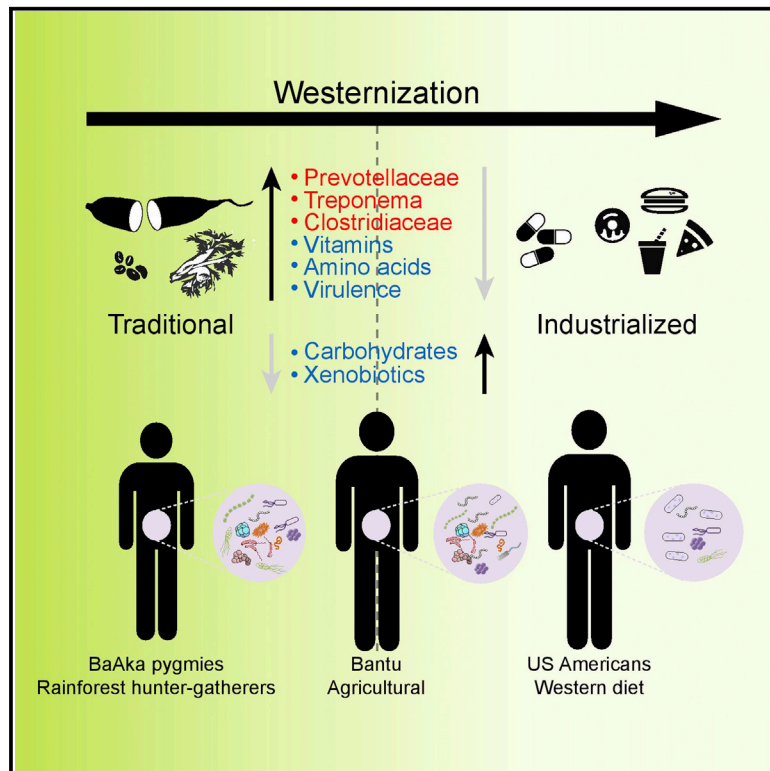

Authors

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Gut Microbiome of Coexisting BaAka Pygmies and Bantu Reflects Gradients of Traditional Subsistence Patterns

Graphical Abstract



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In Brief

Gomez et al. show that coexisting BaAka hunter-gatherers and Bantu agriculturalists have distinct gut microbiomes, which reflect gradients of traditional subsistence strategies. A comparison with US Americans suggests that agriculture and industrialization have triggered loss of traditional microbes and increased carbohydrate and xenobiotic metabolism in humans.

Highlights

- BaAka hunter-gatherers and Bantu agriculturalists have distinct gut microbiomes
- Microbiome profiles follow a gradient that reflects the degree of traditional lifestyle
- Comparisons with US Americans show western-like microbiome patterns in the Bantu
- Westernization drives sugar/xenobiotic processing and loss of traditional microbes



Gut Microbiome of Coexisting BaAka Pygmies and Bantu Reflects Gradients of Traditional Subsistence Patterns

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SUMMARY

To understand how the gut microbiome is impacted by human adaptation to varying environments, we explored gut bacterial communities in the BaAka rainforest hunter-gatherers and their agriculturalist Bantu neighbors in the Central African Republic. Although the microbiome of both groups is compositionally similar, hunter-gatherers harbor increased abundance of Prevotellaceae, *Treponema*, and Clostridiaceae, while the Bantu gut microbiome is dominated by Firmicutes. Comparisons with US Americans reveal microbiome differences between Africans and westerners but show western-like features in the Bantu, including an increased abundance of predictive carbohydrate and xenobiotic metabolic pathways. In contrast, the hunter-gatherer gut shows increased abundance of predicted virulence, amino acid, and vitamin metabolism functions, as well as dominance of lipid and amino-acid-derived metabolites, as determined through metabolomics. Our

results demonstrate gradients of traditional subsistence patterns in two neighboring African groups and highlight the adaptability of the microbiome in response to host ecology.

INTRODUCTION

The recent characterization of gut microbiomes across geographically and culturally diverse human populations has highlighted an integral relationship between lifestyle factors and microbial community composition (Yatsunenko et al., 2012; Obregon-Tito et al., 2015; Martínez et al., 2015; O'Keefe et al., 2015). Specifically, compared to traditional cultures, the gut microbiomes of humans in industrialized societies show a substantial decrease in alpha diversity, loss of bacterial taxa involved in fiber processing, and an emergence of bacterial lineages associated with consumption of agricultural products. Indeed, it has been hypothesized that specific gut microbiome configurations, likely supported by westernized diets and industrialized lifestyles, play a role in the high incidence of metabolic and colonic disorders observed in some modern human populations (Cho and Blaser, 2012; Myles et al., 2013; Manzel et al.,

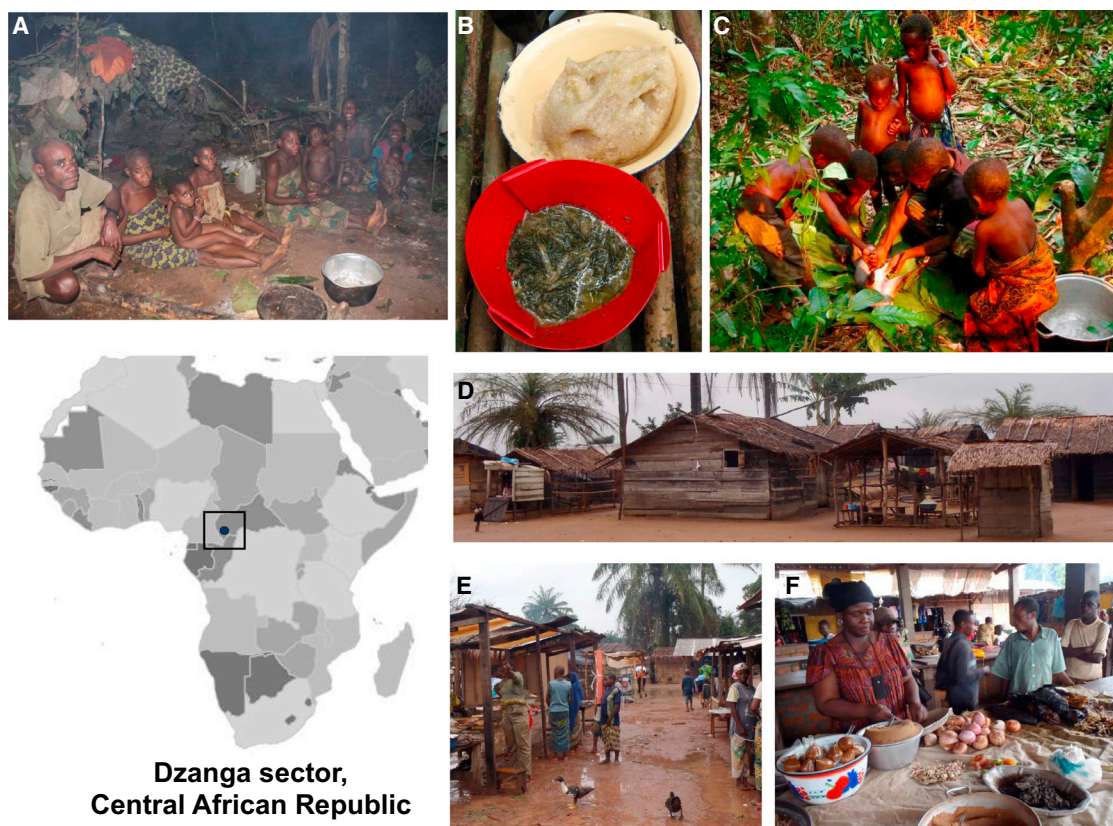


Figure 1. Lifestyle Patterns of the BaAka and Bantu in the Dzanga Sector, Central African Republic

- (A) BaAka family in their traditional village hut.
 (B) Gozo, bitter manioc root (top) and Koko leaves (*Gnetum africanum*) in peanut sauce, two staple foods in the BaAka diet.
 (C) BaAka adult women and children prepare to process a blue duiker (*Philantomba monticola*) after a net hunt.
 (D) Traditional Bantu village.
 (E and F) Bantu market at which agricultural products are sold.

