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Nutritional contribution of plant foods to human diet in evolution

Schnorr, S.L.

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Author: Schnorr, Stephanie

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CHAPTER 4

GUT MICROBIOME OF THE HADZA HUNTER-GATHERERS



Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, Turrone S, Biagi E, Peano C, Severgnini M, Fiori J, Gotti R, De Bellis G, Luiselli D, Brigidi P, Mabulla A, Marlowe F, Henry AG, Crittenden AN.

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ABSTRACT 3

Human gut microbiota directly influences health and provides an extra means of adaptive potential to different lifestyles. To explore variation in gut microbiota and to understand how these bacteria may have co-evolved with humans, here we present the first investigation of phylogenetic diversity and metabolite production of the gut microbiota from a community of human hunter-gatherers, the Hadza of Tanzania. We show that the Hadza have higher levels of microbial richness and biodiversity than Italian urban controls. Further comparisons with two rural farming African groups illustrate other features unique to Hadza that can be linked to a foraging lifestyle. These include absence of *Bifidobacterium* and differences in microbial composition between the sexes that probably reflect sexual division of labor. Furthermore, enrichment in *Prevotella*, *Treponema*, and unclassified *Bacteroidetes*, as well as a peculiar arrangement of *Clostridiales* taxa, may enhance the Hadza's ability to digest and extract valuable nutrition from fibrous plant foods.

4.1 Introduction

The human gut microbiota (GM) is vital for host nutrition, metabolism, pathogen resistance, and immune function (Nicholson et al., 2012), and varies with diet, lifestyle, and environment (De Filippo et al., 2010; Kau et al., 2011; Wu et al., 2011). Together, the host and microbiome have been termed a “supra-organism” whose combined activities represent both a shared target for natural selection and a driver of adaptive responses (Turnbaugh et al., 2007). By studying GM variation across human populations, we are able to explore the limits of our genetic and metabolic potential, and the extent to which GM-host co-evolution is responsible for our physiological flexibility and environmental adaptation (Hehemann et al., 2010; Candela et al., 2012; Rinke et al., 2013).

Comparative studies between unindustrialized rural communities from Africa and South America and industrialized Western communities from Europe and North America have revealed specific GM adaptations to their respective lifestyles. These adaptations include higher biodiversity and enrichment of Bacteroidetes and Actinobacteria in rural communities, and an overall reduction in microbial diversity and stability in Western populations (De Filippo et al., 2010; Yatsunenko et al., 2012). Unindustrialized, small-scale, rural societies are targets for understanding trends in human-GM interactions because they rely less on antibiotics and sterile cleaners, and often consume a greater breadth of unrefined seasonally available foods (Ehlers and Kaufmann, 2010). Yet despite recent focus on rural societies, there remains a significant gap in our knowledge of the microbe-host relationship among hunter-gatherer populations. This is especially problematic because humans have relied on hunting and gathering for 95% of our evolutionary history.

Here, to explore for the first time how a foraging subsistence strategy influences GM profiles, we analyze fecal microbiota from 27 Hadza hunter-gatherers from two separate camp sites (Figure 1).

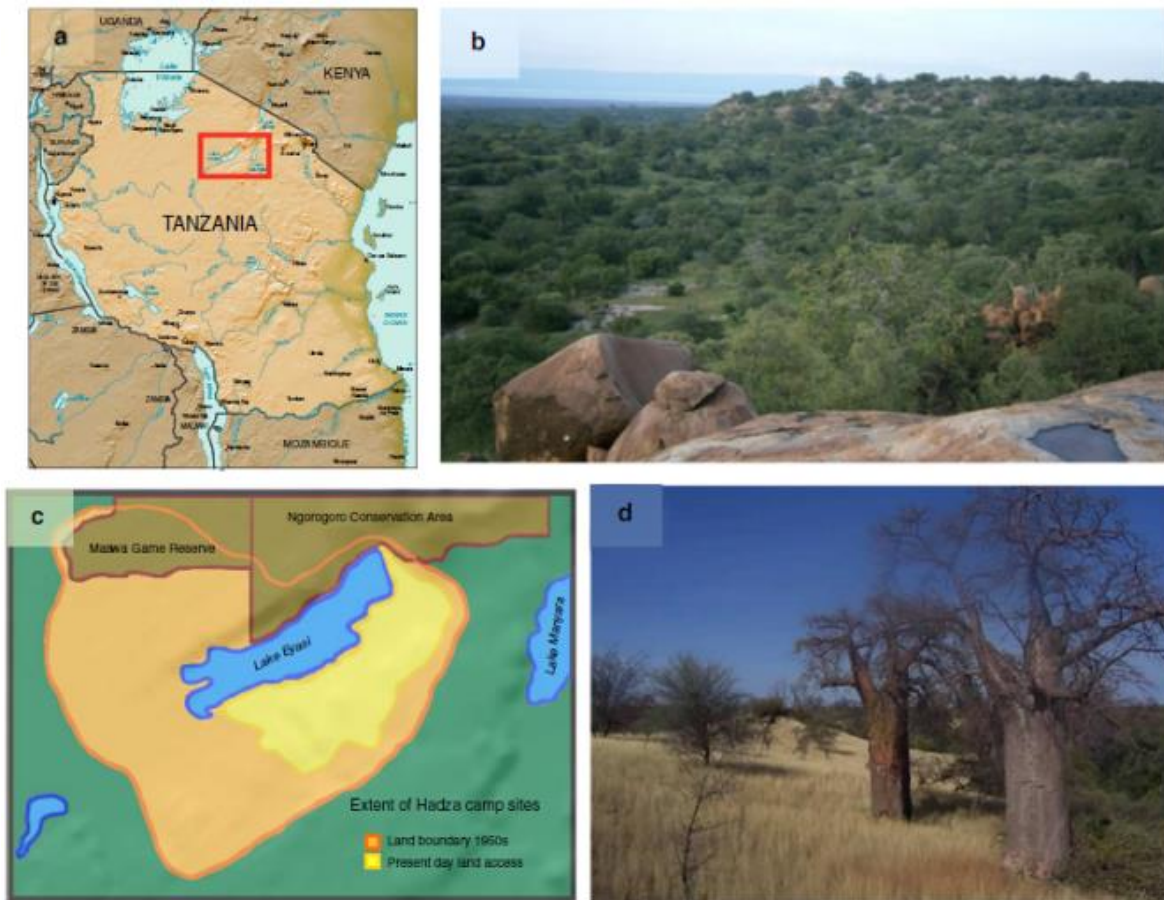


Fig 1. Location and scenery of Hadza land in Tanzania, Africa.

In deep bush camps, hunting and gathering still make up the majority (>90%) of subsistence. **(a)** Location of Hadza land in northern Tanzania; **(b)** top of a rock ridge near Sengele camp overlooking a lush landscape in between two phases of the rainy season; **(c)** extent of the land surrounding Lake Eyasi where Hadza make their campsites, orange line and shading denotes land area in 1950s, and yellow line and shading shows the reduced area Hadza occupy today; **(d)** view of baobab trees within Hadza land during the early dry season. Photos **b** and **d** by S. L. Schnorr and A. N. Crittenden.

The Hadza who chose to participate in this study came from the Dedauko and Sengele camps, situated in the Rift Valley ecosystem around the shores of Lake Eyasi in northwestern Tanzania. These participants are part of the approximately 200-300 traditionally living Hadza, who are one of the last remaining hunting and gathering communities in the world. The Hadza live in small mobile camps with fluid membership, usually comprising a core group of approximately 30 people and target native wild foods, both hunted and foraged, for the bulk of their subsistence (Blurton Jones et al., 1992). While the Hadza are a modern human population, they live in a key geographic region for studies of human evolution and target resources similar to those exploited by our hominin ancestors. The Hadza lifestyle therefore is thought to most closely resemble that of Paleolithic humans.

