findings, our data point to a reduced inter-individual variation in the microbiota of people adhering to a traditional lifeway, with the well-known dominance of bacteria with high potential for fiber degradation (primarily *Prevotella*, *Treponema*, and *Succinivibrio*, but also *Ruminobacter*, *Phascolarctobacterium*, and *Butyrivibrio*), and the underrepresentation or even absence of common members of urban-industrial gut microbiomes (e.g., *Bacteroides*, *Bifidobacterium*, and a series of known SCFA producers, including *Blautia* and *Faecalibacterium*) (De Filippo et al., 2010; Yatsunenko et al., 2012; Schnorr et al., 2014; Martínez et al., 2015; Obregon-Tito et al., 2015; Rampelli et al., 2015; Gomez et al., 2016; Soverini et al., 2016; Smits et al., 2017). Our study also led to the identification of bacteria worthy of further investigation for their possible association with the lifestyle patterns of the study populations, i.e., *Cetobacterium* and *Bulleidia* for rural gut communities, and *Megamonas* and *Oscillospira* for urban microbiotas. *Cetobacterium* is a Fusobacteria genus indigenous to the digestive tract of freshwater fish (Tsuchiya et al., 2008), including tilapia (Standen et al., 2015), which dominates the lower Usuma River reservoir as well as other West African water bodies (Dan-kishiya, 2012). The abundance of *Cetobacterium* in the gut microbial ecosystem of the Bassa individuals sampled in this study may be related to their regular consumption of fish and the close relationship they maintain with the Usuma River. Less information is available for *Bulleidia*, frequently associated with the human oral microbiome but recently identified as exclusive to the intestinal microbiota of Bangladeshi children living in an urban slum compared with upper-middle class suburban children from the United States

(Lin et al., 2013). On the other hand, the *Megamonas* species known so far are listed among Firmicutes members with more limited carbohydrate utilization capabilities (Sakon et al., 2008), and to date they have been identified in urban contexts (Park et al., 2015) and found to be differentially abundant according to ethnicity (Chen et al., 2016). Similarly, *Oscillospira* is shown to increase in abundance with the switch to an animal-based diet, under high-bile conditions (David et al., 2014), thus likely reliant on fermentation products generated by other microbes or on host mucus glycans rather than primary fiber degradation (Konikoff and Gophna, 2016). The high abundance of *Oscillospira* and the exclusive presence of *Megamonas* in the gut microbiota of urban Nigerians may be potential markers of the progressive urbanization and adoption of a Western lifestyle.

According to the metabolomics results, the Bassa, especially infants, show an overall healthy profile with greater proportions of hexoses and fewer amounts of amino acids and biogenic amines compared with urban individuals. As previously discussed (Turroni et al., 2016), the abundance of hexoses may be indicative of a diet high in microbiota-accessible carbohydrates (Sonnenburg and Sonnenburg, 2014), as that of the Bassa, heavily based on tubers, grains, and derived processed foods, as well as a variety of leafy soups, with the microbiota-dependent release of monosaccharides probably exceeding the enteric nutritional demands and thus excreted in the feces. With specific regard to Bassa infants, hexoses may also result from the digestion of the sugary and starchy liquid or semisolid foods they are fed with during weaning. On the other hand, the smaller amounts of amino acids and derivatives in Bassa feces may reflect less protein consumption compared with urban Nigerians and/or altered metabolisms or absorption.