2014; O’Keefe et al., 2015). Thus, cross-cultural comparative microbiome analyses facilitate an understanding of the relative impacts of diverse lifestyles and diets on human adaptation and health.

Previous cross-cultural microbiome studies have compared western and traditional populations; nonetheless, few have analyzed how varying subsistence patterns, such as diet and environment, modulate gut microbiome composition in coexisting, traditional populations, including hunter-gatherers. Moreover, persistent debate has accompanied the question of whether or not foraging lifestyles among hunter-gatherer populations supply adequate nutrition, in the context of a challenging ecology (e.g., food shortage, seasonality, low-energy foods) (Yamauchi et al., 2014; Bailey et al., 1989; Hart and Hart, 1986). As these populations may represent more ancestral subsistence patterns (Cordain et al., 2005), the characterization of microbe-diet interactions in hunter-gatherers and other societies with traditional lifestyles provides potential insight to trace the origins and evolution of modern microbiomes.

To explore how the gut microbiome varies with human adaptation in traditional societies with distinct diets and environment, we explored fecal microbiome composition and its predicted

functional profiles in the BaAka (aka BaAka “Pygmies”) and Bantu, two coexisting human groups in the Dzanga Sangha Protected Areas (DSPA), Central African Republic. The BaAka exhibit hunter-gatherer lifestyles, while their Bantu neighbors engage in more agricultural and western-like subsistence patterns (Figure 1). In addition, to detect how the gut microbiome responds to various subsistence gradients, including those of typically western societies, we integrated data on US Americans, produced by the Human Microbiome Project (Human Microbiome Project Consortium, 2012). Finally, to assess the influence of diet in the BaAka gut, we present data on the fecal metabolome of this hunter-gatherer group. Our results show gradients of traditional subsistence patterns in the two African groups that are reflected in their gut microbiomes.

RESULTS

Comparisons of the BaAka and Bantu Gut Microbiome

Gut microbiome composition was profiled through 16S rRNA gene analysis using 454- pyrosequencing of stool samples from 28 BaAka and 29 Bantu individuals. After quality control, we obtained a total of 504,416 sequence reads, with an average

number of 8,849 reads per sample (range 3,495–15,778). Firmicutes and Bacteroidetes dominated the gut microbiome of both groups. However, the Bantu exhibited a 5:1 Firmicutes: Bacteroidetes ratio, while in the BaAka this ratio was approximately 1:1 ($49.5\% \pm 15.9\%$: $44.4\% \pm 16.9\%$ and $75.5\% \pm 12.4\%$: $15.2\% \pm 9.3\%$ Firmicutes: Bacteroidetes in the BaAka and Bantu, respectively; Wilcoxon rank-sum test $p < 10^{-5}$; [Figure 2A](#)). In addition, BaAka samples showed significantly higher abundances of Cyanobacteria (0.7 ± 0.6 and 0.29 ± 0.32 in BaAka and Bantu, respectively, Wilcoxon rank-sum test, FDR-corrected Q-value = 0.01) and Spirochaetes (1.09 ± 2 in the BaAka versus 0.32 ± 0.65 in the Bantu, FDR-corrected Q-value = 0.04, Wilcoxon rank-sum test). The Bantu also showed higher abundances of Actinobacteria (Wilcoxon rank-sum test, FDR-corrected Q-value = 0.01; [Figure 2A](#)).

Unweighted UniFrac distance ordination (presence/absence of taxa) using taxa clustered at 97% 16S rRNA gene sequence similarity showed that both groups harbor similar gut bacterial communities (PERMANOVA, $R^2 = 0.07$, $p = 0.003$). However, a weighted analysis (based on presence-absence and relative abundance of taxa) shows clear differences between both groups (PERMANOVA, $R^2 = 0.32$, $p < 0.001$) ([Figure 2B](#)). A similar result was obtained with weighted and unweighted Bray-Curtis distances ([Figure S1](#)). A species indicator analysis allowed us to identify taxa driving these differences ([Figure 2C](#)). The BaAka samples were mainly characterized by higher abundance of *Prevotellaceae* (Wilcoxon rank-sum test, FDR $Q = 8.35 \times 10^{-11}$) and *Treponema* (Wilcoxon rank-sum test, FDR $Q = 0.04$), both of which are commonly found in other non-western societies from Africa, Asia, and South America ([Obregon-Tito et al., 2015](#); [Schnorr et al., 2014](#); [Lin et al., 2013](#); [Ou et al., 2013](#)) ([Figure 2C](#)) and whose genomes contain several carbohydrate-degrading modules ([Han et al., 2011](#); [Obregon-Tito et al., 2015](#); [Flint and Bayer, 2008](#)).

Other taxa characteristic of the BaAka over the Bantu were *Sutterella*, *Anaerovibrio*, and unclassified members of the Clostridiaceae and Cyanobacteria (Wilcoxon rank-sum test, FDR $Q < 0.05$; [Figure 2C](#)). 16S rRNA sequences of the three latter taxa matched those of sequences retrieved from the bovine rumen, the gut microbiome of Peruvian hunter-gatherers, and of children from Bangladesh, deposited in NCBI ([Obregon-Tito et al., 2015](#); [Lin et al., 2013](#)). In contrast, the Bantu samples contained a higher abundance of taxa affiliated to the Firmicutes, including Rumino-coccaceae, Mogibacteriaceae, *Faecalibacterium*, *Leuconostoc*, *Lactococcus*, Christenellaceae, and *Dialister*, which are enriched in the gut microbiome of traditional populations reflecting more agricultural economies ([Obregon-Tito et al., 2015](#); [Martínez et al., 2015](#)) (Wilcoxon rank-sum test, FDR $Q < 0.05$; [Figure 2C](#)). Overall, we identified 17 taxa with significantly different abundances between the two populations; the full list is available in [Table S1](#).