Phylogenetic diversity, taxonomic relative abundance, and the short chain fatty acid profile of the Hadza microbiome are compared to those of 16 urban living Italian adults from Bologna, Italy. We then compare these data with previously published data on two different rural African groups from

Burkina Faso and Malawi (De Filippo et al., 2010; Yatsunenکو et al., 2012) to identify GM features unique to the Hadza lifestyle. This study presents the first characterization of any forager GM through work with the Hadza hunter-gatherers, and will allow us to understand how the human microbiota aligns with a foraging lifestyle, one in which all human ancestors participated prior to the Neolithic transition.

4.2 Methods

4.2.1 Subject enrollment

The 27 Hadza volunteers who participated in this study came from the Dedauko and Sengele camps and are part of the approximately 200-300 traditionally living Hadza. Fecal samples were collected over a period of two weeks in January 2013 from consenting healthy participants. All participants were first told of the study, its objectives, and their role as volunteers. Since Hadza are non-literate, verbal consent was obtained by those who agreed to participate, and this was documented by a separate witness. In the case of young Hadza, we obtained verbal assent from the youths and verbal consent from the parents, and again documented by a separate witness. Samples were matched with subject interviews to record age, sex, and health status; but because of ambiguity with regards to age of some of the participants, this information was excluded from further analysis. All work was approved by the University of Leipzig Ethik-Kommission review board on May 29, 2012, reference number 164-12-21052012. Permission for this work was granted from the Tanzanian Commission for Science and Technology (COSTECH), permit No. 2012-315-NA-2000-80.

Sixteen Italian adults (age: 20-40 years) were recruited for this study in the greater Bologna metropolitan area. All subjects were healthy and had not received antibiotics, probiotics or prebiotics for at least 3 months prior to sampling (Table 1). Written informed consent was obtained from the subjects enrolled. Samples were collected between March and April 2013. Twenty-four hour dietary recalls were provided by each enrolled subject for three days. We used the standard method in nutritional science of sampling two week days and one weekend day in an attempt to fully account for dietary habit and fluctuation. Records were entered and analyzed using the Food Processor SQL version 10.13.0 and compiled for summary reporting of the main caloric contributions by food group and macronutrient.

4.2.2 Sample collection and storage

Hadza samples were handled and stored following previously described methods (Nsubuga et al., 2004). Briefly, samples were submerged in 30 mL of 97% ethanol for 24-36 hours, after which the ethanol was carefully poured out and the remaining solid material was transferred to 50 mL tubes containing silica beads (Sigma 10087). All Hadza samples were transported by express to Bologna, Italy where further analysis was performed. Italian samples were collected, dried using the ethanol and silica two-step procedure, and stored at -80 °C in Bologna until further use.



Image 11. Bush lab for fecal sampling and storage in Hadza land.

Table 1. Age, sex and provenience of study enrolled participants.

HADZA				ITALIANS			
SAMPLE ID	AGE	SEX	CAMP	SAMPLE ID	AGE	SEX	PROVENIENCE
H1	43	F	Dedauko	IT1	38	M	Bologna
H2	37	M	Dedauko	IT2	34	F	Bologna
H3	22	M	Dedauko	IT3	29	F	Bologna
H4	24	M	Dedauko	IT4	27	M	Bologna
H5	70	F	Dedauko	IT5	30	M	Bologna
H6	30	M	Dedauko	IT6	32	F	Bologna
H7	38	F	Dedauko	IT7	25	F	Bologna
H8	20	F	Dedauko	IT8	34	M	Bologna
H9	47	F	Dedauko	IT9	39	F	Bologna
H10	40	F	Dedauko	IT10	40	F	Bologna
H11	29	F	Dedauko	IT11	40	F	Bologna
H12	8	F	Dedauko	IT12	30	F	Bologna
H13	34	M	Dedauko	IT13	30	F	Bologna
H14	30	M	Dedauko	IT14	21	F	Bologna
H15	16	M	Dedauko	IT15	29	M	Bologna
H16	21	M	Dedauko	IT16	32	F	Bologna
H17	23	M	Dedauko				
H18	17	M	Dedauko				
H19	15	M	Dedauko				
H20	13	M	Dedauko				
H21	43	M	Sengele				
H22	38*	M	Sengele				
H23	24*	M	Sengele				
H24	65*	M	Sengele				
H25	40*	M	Sengele				
H26	48*	M	Sengele				
H27	29	F	Sengele				

*Asterisks indicate individuals for whom age was less certain.

4.2.3 Comparison of dry and frozen fecal samples

Hadza stool samples could not remain frozen during their removal from Tanzania due to unreliable sourcing of dry ice shipping materials, so we first performed a comparison of DNA extraction and amplification and SCFA quantitation on split samples of Germany-living westerners. Stool samples were split into two segments, one fraction was stored at -80 °C and the second was dried using the two-step ethanol/silica procedure as described above. Total DNA extraction yield, pyrosequencing of the 16S rDNA V4 gene region and SCFA relative abundance quantification were performed (as described below) in parallel from frozen and dried sample aliquots. According to our data, we obtained comparable DNA yield, GM profiles, and SCFA relative abundance profiles from frozen and dry aliquots of the same stool (Table 2). GM profiles were shown to cluster by subject independent of the storage method (Figure 2). Taken together, these data support the reliability of the drying method for use in stool storage.

Table 2. Comparison of dried and frozen storage protocols on test fecal samples shows total DNA yield and absorbance values (a) and SCFA relative abundance (b)

a.

Sample ID	DNA ng ul ⁻¹	A260	A280	260/280	260/230
101 frozen	423.86	8.477	4.494	1.89	1.95
101 dry	610.86	12.217	6.299	1.94	2.06
103 frozen	468.69	9.374	5.213	1.8	1.39
103 dry	260.74	5.215	2.879	1.81	1.54

b.

Sample	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)	Valeric acid (%)
101 frozen	77.23	14.56	6.82	1.39
101 dry	80.45	11.17	7.44	0.94
103 frozen	67.89	20.76	9.53	1.83
103 dry	72.60	18.58	7.87	0.94

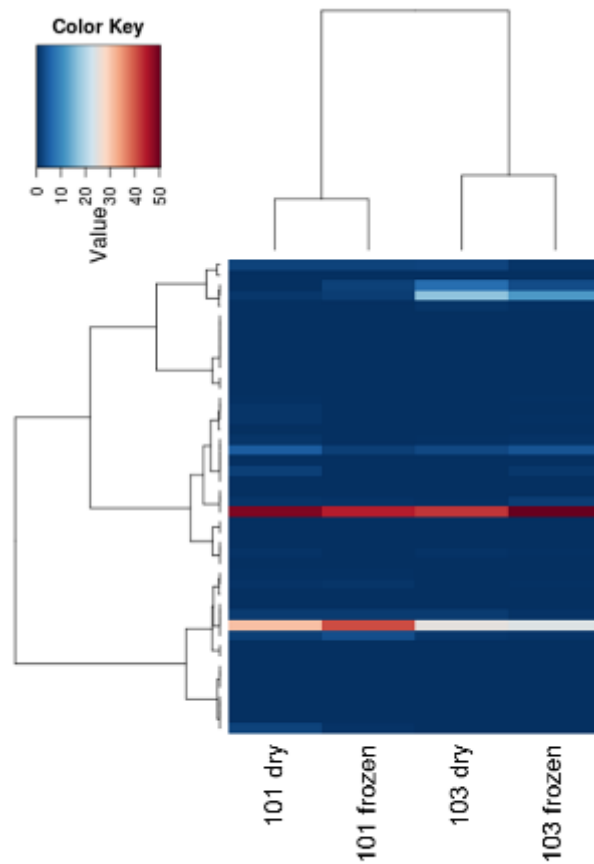


Fig 2. Comparison of dried and frozen storage protocols on test fecal samples. In test experiments, stools from two Western individuals (101 and 103) were collected and split into two samples each. One fraction was stored immediately at -80 °C (frozen) and the second fraction was dried following a two-step ethanol submersion and silica storage procedure (dried). Hierarchical ward-linkage clustering based on the Spearman correlation coefficients of bacterial family proportion is shown. Subjects are clustered in the top of the panel. Families are clustered by the vertical tree.