When focusing on age, our compositional data on urban infants corroborated what is well-known in developed countries, i.e., that the gut microbiota of infants aged <3 years is unstable, with high inter-individual diversity and a taxonomic structure progressively approaching the more complex and stable adult-type microbiota (Yatsunenko et al., 2012). Conversely, different and at times opposite features were observed for the intestinal microbial ecosystem of Bassa infants that, compared with the adult counterparts, showed high biodiversity, lower inter-individual variability, and no difference at the various taxonomic levels. This in turn brought about a finding that lends clues to a lingering question from the work of Schnorr et al. (2014), which is whether bifidobacteria are indeed absent in the kinetics of assembly and development of traditional microbiotas. The data we present here of pre- and peri-weaned Bassa infants confirms that bifidobacteria, which are undoubtedly beneficial for Western human populations, are missing from certain traditional population infant guts. Collectively, these data are in contrast with the work of Yatsunenko et al. (2012), which identified distinctive microbiome features in early childhood in rural populations, including greater inter-individual variation among children than adults, an increasing biodiversity with age, and the dominance of *Bifidobacterium*. While we are aware that the much smaller sample size of our study could have led us to overestimate our results, we believe that our data merit continued consideration of the hypothesis that in a non-Westernized context with vastly different environmental conditions, socio-economic structures, subsistence practices, and external contact with other populations, assumptions about microbiome traits derived from studies on Western populations lead interpretations about human-microbe associations astray. From an ecological standpoint, it is possible to speculate that the extensive sharing of life within the Bassa community (in terms of lifestyle habits, contact with the environment, and usage of untreated river water, which indeed shows traces of microbiota components) results in a high degree of microbial dispersal, thus allowing the human microbiome to behave as a meta-community (Costello et al., 2012). In turn, the establishment of a meta-community, a feature probably common to traditional populations, has the potential to nullify the differences between infant and adult microbiomes, as mainly observed in Western populations, significantly shortening the microbiota assembly process. On the contrary, along with Westernization (involving sanitization, water treatment and other hygienic practices, and reduced life sharing with dispersal limitation), the human microbiome has lost its meta-community feature, resulting in increased individuality, and consequently driving the differentiation of the infant-type microbiome, as well as the trajectory of microbiome assembly, typical of Western populations. Indeed, the acquisition or, more likely, the extension of an infant-type microbiota in modern populations could be the result of neoteny in human evolution, favored by the establishment of profound differences in diet and lifestyle between infants and adults in modern societies. Further supporting this, also metabolic data suggest opposing processes of maturation of the intestinal ecosystem from infancy to adulthood, with the urban metabolome rearranging with increasing age, counter to what is seen among Bassa adults, which remain comparable with the overall Bassa infant profile. In particular, in urban adults, we witnessed a reduction in abundance of several metabolite categories, including hexoses, glycerophospholipids, sphingolipids, and biogenic amines, and a corresponding increase in amino acids. As shown elsewhere, sphingolipids play a crucial, lasting role in the modulation of the intestinal natural killer T cell homeostasis in early life (An et al., 2014), but their presence, as well as that of glycerophospholipids, may be of some relevance even in adults by triggering anti-inflammatory cascades (Stremmel et al., 2005). It is interesting to note that although the age-related increase in fecal amino acids occurred in both populations, the discriminant amino metabolites were yet distinct by population; alanine enriched in Bassa adults, and phenylalanine, tyrosine, and BCAAs in urban Nigerians. Alanine is among the most abundant amino acids in leaves of *Corchorus olitorius* (jute) (https://phytochem.nal.usda.gov/), a native plant of tropical Africa, whose leafy vegetable is popularly used in folk medicine and the preparation of soups (Oboh et al., 2009), such as ayoyo, which is an integral part of Bassa cuisine. Furthermore, alanine is gluconeogenic, and thus may relate to the enrichment in hexoses, as observed in rural subjects. Similarly, the increased phenylalanine and tyrosine amounts in urban adult metabolomes could be related to the diet, because these amino acids are particularly abundant in the leaves of the baobab tree (*A. digitata*) (Yazzie et al., 1994), which serve as the main ingredient of the kuka soup, another staple food in Nigeria.

When placed in an international scenario with compositional data from other human populations with varying subsistence

patterns (Yatsunenko et al., 2012; Schnorr et al., 2014), our data confirm that the way of life (traditional, including hunter-gathering and rural agricultural, versus urban-industrialized), rather than geography, drives structural convergence in gut ecosystem profiles, but with specific gradients in relation to the degree of transition from pre-agricultural to modern lifestyles. Despite the apparent presence of several Western-type taxonomic signatures, urban Nigerians are indeed partly similar to traditional populations and partly to typical urban-industrial communities, indicating an incomplete transition to microbial Westernization. In support of this, the retrieved diet information showed that some urban participants still follow a highly traditional Nigerian diet with limited consumption of processed store-bought foods. In parallel, the degree of schooling was found to vary among the urban subjects enrolled, as well as their hygiene practices, their social class, and the use of antibiotics.

The comparison of metabolite profiles from the Bassa with those from the Hadza hunter-gatherers reveals a shared pattern of enrichment in hexoses and reduction in amino acids and biogenic amines relative to urbanized counterparts. The differences between the populations of the present study are, however, less pronounced as those between Hadza and Italians in Turroni et al. (2016), consistent with the less, and more recently, divergent lifestyle and environmental contexts of Bassa and urban Nigerians.

In summary, the microbial and metabolic characterization of the intestinal ecosystem of rural Bassa and urbanized individuals in Nigeria provided insights into the complex host-microbiome relationships across subsistence strategies, advancing our understanding of the changes in gut microbial communities and metabolic networks that probably accompanied human evolutionary history but, above all, stressing the relevance of the progressive adoption of a Western lifestyle as a major driver selecting for the loss of ancient signatures. Moreover, our findings support the existence of distinct trajectories of development of the intestinal ecosystem in early life, depending on human ecological context.