Next, we used the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) open-source tool ([Langille et al., 2013](#)) to identify 62 predicted metabolic pathways that differed between the gut microbiomes of the BaAka and Bantu (FDR $Q < 0.05$, Wilcoxon rank-sum test; [Figure S2](#); the full list is shown in [Table S2](#)). Among these, pathways involved in glycan metabolism (e.g., lipopolysaccharide biosynthesis), nucleotides, energy (carbon fixation, sulfur, and

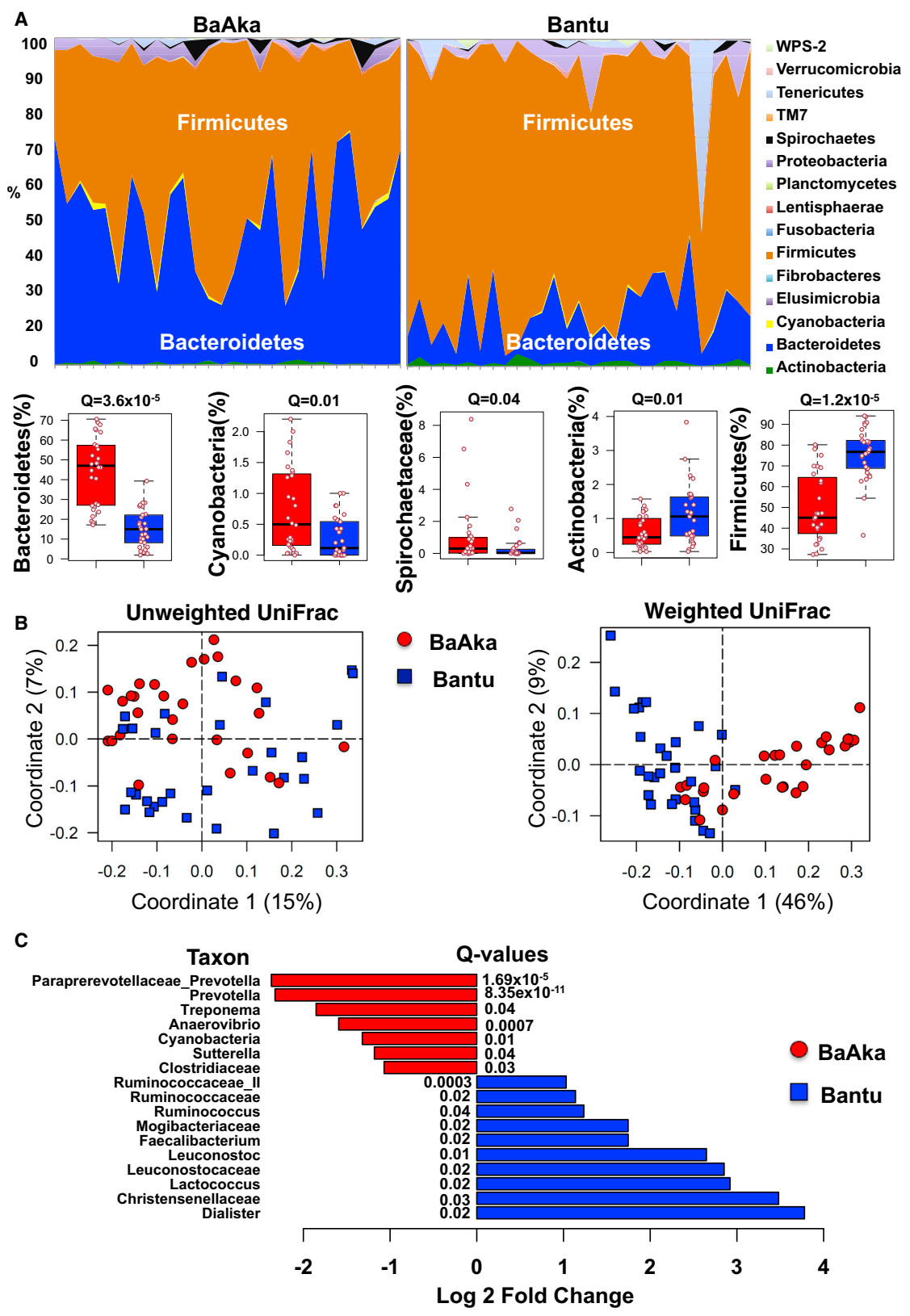
nitrogen), vitamins and cofactors (riboflavin, B6, folate biosynthesis, one carbon pool by folate, nicotinate, and nicotinamide), and the pathogenic cycle of *Vibrio cholerae* were more abundant in the BaAka gut. In contrast, the gut microbiome of the Bantu was characterized by a higher abundance of predicted pathways involved in membrane transport (ABC transporters, phosphotransferase systems, and secretion systems), regulatory signal transduction systems (two-component systems), carbohydrate metabolism (glycolysis and gluconeogenesis, propionate, pentose phosphate and glucuronate interconversions, pyruvate, ascorbate and aldarate, and butanoate), and xenobiotics (degradation of limonene and pinene, nitrotoluene, atrazine, bisphenol, benzoate, chloroalkane, xylene, caprolactam, dioxin, secondary metabolites, and metabolism of xenobiotics by cytochrome P450).

Comparison of the BaAka and Bantu Gut Microbiome with that of Western Populations

To further investigate similarities and differences between the BaAka and Bantu gut microbiomes in the context of traditional and western-like lifestyles, we conducted comparative analyses including the BaAka, Bantu, and US Americans, part of the human microbiome project (HMP). We chose to include the HMP samples because microbiome profiling was conducted using the same sequencing technique (454 pyrosequencing) and targeted the same 16S rRNA variable region (V1-V3). We combined the raw sequencing data from the three studies and analyzed the joint dataset, thus minimizing study-specific confounding effects that could bias our results ([Human Microbiome Project Consortium, 2012](#)).

An unweighted UniFrac distance analysis showed that the gut microbiome of both African groups was equally distant from that of US Americans ([Figures 3A and 3B](#)). However, when taking into account taxa abundance by using a weighted UniFrac metric, the microbiome of the Bantu was more similar to that of US Americans ([Figures 3C and 3D](#)). Indeed, US Americans share more operational taxonomic units (OTUs) with the Bantu than with the BaAka ([Figure S3](#)). Nonetheless, overall, these analyses showed that the gut microbiome of each African group was still more similar to each other than they were to that of US Americans.

Further exploration of the microbiome features distinguishing all groups revealed higher bacterial diversity in both the BaAka and Bantu compared to US Americans (phylogenetic diversity whole tree, Kruskal-Wallis test, FDR $Q < 10^{-4}$), with no differences between the two African groups ([Figure 4A](#)). Additionally, a correspondence analysis based on the abundance of indicator genera characterizing each group (Kruskal-Wallis test, FDR $Q < 0.05$) corroborated the aforementioned discriminant patterns between the BaAka and the Bantu and highlighted the fact that both African groups are largely depleted of *Bacteroides*, *Parabacteroides*, and unclassified Rickenellaceae compared to US Americans ([Figures 4B and 4C](#)). Representative sequences of these taxa were closely related to *Bacteroides vulgatus*, *Parabacteroides distasonis*, and *Allistipes*, respectively. Enrichment of these closely related taxa from the Bacteroidales order seems to provide a common discriminatory pattern between western and traditional societies ([De Filippo et al., 2010](#); [Yatsunenko](#)