4.2.4 DNA extraction from fecal samples

Total DNA from fecal material was extracted using QIAamp DNA Stool Mini Kit (QIAGEN) with a modified protocol. Briefly, 250 milligrams of feces were suspended in 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% SDS). Four 3 mm glass beads and 0.5 g of 0.1 mm zirconia beads (BioSpec Products) were added, and the samples were treated in FastPrep (MP Biomedicals) at 5.5 movements per second for 3 min. Samples were heated at 95 °C for 15 min, then centrifuged for 5 min at full speed to pellet stool particles. Supernatants were collected and 260 µL of 10 M ammonium acetate was added, followed by incubation in ice for 5 min and centrifugation at full speed for 10 min. One volume of isopropanol was added to each supernatant and incubated in ice for 30 min. The precipitated nucleic acids were collected by centrifugation for 15 min at full speed and washed with ethanol 70%. Pellets were resuspended in 100 µL of TE buffer and treated with 2 µL of DNase-free RNase (10 mg mL⁻¹) at 37 °C for 15 min. Protein removal by Proteinase K treatment and DNA purification with QIAamp Mini Spin columns were performed following the kit protocol. Final DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop® Technologies).

4.2.5 16S rDNA gene amplification

For the amplification of the V4 region of the 16S rDNA gene the primer set 520F (5'-AYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (with Y=C/T, D=A/G/T, N=any base, V=A/C/G) was utilized. These primers were designed to include at their 5' end one of the two adaptor sequences used in the 454-sequencing library preparation protocol (adaptor A and B), linked to a unique MID tag barcode of 10 bases allowing the identification of the different samples. PCR mixtures contained 0.5 µM of each forward and reverse primer, 100 ng of template DNA, 2.5 U of GoTaq Flexi Polymerase (Promega), 200 µM of dNTPs and 2 mM of MgCl₂ in a final volume of 50 µL. Thermal cycling consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 40 °C for 30 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 5 min (Claesson et al., 2009). PCR amplifications were carried out in a Biometra Thermal Cycler T Gradient (Biometra).

4.2.6 qPCR for *Bifidobacterium* quantification

qPCR was carried out in a LightCycler instrument (Roche). Quantification of the 16S rRNA gene of *Bifidobacterium* was performed with previously described genus-specific primers bif-164 and bif-662 (Candela et al., 2012). For quantification, standard curves were generated by using 10-fold serial

dilution of genomic DNA from *B. animalis* subsp. *lactis* BI07. Amplification was carried out in a 20 μ l final volume containing 100 ng of fecal DNA, 0.5 μ M of each primer and 4 μ l of LightCycler-FastStart DNA Master SYBR Green I (Roche). Amplifications were done under the following conditions: (i) starting pre-incubation at 95 °C for 10 min; (ii) amplification including 35 cycles of 4 steps each at the temperature transition rate of 20 °C s⁻¹: denaturation at 95 °C for 15 s, annealing at 63 °C for 20 s, extension at 72 °C for 30 s, and fluorescence acquisition at 90 °C for 5 s; (iii) melting curve analysis.

4.2.7 Pyrosequencing of fecal slurries

The PCR products derived from amplification of the specific 16S rDNA V4 hypervariable region were individually purified with MinElute PCR Purification Kit (QIAGEN) and then quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen). After the individual quantification step, amplicons were pooled in equal amounts (thus, creating three 9-plex for Hadza samples and two 8-plex pools for Italian samples) and again purified by 454-Roche Double Ampure size selection protocol with Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH), in order to remove primer dimers, according to the manufacturer's instructions (454 LifeSciences, Roche). Amplicon pools were fixed to microbeads to be clonally amplified by performing an emulsion PCR following the GS-FLX protocol Titanium emPCR LIB-A (454 LifeSciences, Roche). Following this amplification step, the beads were enriched in order to keep only those carrying identical PCR products on their surface, and then loaded onto a picotiter plate for pyrosequencing reactions, according to the GS-FLX Titanium sequencing protocol. All pools were sequenced in one eighth of a plate each.

4.2.8 Bioinformatic analysis of 16S rDNA and statistical methods

Sequencing reads were analyzed using the QIIME pipeline (Caporaso et al., 2010) as described previously (Claesson et al., 2012). Briefly, V4 sequences were filtered according to the following criteria: (i) read length not shorter than 150 bp and not longer than 350 bp; (ii) no ambiguous bases (Ns); (iii) a minimum average quality score over a 50-bp rolling window of 25; (iv) exact match to primer sequences and maximum 1 error in barcode tags. For bacterial taxonomy assignment we utilized RDP-classifier (version 2.2) with 50% as confidence value threshold. Trimmed reads were clustered into OTUs at 97% identity level and further filtered for chimeric sequences using ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Alpha-diversity and rarefaction plots were computed using four different metrics: Shannon, PD whole tree, chao1 and observed species. Weighted and unweighted UniFrac distances and Euclidean distance of genus level relative abundance were used to perform Principal Coordinates Analysis (PCoA). PCoA, and heatmap and bar plots were built using the packages *Made4* (Culhane et al., 2005) and *Vegan* (<http://cran.r-project.org/package=vegan>).

The R packages *Stats* and *Vegan* were used to perform statistical analysis. In particular, to compare gut microbiota structure among different populations for α and β diversity we used a Wilcoxon signed rank test. Data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function [adonis] in the *Vegan* package). Cluster separation in hierarchical clustering analyses was assessed for significance using Fisher's exact test. Significant differences in phylum or genus level abundance between Hadza and Italians, and between Hadza males and females, were assessed by Mann-Whitney U tests, and corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. False discovery rate (FDR) < 0.05 was considered as statistically significant.

Kendall correlation test between SCFA levels and the relative abundance of genera was achieved using function [cor.test] of the package *Stats* of R. Sequences from De Filippo et al. (2010) and Yatsunenکو et al. (2012) were obtained from Metagenomics Rapid Annotation using Subsystem Technology (MGRAST), project I.D. 201, and European Nucleotide Archive (ENA), project number ERP000133, repositories, respectively, and processed and assigned following the QIIME pipeline. Bacterial co-abundance groups (CAGs) were determined as described previously (Claesson et al., 2012). Briefly the associations among the genera were evaluated using Kendall correlation test, visualized using hierarchical Ward clustering with a Spearman correlation distance metrics and used to define co-abundant genera groups. The significant associations were controlled for multiple testing using the qvalue method (FDR<0.05) (Dabney et al., 2013). Permutational MANOVA (Anderson, 2001) was used to determine if the CAGs were significantly different from each other. The Wiggum plot network analysis was created as previously described (Claesson et al., 2012) using cytoscape software (<http://www.cytoscape.org/>). Circle size represents genus abundance and connections between nodes represent positive and significant Kendall correlations between genera (FDR < 0.05).