Further studies on larger worldwide cohorts, possibly with exact age information, are needed to evaluate the scope of these results, in the perspective of establishing the actual impacts on health of the lifestyle contribution to the compositional and functional structure of our gut, and its trajectories across lifespan.

EXPERIMENTAL PROCEDURES

Subject Enrollment and Sample Collection

Eighteen Bassa (nine adults and nine infants) participated in this study. The infants were younger than 3 years old, while the adults were of indeterminable age but presumably younger than 60 years. Sex information was not available. For urban volunteers, 12 infants (<3 years) and 18 adults (5–75 years) were recruited from state capitals of four states in South Western Nigeria (Ilorin, Kwara State; Abeokuta, Ogun State; Ado Ekiti, Ekiti State; Ibadan, Oyo State) and Nigeria capital city (Abuja, Northern Nigeria). Fecal samples were collected in mid-2015 (July–September) upon consent from the adults and assent from the youth with consent granted by parents or guardians. There were series of meetings and interactions with the community to educate them on the purpose of research and to solicit for their participation. Samples were processed in dry form with 97% ethanol, as previously described (Schnorr et al., 2014), and then transported to Bologna (Italy) for analysis. Information on diet, hygiene practices, and the use of antibiotics was collected. Hygiene practices were defined as low or high, based on community crowding (slum conditions with pit latrines versus three or four-bedroom apartments with water closet toilet facilities), frequency of washing utensils before using them, etc. The antibiotic use was regarded as moderate when occurred as needed, under medical, or self-prescription. The study was approved by the Institute of Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria, with ethical approval number UI/EC/15/0050.

DNA Extraction and Sequencing of V3–V4 16S rRNA Region

Microbial DNA was extracted from feces using the repeated bead-beating plus column method (Yu and Morrison, 2004), with only a few changes (Biagi et al., 2016). In brief, fecal suspensions in lysis buffer (500 mM NaCl, 50 mM Tris-HCl [pH 8], 50 mM EDTA, 4% [w/v] SDS) were treated three times in a FastPrep instrument (MP Biomedicals, Irvine, CA, USA) at 5.5 movements/s for 1 min, in the presence of four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA). After 15-min incubation at 95°C and pelleting stool particles, nucleic acids were precipitated by adding 10 M ammonium acetate and 1 vol of isopropanol. Pellets were washed with 70% ethanol and then resuspended in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 (TE) buffer. After treatment with 10 mg/mL DNase-free RNase at 37°C for 15 min, samples were subjected to protein removal and column-based DNA purification following the manufacturer's instructions (QIAamp DNA Stool Mini Kit; QIAGEN, Hilden, Germany).

One liter of water from the Usuma River was filtered through a 0.4-μm polycarbonate membrane. The membrane was resuspended in 10 mL of sterile deionized water and vortexed for 3 min to release cells. After centrifugation at 12,000 rpm for 10 min, the pellet was resuspended in 300 μL of InstaGene Purification Matrix (Bio-Rad, Hercules, CA, USA), followed by 30-min incubation at 56°C. The sample was vortexed at high speed for 10 s, placed in a 100°C heat block for 8 min, vortexed again, and spun down at 10,000–12,000 rpm for 2–3 min to collect the supernatant-containing DNA.

The V3–V4 region of the 16S rRNA gene was amplified using the 341F and 805R primers with added Illumina adaptor overhang sequences as previously reported (Candela et al., 2016). Amplicons were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology, further cleaned up, and pooled at equimolar concentrations. The final library was denatured with 0.2 N NaOH and diluted to 6 pM with a 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2 × 300 bp paired-end protocol, according to the manufacturer's instructions.

Metabolomics

Two quantitative metabolomics approaches were undertaken in order to gather information about key metabolites, including, but not limited to, those possibly contributed by intestinal bacteria or attributable to microbial activity in the gut.

The first, described in Schnorr et al. (2014), was applied to quantify the fecal levels of SCFAs. In brief, feces were homogenized in 10% perchloric acid and centrifuged at full speed for 5 min at 4°C. Supernatants were diluted 1:10 in water and added with D8-butyric acid (internal standard) to 20 μg/mL. Headspace solid-phase microextraction was performed using a 75-μm carboxen-polydimethylsiloxane fiber (Supelco; Sigma-Aldrich, Milan, Italy) under the following conditions: 70°C, 10-min equilibration and 30-min extraction. Analytes were desorbed into the gas chromatograph (GC) injector port at 250°C for 10 min. GC-MS analysis was carried out on a TRACE GC 2000 Series (Thermo Fisher Scientific, Waltham, MA, USA) GC, interfaced with GCQ Plus (Thermo Fisher Scientific) mass detector with ion trap analyzer, operating in electron ionization (EI) mode (70 eV). A Phenomenex ZB-WAX column (100% polyethylene glycol; 30 m × 0.25 mm ID, 0.15-μm film thickness) was used. GC was operated in splitless mode, with helium as the carrier gas, at 1.0 mL/min flow rate. The GC oven temperature program was as follows: 40°C (hold time, 5 min), ramped by 10°C/min to 220°C (5 min). The injector base, transfer line, and ionization source temperatures were set to 250°C, 250°C, and 200°C, respectively. Mass spectra were recorded in full scan mode to collect the total ion current chromatograms. Quantitative analysis was carried out by