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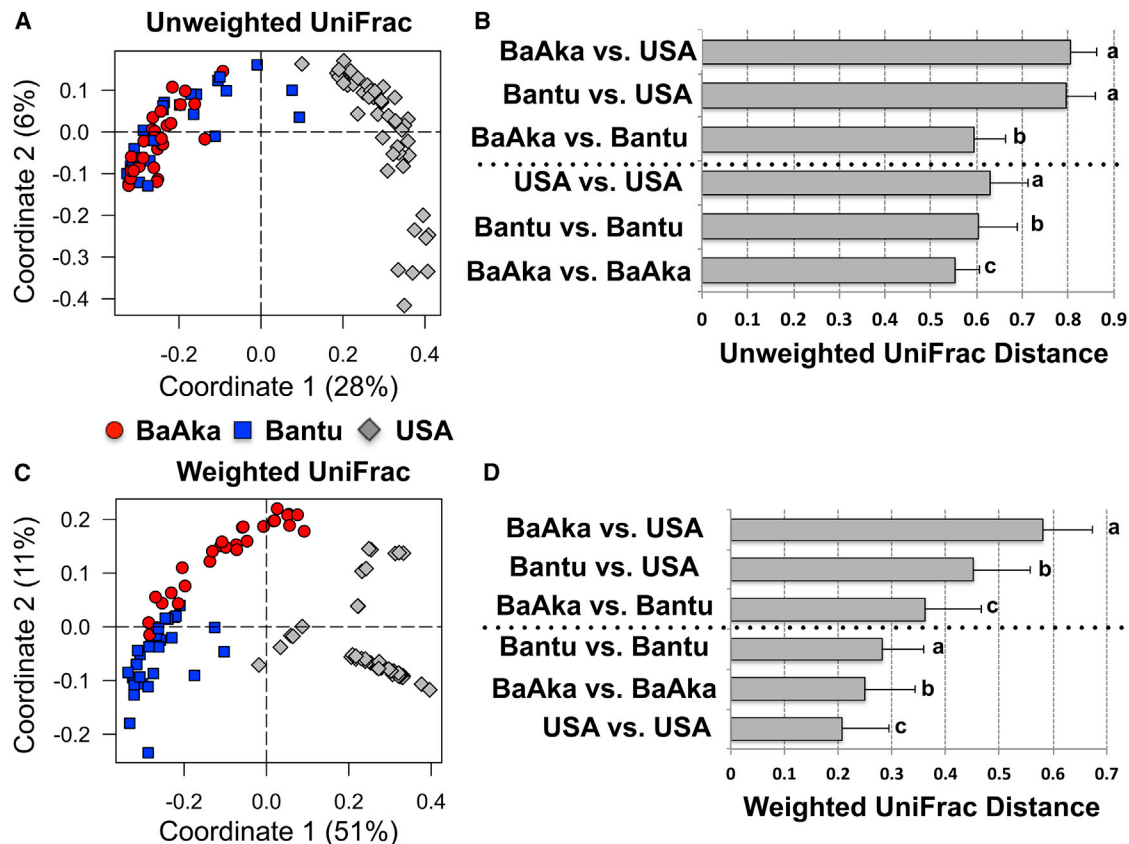


Figure 3. Comparison of the BaAka and Bantu Gut Microbiome Relative to that of US Americans

(A and B) Principal coordinate analyses based on unweighted UniFrac distances (A) and average distance differences among groups (B). Bar plots labeled with different letters (a, b, and c) denote UniFrac distances between and within groups (BaAka, Bantu, and US Americans) are significantly different (Kruskal-Wallis tests, FDR-adjusted $Q < 0.05$).

(C and D) Principal coordinate analyses based on weighted UniFrac distances (C) and average distance differences among groups (D).

See also [Figures S3](#) and [S4](#) and [Table S2](#).

[et al., 2012](#); [Ou et al., 2013](#); [Martínez et al., 2015](#)). Interestingly, OTUs classified as Rickenellaceae and *Bacteroides*, were among the most abundant taxa shared between the Bantu and US Americans ([Figure S3](#)), which would imply that Bantu microbiomes might harbor more western-like patterns, than those of the BaAka do. However, as some species affiliated with the *Bacteroides* lineage possess several known carbohydrate-processing modules for resistant starch and hemicellulose degradation ([Flint et al., 2008](#)), the existence of a typically western microbiome should be validated with more detailed functional approaches.

Additionally, we detected a gradient in the abundance of taxa characterizing the BaAka across populations ([Figure 4C](#)). For instance, *Prevotella*, unclassified Clostridiaceae, and *Treponema* showed the highest abundances in the BaAka and intermediate levels in the Bantu, but were significantly depleted in US Americans ([Figure 4C](#)) (Kruskal-Wallis test, FDR $Q < 0.05$). Likewise, the abundance of some predicted microbiome functions via PICRUST was more similar between the Bantus and US Americans than between the BaAka and US Americans ([Figure S4](#); [Table S2](#)). For instance, an ordination plot based on Bray-Curtis dissimilarities on predicted modules (enzymes) shows

Figure 2. Gut Microbiome Composition of the BaAka and Bantu

(A) Relative abundance of taxa at phyla level shows sharp shifts in the Firmicutes: Bacteroidetes ratios between the two groups. The boxplots show specific differences in the relative abundance of discriminant phyla between the BaAka and Bantu gut microbiomes (indicator values > 0.5 and Wilcoxon rank-sum tests, FDR-adjusted $Q < 0.05$; [Table S1](#)).

(B) Principal coordinate analyses based on unweighted (left) and weighted (right) UniFrac distances between gut bacterial communities (relative abundance of taxa clustered at 97% 16S rRNA sequence similarity) of the BaAka and Bantu.

(C) Fold changes of the main discriminant genera between the two groups (indicator value > 0.5 , Wilcoxon rank-sum tests, FDR-adjusted $Q < 0.05$; [Table S1](#)). Family taxa names represent those of unclassified genera.

See also [Figures S1](#) and [S2](#) and [Table S1](#).