4.2.9 GC-MS determination of SCFAs in fecal samples

Aliquots of dried fecal samples (about 250 mg) were briefly homogenized after the addition of 1 mL of 10% perchloric acid in water and centrifuged at 15,000 x g for 5 min at 4 °C. 500 μ L of supernatant were diluted 1:10 in water, 10 μ L of D8-butyric acid (internal standard, IS) were added to the sample at the final concentration of 20 μ g mL⁻¹. The calibration curves were prepared adding the IS to scalar amounts of the acids in diluted samples or water (for external standardization). All the standards (purity > 99%), acetic, propionic, butyric, valeric acids and IS were provided by Sigma and were used to prepare calibration solutions for quantitation (linear response) and identification. HS-SPME was performed by using a 75 μ m CarboxenTM/polydimethylsiloxane fiber (Supelco). The optimized final extraction conditions were: temperature 70 °C, 10 min of equilibration time, 30 min of extraction time. The analytes were desorbed into the GC injector port at 250 °C for 10 min, including fiber cleaning. GC-MS analysis was carried out on a TRACE GC 2000 Series (ThermoQuest CE Instruments) gas

chromatograph, interfaced with GCQ Plus (ThermoQuest) mass detector with ion trap analyzer, operating in EI mode (70 eV). The capillary GC column was a Phenomenex ZB-WAX (30 m x 0.25 mm ID, 0.15 μm film thickness), consisting of 100% Polyethylene Glycol. Helium (He) was the carrier gas at a flow rate of 1.0 mL min^{-1} . An oven temperature program was adopted: initial 40 $^{\circ}\text{C}$ (hold time: 5 min), then ramped by 10 $^{\circ}\text{C min}^{-1}$ to 220 $^{\circ}\text{C}$ (hold time: 5 min). The temperature of transfer line and ionization source was maintained at 250 and 200 $^{\circ}\text{C}$, respectively.

The GC was operated in splitless mode; the injector base temperature was set at 250 $^{\circ}\text{C}$. The mass spectra were recorded in full scan mode (34-200 amu) to collect the total ion current (TIC) chromatograms. Quantitation was carried out by using the extracted ion chromatograms (XIC) by selecting fragment ions of the studied analytes (43 and 60 amu for acetic acid, 55 and 73 amu for propionic acid, 60 and 73 amu for butyric and valeric acids and 63 and 77 amu for IS). The SCFAs concentration in fecal samples was expressed in micromoles per gram ($\mu\text{mol g}^{-1}$) of feces. Limit of detection (LOD) ranged from 4 to 68 nmol g^{-1} .

4.3 Results

4.3.1 Dietary information for sampled cohorts

The Hadza diet consists of wild foods that fall into five main categories: meat, honey, baobab, berries, and tubers (Table 3) (Murray et al., 2001; Schoeninger et al., 2001; Marlowe and Berbesque, 2009). They practice no cultivation or domestication of plants and animals and receive minimal amounts of agricultural products (<5% of calories) from external sources (Marlowe, 2010). By comparison, the diet of the Italian cohort derives almost entirely from commercial agricultural products and adheres largely to the Mediterranean diet: abundant plant foods, fresh fruit, pasta, bread, and olive oil; low to moderate amounts of dairy, poultry, fish, and red meat (Table 4). Additionally, the majority of carbohydrates (based on gram amount) came from easily digestible starch (54%) and sugar (36%) while very little was derived from fiber- soluble or insoluble (10%) (Figure 3).

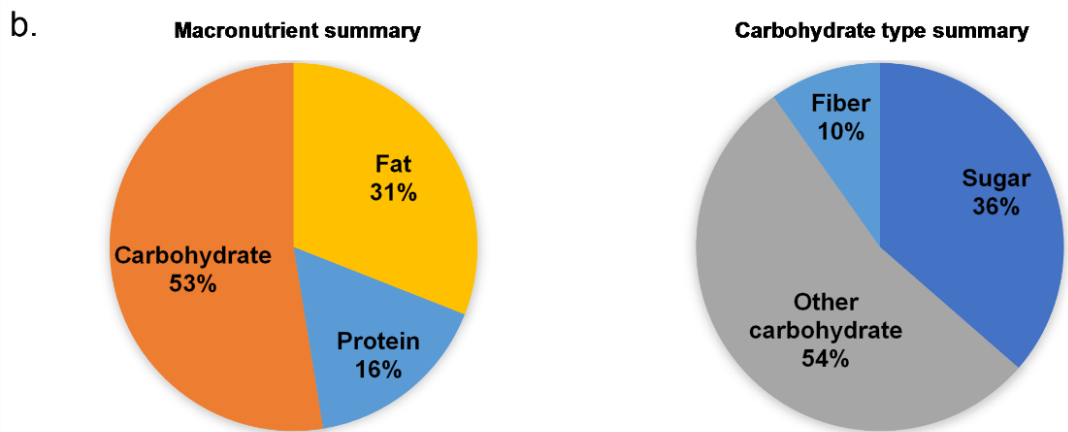
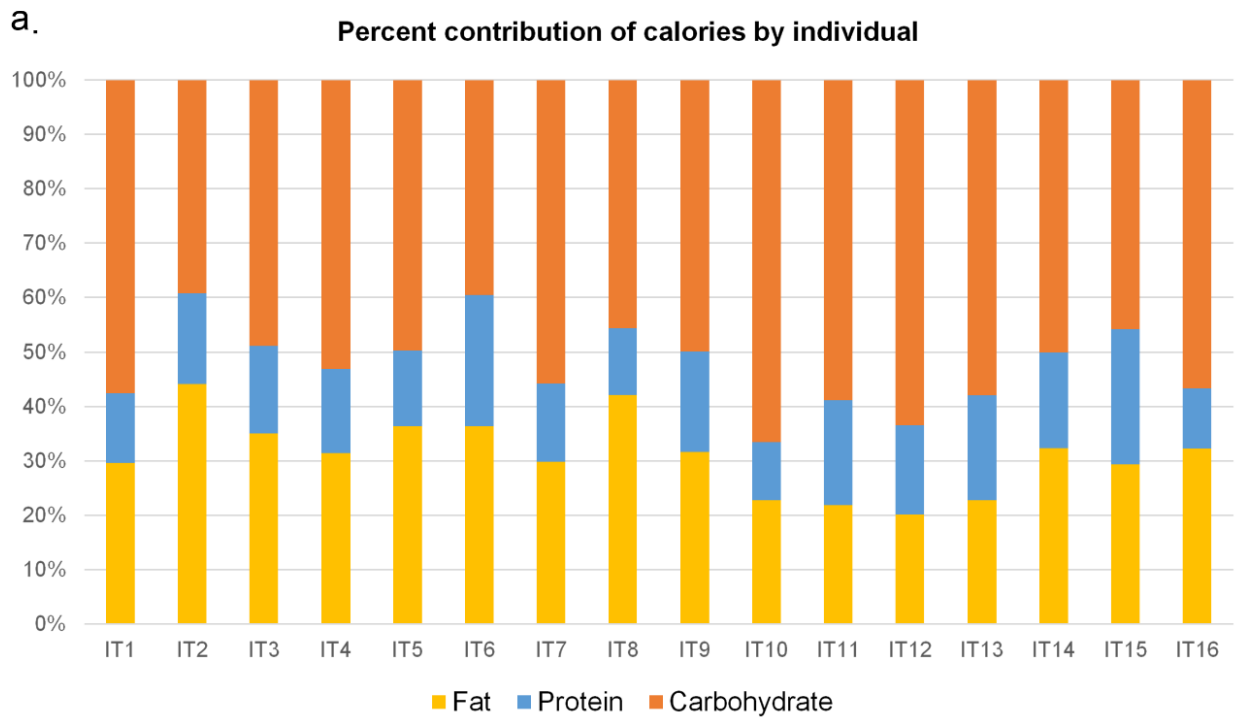


Fig 3. Macronutrient summaries of Italian cohort diet. **(a)** The percent macronutrient intake is reported by subject based upon three days of dietary recalls collected during two week days and one weekend day. Recorded diets represent the typical daily diet of each subject. **(b)** Pie graphs show the summary of the average macronutrient intake by percent of kilocalories consumed for the entire subject cohort as well as a breakdown of carbohydrate type.