using the extracted-ion chromatograms on quantifier and qualifier ions of each analyte, as previously described (Schnorr et al., 2014). Due to insufficient fecal material, 11 rural Bassa (3 infants, 8 adults) and 23 urban Nigerians (8 infants, 15 adults) were analyzed.

The latter, semi-untargeted approach was based on the Absolute*IDQ* p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) that allows the quantification of key metabolites from main metabolic pathways, including acylcarnitines, amino acids, biogenic amines, hexoses, sphingolipids, and glycerophospholipids (Table S3). As reported in Turroni et al. (2016), about 200 mg of feces was extracted by adding three equivalents (w/v) of methanol, followed by vortex-mixing (3 s) and 10-min sonication. After centrifugation at 14,000 rpm for 10 min at 4°C and filtration through 0.22-μm polyethersulfone (PES) membranes, fecal extracts were analyzed in a combined flow injection (FIA) and LC-MS/MS assay, based on phenylisothiocyanate-derivatization in the presence of isotopically labeled internal standards, according to the manufacturer's instructions. LC-MS/MS and FIA plates were run on a Series 200 HPLC (PerkinElmer, Waltham, MA) coupled with a 4000QTrap mass spectrometer operated in triple quadruple mode (AB-Sciex, Toronto, ON, Canada). Data were processed by Analyst 1.6.3. Due to insufficient fecal material, 9 rural Bassa (3 infants, 6 adults) and 21 urban Nigerians (7 infants, 14 adults) were analyzed.

Bioinformatics and Statistics

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME (Caporaso et al., 2010). High-quality reads were binned into OTUs at 97% similarity using UCLUST (Edgar, 2010). Taxonomy was assigned using the RDP classifier against Greengenes database (May 2013 release). All singleton OTUs were discarded. Alpha diversity was computed after rarefaction to 8,480 sequences per sample (minimum sampling depth) using observed OTUs, Shannon and Faith's phylogenetic diversity (PD) indices. Beta diversity was estimated by computing weighted and unweighted UniFrac (16S rRNA data), Euclidean (metabolome), and Bray-Curtis (16S rRNA data and genus tables from worldwide populations) distances. Bacterial and metabolic CAGs were determined as previously described (Claesson et al., 2012; Turroni et al., 2016); Wiggum plots were created using Cytoscape 3.2.1. For bacterial CAGs, genera with $\geq$0.1% relative abundance in at least 30% of subjects were considered. For metabolic CAGs, metabolites with $\geq$0.1% relative abundance in at least two subjects were included. Discriminatory metabolites between study populations were identified using Random Forests (Breiman, 2001). SourceTracker (Knights et al., 2011) was used to estimate the proportional contributions of traditional or urban sources to the microbiota of Bassa and urban Nigerians.

All statistical analysis was performed in R 3.3.2 using R studio 1.0.136. Principal coordinate analysis (PCoA), PCA, Procrustes, adonis (permutation test with pseudo-F ratios), and ANOSIM tests were performed using the vegan package; the Random Forests analysis was carried out using the library package randomForest, SourceTracker using the corresponding package SourceTracker, and correlation (Spearman and Kendall tau) tests and non-parametric tests (Wilcoxon rank-sum test or Kruskal-Wallis test) were achieved using the stats package. p values were corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. A corrected p value <0.05 was considered statistically significant.

DATA AND SOFTWARE AVAILABILITY

The accession number for the sequencing reads reported in this paper is MG-RAST: mgp83994.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.05.018.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.A.A., P.B., M.C., E.B., and S.T.; Methodology, F.A.A., M.C., E.B., S.T., and S.L.S.; Software, S.R. and M.S.; Formal Analysis, S.R., M.S., E.B., F.A.A., and S.T.; Investigation, H.J.A., F.A.A., E.B., J.F., S.C., L.C., and S.T.; Resources, V.C. and P.B.; Writing – Original Draft, S.T.; Writing – Review & Editing, M.C., E.B., F.A.A., H.J.A., S.R., J.F., S.L.S., and S.T.; Visualization, S.R., E.B., M.C., and S.T.; Supervision, M.C., F.A.A., E.B., and S.T.; Project Administration, M.C., F.A.A., E.B., and S.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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