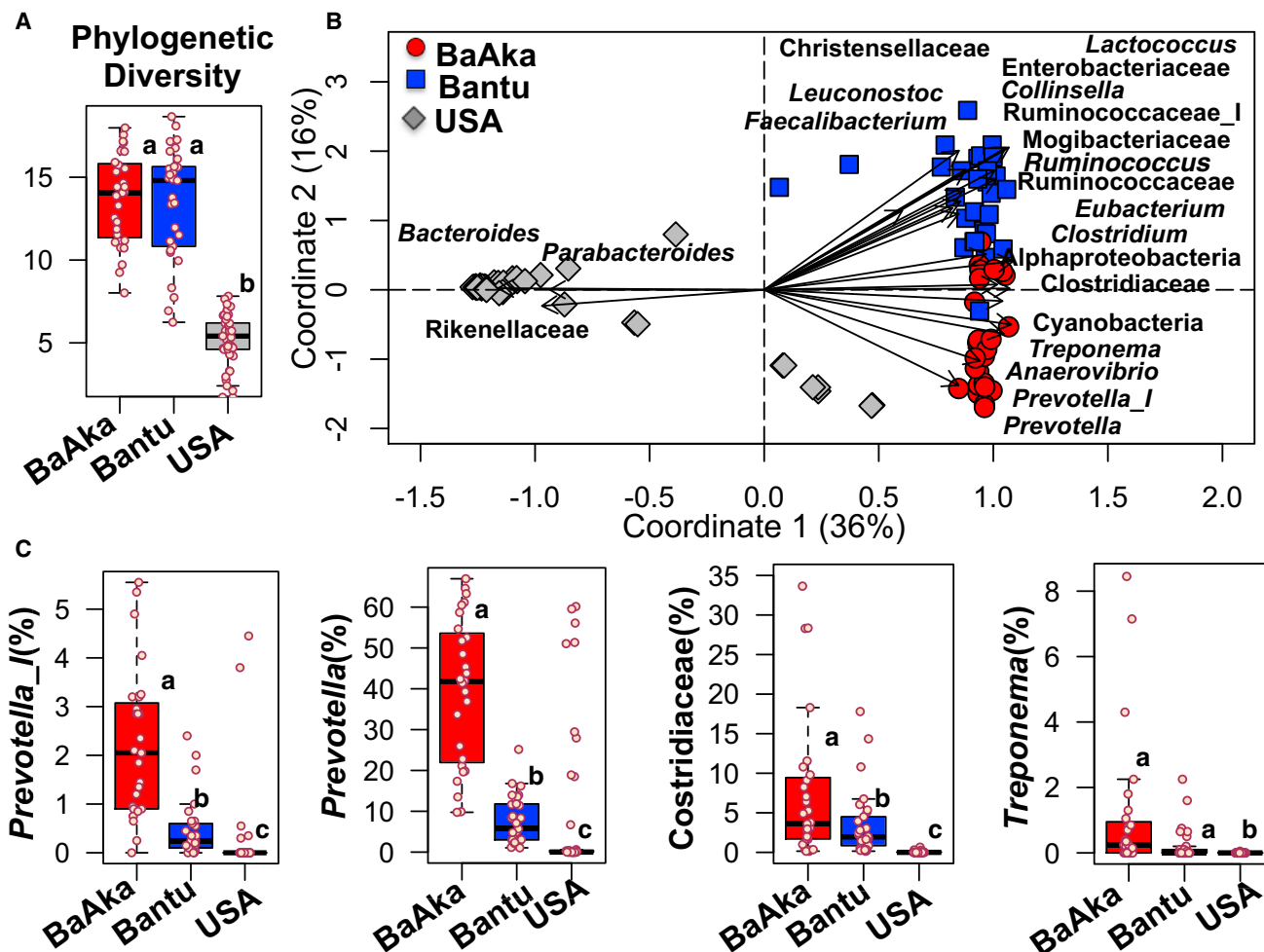


Figure 4. Features Characterizing the Gut Microbiome of the BaAka, Bantu, and US Americans

(A) Phylogenetic diversity comparisons.

(B) Correspondence analysis (CA) showing indicator genera driving gut microbiome differences between the three groups. The distance between vectors (arrows) and the symbols (circles, squares, and diamonds) that represent each taxon gives an estimate of the taxon's relative abundance in a given sample.

(C) Boxplots showing differences in the relative abundance of some indicator genera driving gut microbiome differences among the BaAka, Bantu, and US Americans. Boxplots labeled with different letters (a, b, and c) denote taxa abundances are significantly different between groups (Kruskal-Wallis tests, FDR-adjusted $Q < 0.05$). Family taxa names represent those of unclassified genera.

See also [Figures S3](#) and [S4](#).

clustering between these two groups along the second principal coordinate (Figure S4A). Likewise, predicted gene pathways involved in carbohydrate metabolism, such as glycolysis and gluconeogenesis, pentose and glucuronate interconversions, pyruvate metabolism, and the pentose phosphate pathway were significantly depleted in the BaAka, whereas they were enriched in the Bantu and US Americans (Kruskal-Wallis test, FDR $Q < 0.05$). A similar pattern was found with the abundance of genes involved in the bisphenol degradation pathway and in histidine and methane metabolism (Figure S4B).

The BaAka Gut Metabolome and Metabolite-Microbiome Networks

Of the 28 BaAka samples analyzed, 20 yielded usable gas chromatography/mass spectrometry (GC/MS) spectra. Anal-

ysis of polar and non-polar fractions detected a total of ~2,500 mass spectral data, of which 260 compounds were positively identified. After eliminating metabolites found in less than 50% of samples, the stool metabolomes of the BaAka were mainly grouped into lipids (40.8% of all metabolites), carbohydrates (19%), sterols (18.9%), phosphates (8%), organic acids (7.9%), amino acids and amines (3.3%), and bile acids (1.7%) (Figure 5A). Coprostan-3-ol, the main conversion product from cholesterol metabolism in the distal colon (Sekimoto et al., 1983), was the most abundant metabolite ($15.6\% \pm 10.6\%$), followed by glycerol ($12.7\% \pm 11.74\%$), stearic acid ($11.9\% \pm 8.7\%$), palmitic acid ($10\% \pm 6.5\%$), phosphoric acid ($8\% \pm 8.5\%$), and lactic acid ($4.5\% \pm 5\%$). Other metabolites comprised less than 2% of abundance and were mostly long chain fatty acids, cholesterol, secondary