Table 3. Summary of Hadza foods and nutrition.

Food type	Hadza name	English name	Species name	Moisture (%)	g 100g ⁻¹ dry wt.					kcal 100g ⁻¹ dry wt.	References
					Edible portion	Carbohydrate	Protein	Fat	Fiber		
Baobab	N//obabe (pulp)**	Baobab	<i>Adansonia digitata</i>	4.7	na	46.6	2.5	0.7	45.1	203.0	1
	N//obabe (seed)**	Baobab	<i>Adansonia digitata</i>	4.8	na	11.2	36.3	29.3	14.1	454.0	1
Berry	Kisinubi	na	<i>Cordia sinensis</i>	73.0	na	68.8	12.6	1.8	11.6	342.0	1
	Undushibi**	na	<i>Cordia sinensis</i>	71.0	na	61.4	15.2	na	13.6	324.0	1
	Masakapi	na	<i>Cordia crenata</i>	69.0	na	62.5	12.7	1.9	17.8	318.0	1
	Hlukayebe	na	<i>Grewia villosa</i>	24.0	na	72.7	7.1	na	13.4	337.0	1
	Kongolubi	na	<i>Grewia bicolor</i>	26.0	na	66.1	12.0	2.0	13.2	330.0	1
	Pawe	na	<i>Sclerocarya birrea</i>	83.0	na	49.9	3.6	na	37.7	232.0	1
	Honey	Ba'alako**	Honey (stinging)	<i>Apis mellifera</i>	15.1	na	89.1	3.3	7.2	na	434.0
N//ateko**		Honey (stingless)	<i>Meliponinae sp.</i>	23.5	na	92.8	3.2	3.2	na	412.5	1
Meat	Gewedako**	Dik-dik	<i>Madoqua kirkii</i>	na	na	na	na	na	na	na	2
	Tsokwonako**	Giraffe	<i>Giraffa camelopardalis</i>	na	na	na	na	na	na	na	2
	Chacha**	Galago (lesser)	<i>Galago senegalensis</i>	na	na	na	na	na	na	na	2
	Ndonoko**	Galago (greater)	<i>Otolemur crassicaudatus</i>	na	na	na	na	na	na	na	2
	Tsunako**	Bee larvae	<i>Apis mellifera</i>	na	na	na	na	na	na	na	2
Tuber	//Ekwa hasa**	na	<i>Vigna frutescens</i>	68.8	39.4	60.8	7.2	0.8	28.2	282.3	3
	//Ekwa gadabi	na	<i>Vigna frutescens</i>	70.0	na	60.6	8.0	1.9	22.7	84.5	4
	Shumuko**	na	<i>Vatoraea pseudolablab</i>	90.1	na	50.8	3.3	0.6	35.2	225.0	4
	Do'aiko	na	<i>Vigna macrorhyncha</i>	85.0	na	58.6	10.4	0.1	22.4	292.0	4
	Matukwaiko	na	<i>Coccinea surantiaca</i>	86.5	na	69.5	12.4	0.0	13.3	337.0	4
	Mak'alitako**	na	<i>Eminia entennulifa</i>	71.7	56.9	26.4	4.1	1.1	17.1	110.1	3, 4
	Panjuko**	na	<i>Ipomoea transvaalensis</i>	70.6	91.8	54.8	4.2	1.7	2.6	233.0	4

¹ Murray et al., (2001); ² Marlowe, (2010); ³ Schoeninger et al., (2001); ⁴ Vincent, (1985)

Table 4. Percent contribution of food categories and corresponding nutrients to average daily Italian cohort diet¹.

Food Category	Most common food items	Energy (kcal)	Fat (g)	Protein (g)	All carbohydrate (g)	Fiber (g)	Sugar (g)	Other carbohydrate (g)
Bread, cereal, pasta, grain		37.2%	25.3%	33.6%	48.3%	45.2%	16.0%	74.0%
	flatbread, brioche, brown bread							
	pasta: tortellini, lasagna, noodles							
	cereals, biscuits, crackers, pizza crust							
Other starch		3.7%	1.2%	2.3%	5.7%	6.3%	0.7%	8.5%
	beans, legumes							
	rice, potatoes, couscous							
Meats, egg, meat broths		10.7%	16.0%	31.2%	0.7%	0.4%	1.0%	0.5%
	pork: cured, steak, roast, salami							
	chicken: baked, roasted							
	beef: steak, roast, sausage,							
	fish: tuna, sole, cod, bass, flounder							
Dairy		13.0%	22.3%	19.9%	5.1%	0.5%	13.0%	0.7%
	milk, cream, yogurt							
	cheese: parmesan, mozzarella, sheep							
Fruit, fruit juice		8.8%	0.8%	2.4%	17.2%	19.9%	29.4%	5.6%
	banana, apple, pear, persimmon, citrus							
	orange juice, pear juice							
Vegetables, sauces, herbs		4.6%	3.1%	5.4%	6.1%	23.1%	4.9%	4.6%
	lettuce, carrot, onion							
	tomato sauce, mixed vegetables							
	boiled cabbage, squash							
Desserts, sugary treats		12.6%	14.6%	4.0%	14.6%	2.8%	33.3%	4.8%
	sugar, honey, cake, jam, soda							
	chocolate, pastries, ice cream							
Oil, butter, fat		4.4%	13.6%	0.0%	0.0%	0.0%	0.0%	0.0%
	olive oil, butter							
Alcohol		3.5%	0.0%	0.3%	1.4%	0.0%	0.3%	0.8%
	beer, wine							

Nuts		1.1%	3.1%	0.8%	0.2%	1.2%	0.1%	0.2%
	walnut, peanut							
Other		0.4%	0.1%	0.1%	0.6%	0.5%	1.3%	0.2%
	espresso, coffee, barley coffee, tea							
	vinegar, mustard, salt							
Average daily amount		1852.3	66.3	75.0	236.5	20.0	84.5	120.6

¹Percent contribution of each food group for energy (kcal), fat (g), protein (g), and carbohydrate (g). Carbohydrate is further broken down into types of carbohydrate with percent contribution by gram of the following: fiber, sugar, and other carbohydrate (oligosaccharides). An average daily intake for each category is reported in the last row.

4.3.2 Characterization of Hadza microbiota

Fecal samples from 27 Hadza, aged 8-70 years, mean age 32 years, and 16 Italians aged 20-40 years, mean age also 32 years (Table 1), were collected and pyrosequenced in the V4 gene region of bacterial 16S rDNA, resulting in 309,952 high-quality reads and an average of 7208 ± 2650 reads per subject. Reads were clustered into 11,967 operational taxonomic units (OTUs) at 97% identity. We used several different metrics to calculate α -diversity, including phylogenetic diversity (Faith, 1992), OTU species count, the Chao1 index for microbial richness, and the Shannon index for biodiversity (Figure 4). Rarefaction curves for phylogenetic diversity plateaued after 4000 reads per sample, approximating a saturation phase. All measures indicate a much higher GM diversity within the Hadza than in Italian samples ($P < 0.001$, Mann-Whitney U test).

The Hadza and Italian samples show many notable differences in microbiota relative abundance, as a percent of reads assigned, at both phylum and genus levels (Figure 5, Table 5). In particular, the Hadza GM is largely dominated by Firmicutes ($72\% \pm 1.9\%$) and Bacteroidetes ($17\% \pm 1.1\%$). Other represented phyla are Proteobacteria ($6\% \pm 1.2\%$) and Spirochaetes ($3\% \pm 0.9\%$), with 2% of phylum level OTUs remaining unclassified. The most represented families in the Hadza GM are *Ruminococcaceae* (34%), *Lachnospiraceae* (10%), *Prevotellaceae* (6%) Clostridiales Incertae Sedis XIV (3%), *Succinivibrionaceae* (3%), *Spirochetaceae* (2%) and *Eubacteriaceae* (2%). Interestingly, a large number of taxa, the majority belonging to Bacteroidetes, Clostridiales, Bacteroidales, and *Lachnospiraceae*, are unassigned at the level of family and genus, together representing 22% of the total community.

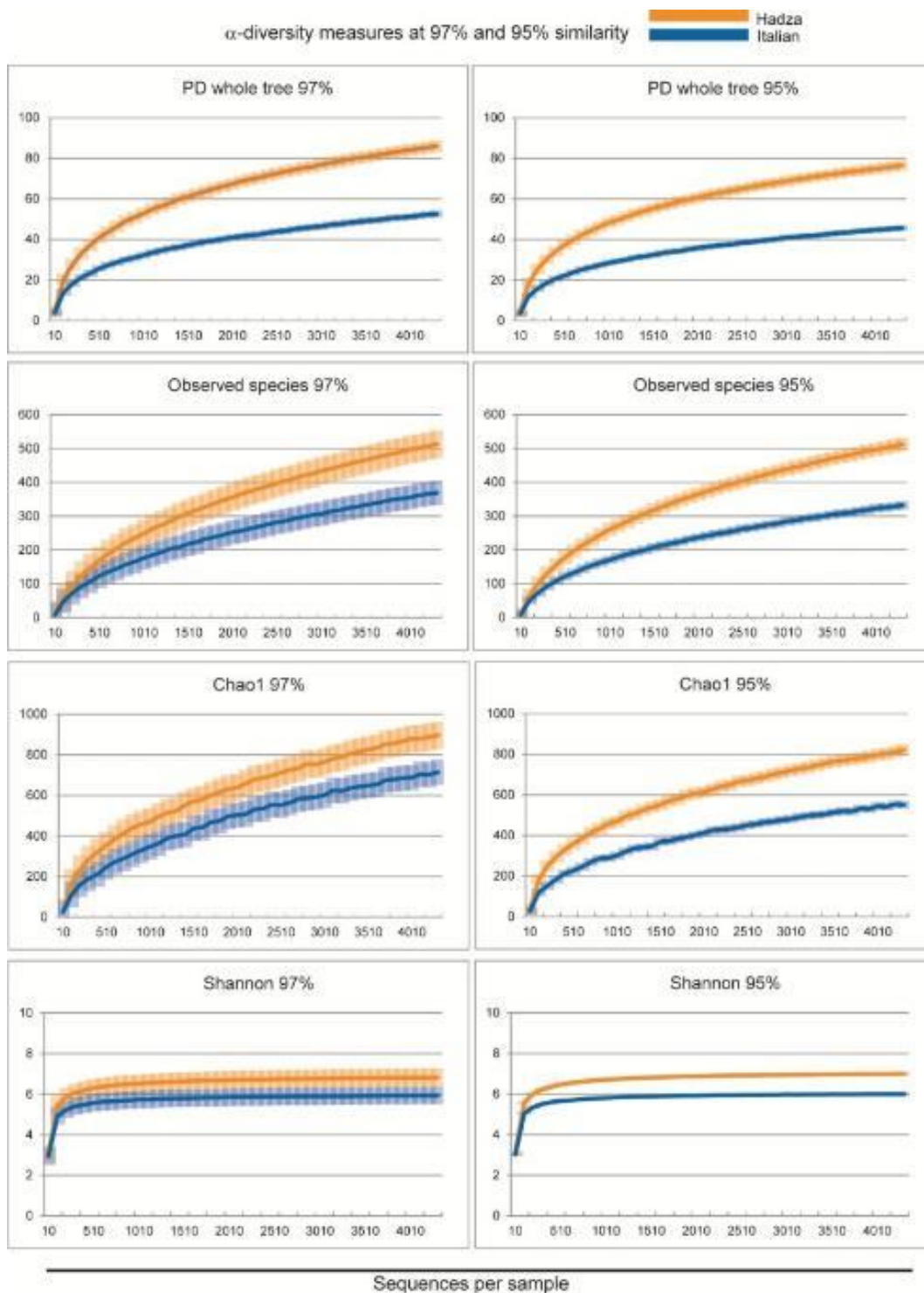


Fig 4. Rarefaction curves of different α -diversity metrics. The OTU table at 0.05 and 0.03 similarity thresholds was rarefied up to 4500 reads per sample and analyzed using various diversity metrics for both Hadza and Italian subjects. Similarity thresholds of 95% (family level) and 97% (genus level) were considered. Metrics used were Faith’s Phylogenetic Diversity (PD whole tree), observed OTUs, the Chao1 measure of microbial richness, and the Shannon index of biodiversity. All four diversity metrics showed greater diversity in the Hadza microbiota compared to the Italian microbiota. Plateaus occurred by about 4000 reads per sample, approximating a saturation phase and indicating that the breadth of diversity has been well captured in the sequencing.