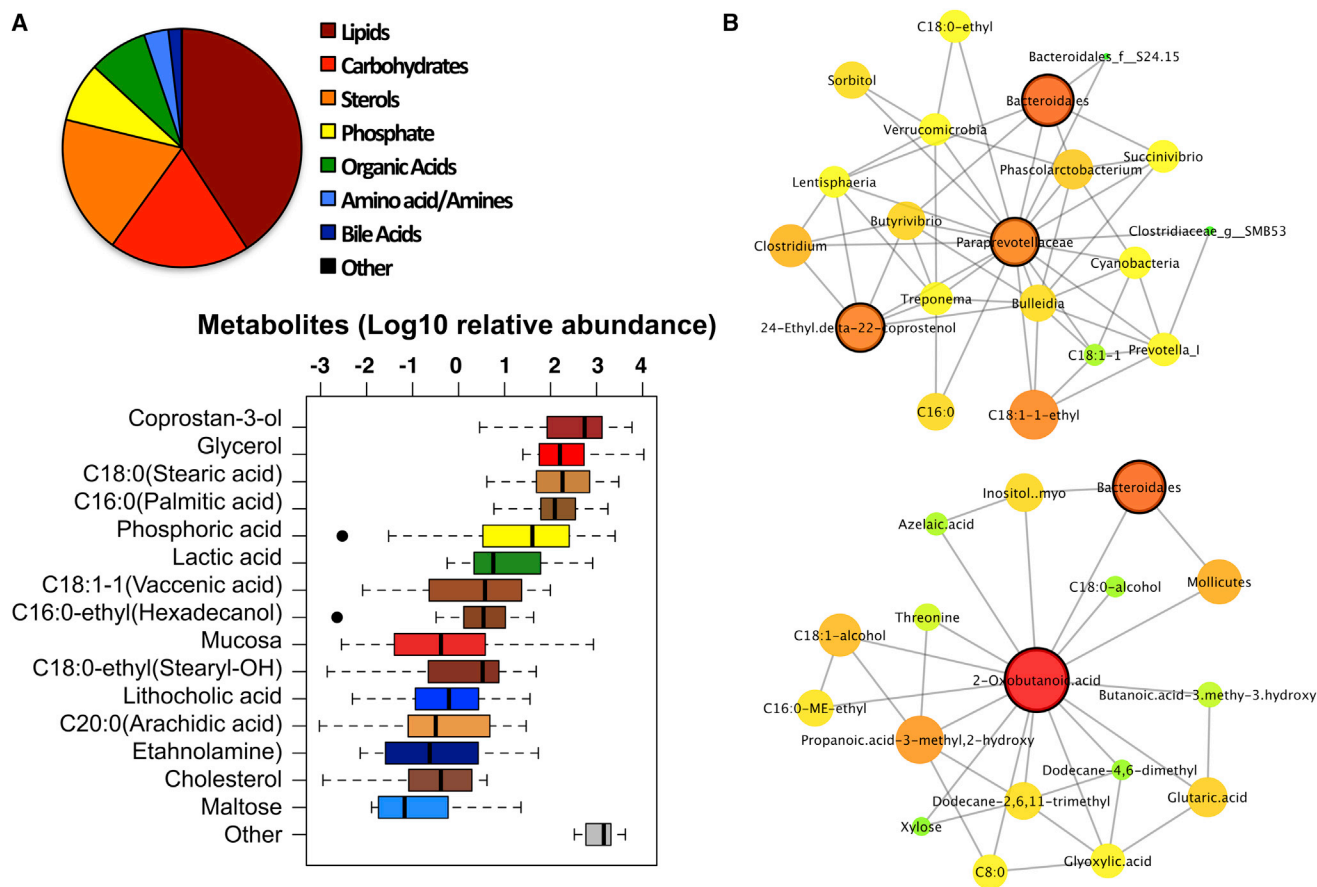


Figure 5. Gut Metabolome Composition in the BaAka and Metabolite-Microbiome Interaction Networks

(A) Relative abundance of metabolites according to broad classifications (upper) and dominant metabolites (lower).

(B) Sub-network view of interactions between bacterial taxa (at the genus level) and metabolites (nodes). Edges represent Spearman correlation coefficients ($0.5 < \rho < 0.94$). Node size is proportional to the betweenness centrality of the node, which is an indirect measure of functional relevance of a node in a network and its capacity to hold together communicating nodes. Nodes with the highest betweenness centrality values are also highlighted with increasing red tones. The two networks highlight the nodes with the highest betweenness centrality (Paraprevotellaceae and 2-oxobutanoic acid) and their direct interactions with other taxa and metabolites. The full network is described in Figure S5, and network statistics are available in Table S3. Family and order taxa names represent those of unclassified genera.

See also Figure S5 and Table S3.

bile acids (lithocholic acid, deoxycholic acid, and hydrocholestone), and plant phenolics (for example, benzoic acid and benzene-acetic acid).

A network analysis based on Spearman correlation coefficients ($\rho > 0.5$) between genus-level bacterial taxa and metabolites was used to highlight metabolites or taxa with functional relevance in the BaAka gut. Stress centrality is an indirect measure of regulatory relevance of a node within a network; for instance, 2-oxobutanoic acid, a compound involved in the metabolism of many amino acids (glycine, methionine, valine, leucine, serine, threonine, and isoleucine), and unclassified Bacteroidales were the nodes with the highest stress in the network (Figures 5B and S5; network statistics are available in Table S3). Other nodes with high stress were represented by 24-ethyl-delta-22-coprostenol (a metabolite derived from cholesterol metabolism) and unclassified Paraprevotellaceae (related to sequences retrieved from the gut of children from Bangladesh

(Lin et al., 2013; ntblast, ID sequence similarity $>97\%$, and 100% coverage).

DISCUSSION

Here, we present data demonstrating that the distal gut microbiome of the BaAka hunter-gatherers (aka BaAka pygmies) and Bantu, two African groups who coexist in Central Africa, harbor unique compositional and functional features that likely reflect adaptations to different lifestyles. Through comparisons with US Americans, the data corroborate group-specific microbiome composition and reflect a gradient in subsistence patterns from more traditional to more western (or industrialized) in these African populations. We also propose that specific features of gut microbiome composition in the BaAka reflect adaptations to evolutionary and ecological constraints, associated with both the hunter-gatherer lifestyle and BaAka-specific phenotypes.

The Gut Microbiome of African Groups Reflects Particular Subsistence Patterns

The microbiome patterns detected in the BaAka are consistent with those recently reported in other hunter-gatherer cohorts, such as the Matsés from Peru and the Hadza from Tanzania (Schnorr et al., 2014; Obregon-Tito et al., 2015). It is likely that particular microbiome patterns in the BaAka and other hunter-gatherers arise in response to high consumption of fibrous tubers, such as bitter manioc root and other highly fibrous starches, such as wild yams (Figure S1) (*Discorea* spp.) (Remis and Jost Robinson, 2014; Dounias, 2001; Schnorr et al., 2014)

The BaAka consume high quantities of *Gnetum africanum* (koko) leaves (Remis and Jost Robinson, 2014; Yamauchi et al., 2000) (Figure 1). This resource is high in fiber and tannins (Isong et al., 1999), which could explain the enrichment of other potentially fibrolytic taxa in their microbiome. For instance, *Prevotella*, the most dominant taxon in the BaAka gut microbiome, is an established polysaccharide degrading bacterium that inhabits the bovine rumen. Members of the genus *Prevotella* can exhibit important β -D-xylanase and β -D-xylosidase activities (Gasparic et al., 1995), suggesting a high influx of substrates with high content of hemicellulose to the colonic ecosystem of the BaAka.

Notably, predicted gene pathways involved in the metabolism of cofactors and vitamins showed higher abundance in the BaAka microbiome compared to that of the Bantu. Despite reports of possible colonic mechanisms for absorption of vitamins (for example, folate) (Dudeja et al., 2001), it is likely that the contribution of gut bacteria to host vitamin landscape is negligible (Hume, 1997), and other dietary sources, such as meat, provide a much greater contribution. Thus, increased vitamin and cofactor metabolism in the BaAka gut microbiome may support increased metabolic (enzymatic) activity for microbial cell growth, amino acid metabolism (e.g., one carbon pool by folate), or even fiber processing.

It is interesting that taxa, such as *Prevotella*, *Treponema*, unclassified Clostridiaceae, and Cyanobacteria, are also present in the Bantu, but at lower abundances compared to the BaAka. One plausible explanation is that the Bantu consume less fibrous tubers, leaves, and other plant structural materials compared with the BaAka. Thus, the patterns observed in the gut microbiome of the Bantu may reflect more recent transitions from traditional to modern, agricultural, or western-like lifestyles, resulting in a gradual decline in the abundance of traditional microbiome features, such as *Prevotella* and *Treponema*. Furthermore, enrichment and coabundance of *Faecalibacterium* and lactic acid bacteria (*Leuconostoc*, *Lactococcus*) in the Bantu may reflect higher availability of digestible sugars in their colonic ecosystem, associated with active transition to western diets. This observation is also consistent with the higher abundance of predicted gene pathways involved in methane metabolism, transporters, signal transduction mechanisms, and an increased Firmicutes:Bacteroidetes ratio in the Bantu. These traits have been persistently found in humans and mice with increased capacity to harvest dietary energy (Ley et al., 2006; Turnbaugh et al., 2006). Moreover, a high abundance of predicted metabolic pathways involved in carbohydrate metabolism (e.g., pyruvate, pentose, and glycolysis pathways) in US Americans and Bantu compared to the BaAka is also consistent with the Bantu

microbial ecosystem being influenced by transitions to more western-like diets. However, more detailed data on nutritional differences between both groups is needed to confirm these patterns.