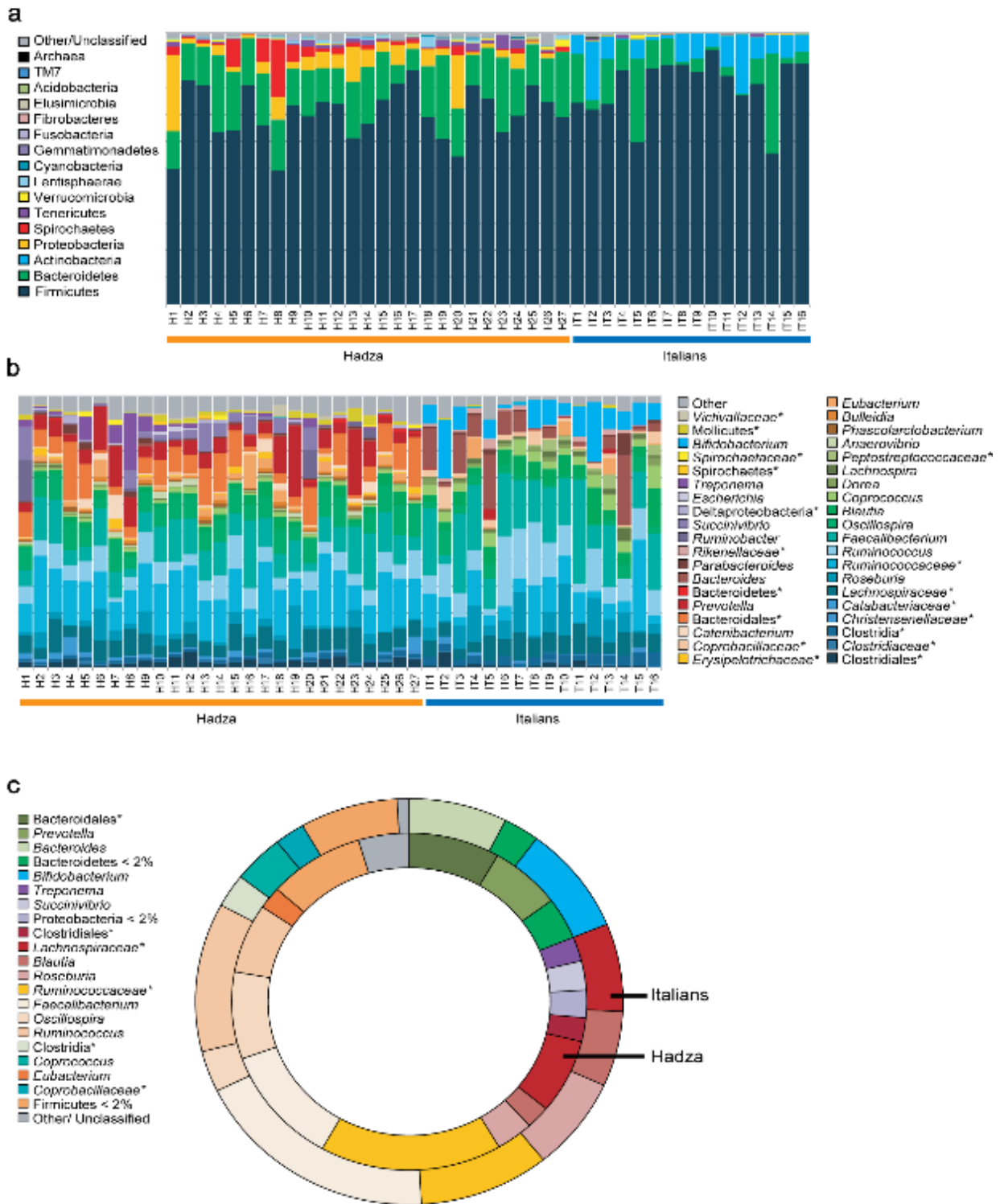


Fig 5. Bacterial relative abundance of Hadza and Italian subjects. 16S rDNA gene survey of the fecal microbiota of 27 Hadza (H1-H27) and 16 Italian (IT1-IT16) adults. Relative abundance of (a) phylum and (b) genus classified fecal microbiota is reported. Histograms are based on the proportion of OTUs per subject. Colors were assigned for all phyla detected, and for genera with a relative abundance $\geq 1\%$ in at least 10% of subjects. (c) Donut charts summarizing genera relative abundance for Italians (outer donut) and Hadza (inner donut). Genera were filtered for those with $\geq 2\%$ of total abundance in at least 10% of subjects. *denotes unclassified OTU reported at higher taxonomic level.

Table 5. Summary of taxa differences between Hadza and Italian subjects¹**a.**

over-represented in Hadza

Phylum	Italians ±		Hadza ±		FDR
	SEM		SEM		
Bacteroidetes	10.1	2.7	17.4	1.1	0.002
Bacteria unclassified	0.2	0.05	1.8	0.2	< 0.001
Proteobacteria	0.3	0.04	5.9	1.2	< 0.001
Spirochaetes	0	0	2.8	0.9	< 0.001
Genus					
<i>Anaerophaga</i>	0	0	0.6	0.1	< 0.001
Bacteroidales unclassified	0.07	0.02	0.8	0.1	< 0.001
<i>Porphyromonadaceae</i> unclassified	0.03	0.02	0.9	0.3	< 0.001
<i>Prevotella</i>	0.4	0.3	6.2	1.1	< 0.001
Bacteroidetes unclassified	0.2	0.07	6.6	0.8	< 0.001
Sphingobacteriales unclassified	0	0	0.6	0.1	< 0.001
<i>Eubacterium</i>	1.4	0.6	2.2	0.5	0.013
<i>Robinsoniella</i>	0.02	0	0.9	0.2	< 0.001
Clostridiales unclassified	7.4	0.8	11.1	0.6	< 0.001
<i>Butyricoccus</i>	0.4	0.05	2.6	0.5	< 0.001
<i>Hydrogenoanaerobacterium</i>	0.07	0.03	1	0.1	< 0.001
<i>Oscillibacter</i>	1	0.3	3.8	0.4	< 0.001
<i>Ruminococcaceae</i> unclassified	3.6	0.6	8.6	0.7	< 0.001
<i>Sporobacter</i>	0.8	0.2	3.7	0.4	< 0.001
<i>Veillonellaceae</i> unclassified	0.02	0.01	0.8	0.2	< 0.001
<i>Bulleidia</i>	0	0	0.7	0.07	< 0.001
<i>Catenibacterium</i>	0.7	0.4	1.4	0.4	0.001
Firmicutes unclassified	0.3	0.1	0.8	0.1	< 0.001
<i>Ruminobacter</i>	0	0	1.2	0.7	0.045
<i>Succinivibrio</i>	0.02	0.02	2.8	0.7	< 0.001
Proteobacteria unclassified	0.02	0.01	0.5	0.2	< 0.001
<i>Treponema</i>	0	0	2.5	0.7	< 0.001

b.

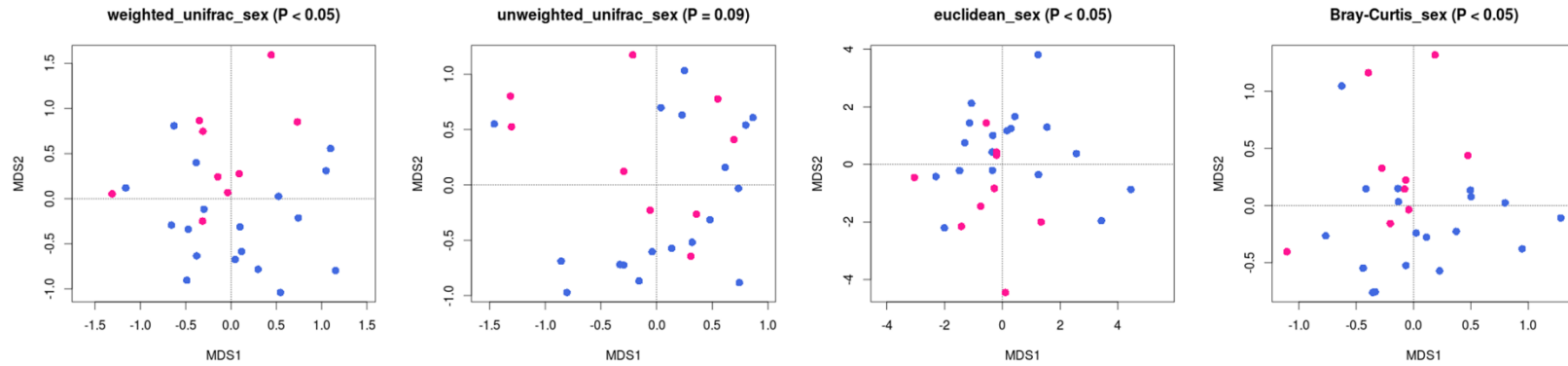
over-represented in Italians

Phylum	Italians \pm		Hadza \pm		FDR
	SEM		SEM		
Actinobacteria	8.3	1.5	0.1	0.02	< 0.001
Firmicutes	80.9	2.7	71.8	1.9	0.006
Genus					
<i>Bifidobacterium</i>	8.1	1.5	0.02	0.01	< 0.001
<i>Bacteroides</i>	7.1	2	0.2	0.04	< 0.001
<i>Alistipes</i>	0.9	0.2	0	0	< 0.001
<i>Blautia</i>	9.5	1.3	3.5	0.4	< 0.001
<i>Anaerosporebacter</i>	0.6	0.1	0.2	0.04	0.003
<i>Coprococcus</i>	3.6	0.7	1.4	0.2	0.005
<i>Dorea</i>	1.4	0.2	0.4	0.06	< 0.001
<i>Lachnospiraceae</i> unclassified	6.4	0.7	3.9	0.2	0.001
<i>Roseburia</i>	7.7	1.3	3.9	0.8	0.014
<i>Faecalibacterium</i>	18.5	2.4	11.8	1	0.022
<i>Ruminococcus</i>	8.6	1.8	2.1	0.3	0.005
<i>Erysipelotrichaceae</i> unclassified	1.5	0.3	0.7	0.2	0.026