In this regard, the increased abundance of predicted gene pathways involved in xenobiotic degradation in the Bantu is noteworthy. For instance, Bantu individuals exhibited higher abundances of predicted pathways involved in the degradation of bisphenol (a common food additive), similar to those seen in US Americans, and higher compared with the BaAka. These observations are consistent with the impact the metabolism of drugs (including antibiotics) and dietary additives has on the gut microbiome (Haider and Turnbaugh, 2013; Sousa et al., 2008) and may support the fact that the Bantu have easier access to therapeutic drugs, a signature of industrialized lifestyles. In fact, although there are slight differences in predicted gene pathways involved in beta-lactamase resistance between the BaAka and Bantu ($p = 0.06$), the occurrence of this predicted pathway in the Bantu is higher and similar to that seen in US Americans.

Despite convergence of some microbiome features between the Bantu and those of US Americans, the gut microbiomes of the BaAka and Bantu are more similar to each other than to that of westerners. These data support previous observations of a loss of microbial diversity in industrialized human populations compared with traditional groups (Moeller et al., 2014; Yatsunenkov et al., 2012). The reasons for these distinct gut microbiome differences between traditional and westernized populations have been mainly attributed to lifestyles factors (Cho and Blaser, 2012). However, gut microbiome differences may also be shaped by distinct genetic backgrounds between the populations, as has been reported recently (Goodrich et al., 2014; Blekhan et al., 2015). Finally, although our western versus non-western microbiome analyses coincide with the aforementioned patterns, these cross-study comparisons (Bantu and BaAka versus US Americans) should be taken with care, as differences in sample collection and sequencing protocols may introduce potential bias.

Evolutionary and Ecological Adaptations Reflected in the BaAka Gut Microbiome

Lipid-derived metabolites, such as coprostanol and fatty acids, were predominant in the colonic ecosystem of the BaAka, possibly reflecting a tendency to incorporate in their diet significant amounts of animal prey or even lipid-rich nuts, such as peanuts and *Irvingia* nuts (Remis and Jost Robinson, 2014). Consumption of meat and nuts by the BaAka could also reflect the prevalence of amino-acid-derived metabolites (e.g., 2-oxobutanoic acid, propanoic acid 3-methyl, 2-hydroxy, and butanoic acid 2-hydroxy). Increased amino acid turnover and secondary bile acids in the colon are often associated with colonic inflammation (Louis et al., 2014; Windey et al., 2012; Carbonero et al., 2012). Thus, it is intriguing that hunter-gatherer populations incorporate significant amounts of lipids and protein in their diets, without showing the detrimental metabolic patterns characterizing western societies (Cordain et al., 2002; Ulijaszek and Lofink, 2006). A possible explanation is that the BaAka exploit polyunsaturated and monounsaturated fats from wild game

and fish, as opposed to the predominantly saturated fats found in domestic cattle (Cordain et al., 2005). Also, diets rich in fiber and polyphenolics are reported to have hypolipidemic and antioxidant effects (Zhang et al., 2013). However, an effect of lipid-rich diets in health status of the BaAka has never been assessed and, as we lack metabolomic data on the Bantu or US Americans, it is unclear whether this lipid-rich metabolome is a generalized trait in the BaAka or other hunter-gatherers.

These metabolome-microbiome markers also motivate questions regarding energy storage and expenditure patterns in the BaAka, particularly in the context of the pygmy phenotype (short stature). Metabolomes high in long-chain fatty acids and cholesterol-derived markers have been observed in mice and humans consuming high-fat and high-energy diets, with significant contributions to energy budgets mediated by gut microbes (Daniel et al., 2014). Although energy intake has been suggested to be low in the wider BaAka community (Yamauchi et al., 2000), due to limited food access (Perry et al., 2014; Hewlett, 2014), our microbiome-metabolome data suggest that the BaAka may not be energetically deficient. Thus, a question that warrants further exploration is whether the BaAka gut microbiomes facilitate energy harvest from plant matter and animal consumption to compensate for the energetic constraints reflected in their phenotype.

The BaAka microbiomes had a high abundance of several predicted gene pathways involved in pathogenicity. For instance, predicted *luxS*, S-ribosyl homocysteine lyase, involved in the pathogenic cycle of *V. cholerae* (Vendeville et al., 2005), showed higher abundance in the BaAka compared with the Bantu (Wilcoxon rank-sum test, $p = 4.954 \times 10^{-8}$). Also, *Sutterella*, a taxon associated with gastrointestinal distress (Mukhopadhyaya et al., 2011), was more abundant in the hunter-gatherers, consistent with high pathogen loads in the gastrointestinal tract of pygmies (Migliano et al., 2007; Jarvis et al., 2012). In this regard, the BaAka population studied herein suffers from multiple infections of pathogenic gastrointestinal parasites, such as *Trichuris trichiura*, *Necator* spp., *Ascaris lumbricoides*, and *Strongyloides* spp. (Hasegawa et al., 2014). This is noteworthy in the context of recent reports indicating that parasitism is correlated with the composition of the gut microbiome of rural African populations (Morton et al., 2015). In addition, evolutionary adaptations in genes causing negative regulation of cytokine signaling in the BaAka, potentially increasing susceptibility to infection, have been previously reported (Jarvis et al., 2012). Further research is needed to determine whether prevalence of infection in the BaAka is related to their unique environment and diet or perhaps represents long-term, coevolutionary host-microbe interactions, resulting in more tolerant immune mechanisms (Van den Abbeele et al., 2011). Thus, a more robust molecular screening of potential pathogens and virulence-associated genes, along with simultaneous characterization of host genes associated with immune responses, would help clarify this issue.

In addition, the existence of the patterns found here should be evaluated over a wider sample, including multiple hunter-gatherer and traditional populations experiencing dietary fluctuations and varying degrees of transition to agriculture and industrialization. Importantly, this approach will be key to dissecting the specific time points in which traditional microbiome features,

such as *Prevotella* and *Treponema*, were lost, and western patterns, such as high abundances of *Bacteroides*-like taxa, emerged. Along these lines, the observation that *Prevotella* and *Treponema* were detected in coprolites from extinct humans in northern Mexico from 1,400 years BP (Tito et al., 2012), and are also enriched in wild apes (Gomez et al., 2015, 2016), raises questions as to the role of these taxa in the evolution of the human microbiome.

Studying the gut microbiome of hunter-gatherers and traditional human populations constitutes an opportunity to trace “ancestral” microbiome traits and to unravel the influence of the gut microbiome in human evolution. These analyses should include efforts to reconstruct the vast uncharacterized diversity in the microbiome of these human populations, using culture-dependent and genome reconstruction approaches (Obregon-Tito et al., 2015), and comparative analyses that include nonhuman primates (Moeller et al., 2014). Likewise, detailed characterization of the molecular interactions between dietary components, the gut microbiome, and host genetic variation across traditional and industrialized populations has the potential to shed light on the forces shaping the gut microbiome, in the context of human evolution and health.

EXPERIMENTAL PROCEDURES

Study Site, Subjects, and Sample Collection

Stool samples were collected from 28 BaAka and 29 Bantu inhabiting Dzanga Sangha Protected Areas (DSPA), Central African Republic. The study subjects included personnel hired as gorilla trackers (BaAka) hired by the Primate Habituation Program (PHP) and park ecoguards hired by DSPA. Samples from the BaAka also included seven female partners. The BaAka males alternated periods of time between the research camps, tracking the gorillas in the Park and their villages. Both trackers and their partners frequently did either day hunts from the villages or longer hunting trips within the Special Reserve.

The samples were collected from June to August of 2010 and 2011 upon consent from all individuals and also as part of an effort to characterize their parasite loads. Approximately 1 g of feces was collected in 2-ml Eppendorf tubes containing RNAlater (Invitrogen, Life Technologies) for microbiome analysis. For metabolomic analyses, approximately 1 g of feces was collected in a separate tube containing ethanol (95%). Both sets of samples were kept at room temperature for a maximum of 1 month before transport to the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, where they were kept at -20°C , until they were shipped to the University of Illinois at Urbana-Champaign, where DNA was extracted.

All work was approved according to the rules and regulations from the Ministre de l'Education Nationale, de l'Alphabetisation, de l'Enseignement Supérieur, and de la Recherche (Central African Republic), as well as the Institutional review board for the protection of human subjects from the University of Illinois at Urbana-Champaign (permit number 13045, September 4, 2014).

Sample Processing and DNA Analyses

DNA was extracted from stool samples using the MoBio Ultraclean Soil Kit (MoBio Laboratories). The V1-V3 region of the 16S rRNA gene was PCR amplified (20 cycles at 94°C for 30 s, at 48°C for 30 s, and at 72°C for 2 min) using primers 27f (5'-AGAGTTTGATYMTGGCTCAG-3'), corresponding to nucleotides 8–27 of the *Escherichia coli* 16S rRNA gene) and 534r (5'-ATACCGCGCTGCTGGCA-3'), tagged with identifying barcodes, MID tag 1–50). The amplicons were multiplexed and pyrosequenced using 454 FLX-Titanium technology at the J. Craig Venter Institute (Rockville). After removing low-quality sequences ($< Q30$, sequences shorter than 250 nt, sequences with homopolymers longer than 6 nt, and sequences containing ambiguous base calls or incorrect primer sequences) with custom perl scripts, reads were processed using the online tool QIIME, and operational taxonomic units (OTUs)

(97% 16S rRNA sequence identity) were picked using the closed reference OTU picking script against the Greengenes database (v.13_5) (Caporaso et al., 2010). This pipeline was also used to calculate phylogenetic diversity and UniFrac distances. 16S rRNA sequences from US Americans, part of the HMP, correspond to the V1-V3 16SrRNA variable region, also obtained through 454 pyrosequencing. The closed reference OTU table obtained was also used to predict functionality based on 16S rRNA data abundances, using the online open source tool PICRUSt (Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013). Predicted data were collapsed at different KEGG (Kyoto Encyclopedia of Genes and Genomes) hierarchical levels, including pathways and modules.

Metabolomic Analyses

Extraction for polar and non-polar metabolites was performed separately. Metabolites were extracted with 1 ml of 70% methanol and sonication in QSonica Microson XL2000 Ultrasonic Homogenizer (QSonica). Lysed cell pellets were subsequently fractionated at room temperature with 5 ml of 70% methanol and chloroform, accompanied by centrifugation (10 min at maximum speed). 1 ml of each extract was evaporated under vacuum at -60°C , and then dried extracts were derivatized. The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (NIST), W8N08 (Palisade Corporation), and a custom-built library of 520 unique metabolites. All known artificial peaks were identified and removed. To allow comparison between samples, all data were normalized to the internal standard in each chromatogram and the sample dry weight (DW). The spectra of all chromatogram peaks were evaluated using the AMDIS 2.71 (NIST) program. Metabolite concentrations are reported as “(analyte concentration relative to hentriacontanoic acid) per gram DW” (relative concentration), e.g., as target compound peak area, divided by the internal standard (IS) peak area (IS concentration is the same in all samples): $N_i = X_i \times X^{-1}_{\text{IS}} \times g \text{ wet w.}$

Statistical Analyses

All multivariate and community analyses were conducted using the *ca*, *vegan*, and *labdsv* packages of R (R Core Team, 2014) on the relative abundance of each taxon (Nenadic and Greenacre, 2007; Oksanen et al., 2012; Roberts, 2012). Indicator species analysis (Dufrene and Legendre, 1997) characterized the most prominent taxa and pathways in the gut microbiome of each group. Briefly, the indicator species analyses selects the most representative features in each cluster or group and splits these features into as many clusters being compared. These features are chosen by assigning an indicator value to each feature (taxon or pathway). This indicator value is the product of the relative average abundance and relative frequency of that feature in a group. Thus, indicator values closer to one (1.0) indicate that a feature has high abundances within a group, compared to others, and that it is present in most or all of its members. Significance of the indicator value is assessed through permutation tests (probability) (Roberts, 2012). Here, we selected indicator values >0.5 for discriminant taxa and >0.3 for discriminant pathways. Kruskal-Wallis tests adjusted for multiple comparisons were used to confirm significance of all discriminant features, using the *pgirmess* packages of R (Revelle, 2014; Giraudoux, 2014). The CCREPE (Compositionally Corrected by Renormalization and Permutation) package of R (Schwager et al., 2016; <https://bitbucket.org/biobakery/biobakery/wiki/ccrepe>) was used to assess the existence of true correlations between microbiome and metabolome data, avoiding spurious relationships due to sparsity, non-independence and non-normal distribution of compositional data. Network analyses were carried out with Cytoscape (Shannon et al., 2003). GC/MS spectra data from metabolomic analyses were transformed as follows: (1) metabolites with $>50\%$ of missing data were removed from the set and (2) the relative abundance of a given metabolite was expressed relative to the sum of all the spectra obtained for a given sample.

ACCESSION NUMBERS

The accession number for the BaAka and Bantu gut microbiomes reported in this paper is MG-RAST:16608.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.02.013>.

AUTHOR CONTRIBUTIONS

Conceptualization, A.G., K.J.P., B.A. White, S.R.L., and R.B.; Methodology, A.G.; Software, C.J.Y.; Formal Analysis, A.G.; Investigation, A.G., K.J.P., K.V., M.G.T., and J.D.U.; Resources, K.J.P., D.M., A.T., M.G.T., M.B.B., K.E.N., F.C., H.R.G., B.A. Wilson, S.R.L., B.A. White, and R.B.; Writing – Original Draft, A.G.; Writing – Review & Editing, A.G., M.B.B., K.J.P., C.J.Y., K.R.A., D.M., A.T., C.A.J.R., M.J.R., K.E.N., H.R.G., B.A. Wilson, E.M., R.M.S., B.A. White, S.R.L., and R.B.; Visualization, A.G. and M.B.B.; Supervision, A.G., B.A. White, S.R.L., and R.B.; Project Administration, K.J.P., D.M., A.G., S.R.L., B.A. White, and R.B.; Funding Acquisition, K.J.P., D.M., K.E.N., B.A. Wilson, B.A. White, S.R.L., and R.B.

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