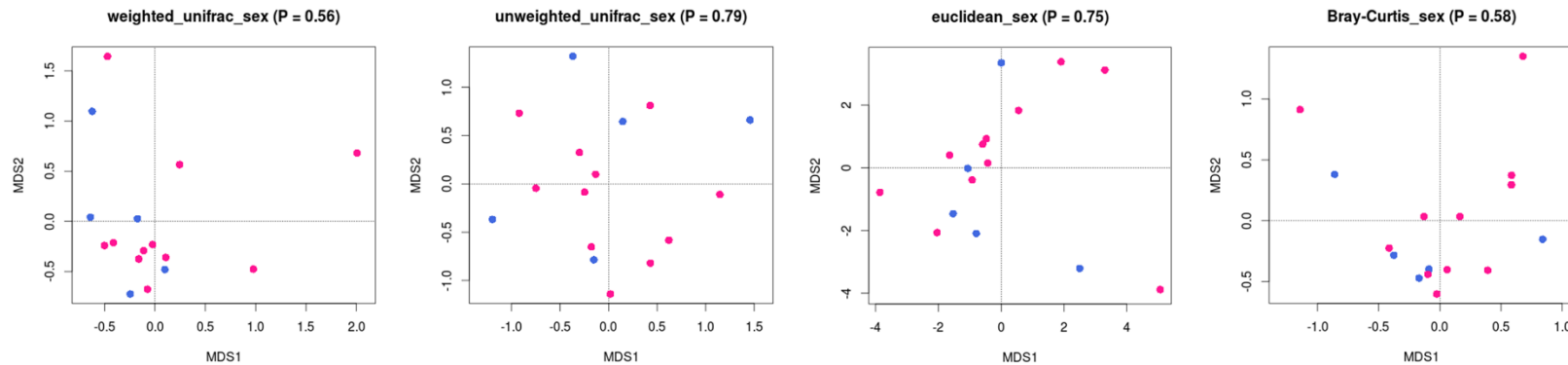
[†]Bacterial phyla and genera significantly over-represented in the gut ecosystem of Hadza (a) and Italians (b). For each microbial group, the mean relative abundance (%) \pm SEM and *P*-value of the differences between the two populations (Mann-Whitney U test) are reported.

To explore variation within the Hadza GM, we used weighted and unweighted UniFrac distances to assess differences based on camp location and sex. We found no significant difference in phylogenetic diversity or relative abundance between camps (Supplementary Figure 1). However, unlike the Italian cohort, the Hadza GM does show significant separation by sex based on weighted UniFrac distance ($P < 0.05$, permutation test with pseudo F-ratio). Analogous results were obtained when Euclidean and Bray-Curtis distance of genera relative abundance were considered ($P < 0.05$) (Figure 6).

Hadza



Italians



Female

Male

Fig 6. Sex difference in GM structure among Hadza and Italians. PCoAs based on unweighted and weighted UniFrac distances as well as Euclidean and Bray-Curtis distances show patterns of separation by sex within each subject cohort and their respective P-values. Significance was calculated by permutation test with pseudo F-ratio. Pink, females; blue, males.

To determine a structural basis for the observed separation, we compared genera relative abundance between Hadza men and women using a Mann-Whitney U test, and found a significantly increased abundance of *Treponema* ($P < 0.05$) in women and increased *Eubacterium* ($P < 0.05$) and *Blautia* ($P < 0.001$) in men. These differences may result from the pronounced sexual division of labor and sex differences in diet composition among the Hadza (Marlowe, 2001). Women selectively forage for tubers and plant foods, and spend a great deal of time in camp with children, family members, and close friends. Men are highly mobile foragers and range far from the central camp site in order to obtain game meat and honey (Pontzer et al., 2012). Though all foods are brought back to camp and shared, men and women consume slightly more of their targeted foods from snacking throughout the day (Berbesque et al., 2011). The increased *Treponema* among women may be an adaptation to the higher amount of plant fiber in their diet, especially from tubers. *Treponema* is considered an opportunistic pathogen in industrialized populations due to *T. pallidum*, the bacterium responsible for syphilis and yaws (Antal et al., 2002). However, this genus also includes proficient cellulose and xylan hydrolyzers (Warnecke et al., 2007), and it is possible *Treponema* acts as a mutualistic component of the Hadza GM to help with fiber degradation. The sex-based divisions in the Hadza lifestyle likely play a role in altering composition abundance of the GM through different patterns of environmental and community exposure, such as those previously viewed across age, geography or diet (De Filippo et al., 2010; Yatsunenkov et al., 2012). Further clarification of this division would require the inclusion of more Hadza women in the sample pool.

4.3.3 Detailed comparison to Italian controls

The Hadza and Italian GM profiles are quite distinct. Community structure visualized using PCoA of weighted and unweighted UniFrac distances reveal a sharp segregation along PCo1, indicating a strong core division in GM phylogeny between Hadza and Italian individuals ($P < 0.001$, permutation test with pseudo F-ratio) (Figure 7). Mean values of unweighted UniFrac distances also reveal lower within-group variability of taxonomic diversity among Hadza than Italians ($P < 0.001$). This similarity in breadth of phylogenetic diversity among Hadza is likely a result of close proximity community living with food sharing. Camp movement is usually resource driven (food and water) and the size and duration of camps vary greatly by season. In the dry season, many groups congregate around water holes, which also make hunting more productive. During the wet season groups are small and much more scattered with often five or fewer adults.

Though Firmicutes and Bacteroidetes are the dominant phyla in both Hadza and Italian GM, Hadza are characterized by a relatively higher abundance of Bacteroidetes and a lower abundance of Firmicutes (Table 5). The two GM ecosystems are remarkably different with respect to subdominant phyla (<10% relative abundance). Hadza are largely enriched in Proteobacteria and Spirochaetes, which are extremely rare in the Italian GM, while Actinobacteria, an important subdominant component of

the Italian GM, are almost completely absent from the Hadza microbiome. At the genus level, the Hadza GM is comparatively enriched in *Prevotella*, *Eubacterium*, *Oscillibacter*, *Butyrivibrio*, *Sporobacter*, *Succinivibrio* and *Treponema* and correspondingly depleted in *Bifidobacterium*, *Bacteroides*, *Blautia*, *Dorea*, unclassified *Lachnospiraceae*, *Roseburia*, *Faecalibacterium*, *Ruminococcus* and unclassified *Erysipelotrichaceae*. Moreover, there are many unclassified genera belonging to Bacteroidetes, Clostridiales and *Ruminococcaceae* in the Hadza GM, emphasizing our still limited ability to identify community dependent bacteria. The absence of *Bifidobacterium* in the Hadza GM was confirmed by qPCR (Supplementary Table 1). Taken together, data from our GM comparative analysis indicate a characteristic configuration for the Hadza gut microbial ecosystem that is profoundly depleted in *Bifidobacterium*, enriched in Bacteroidetes and *Prevotella*, and comprising an unusual arrangement of Clostridiales. This arrangement is defined by a general reduction of well-known butyrate producers, members of the *Clostridium* clusters IV and XIVa (den Besten et al., 2013), and a corresponding increase in unclassified Clostridiales and *Ruminococcaceae*. Interestingly, the Hadza GM is also characterized by a relevant enrichment in what are generally considered opportunistic microorganisms, such as members of Proteobacteria, *Succinivibrio*, and *Treponema*.

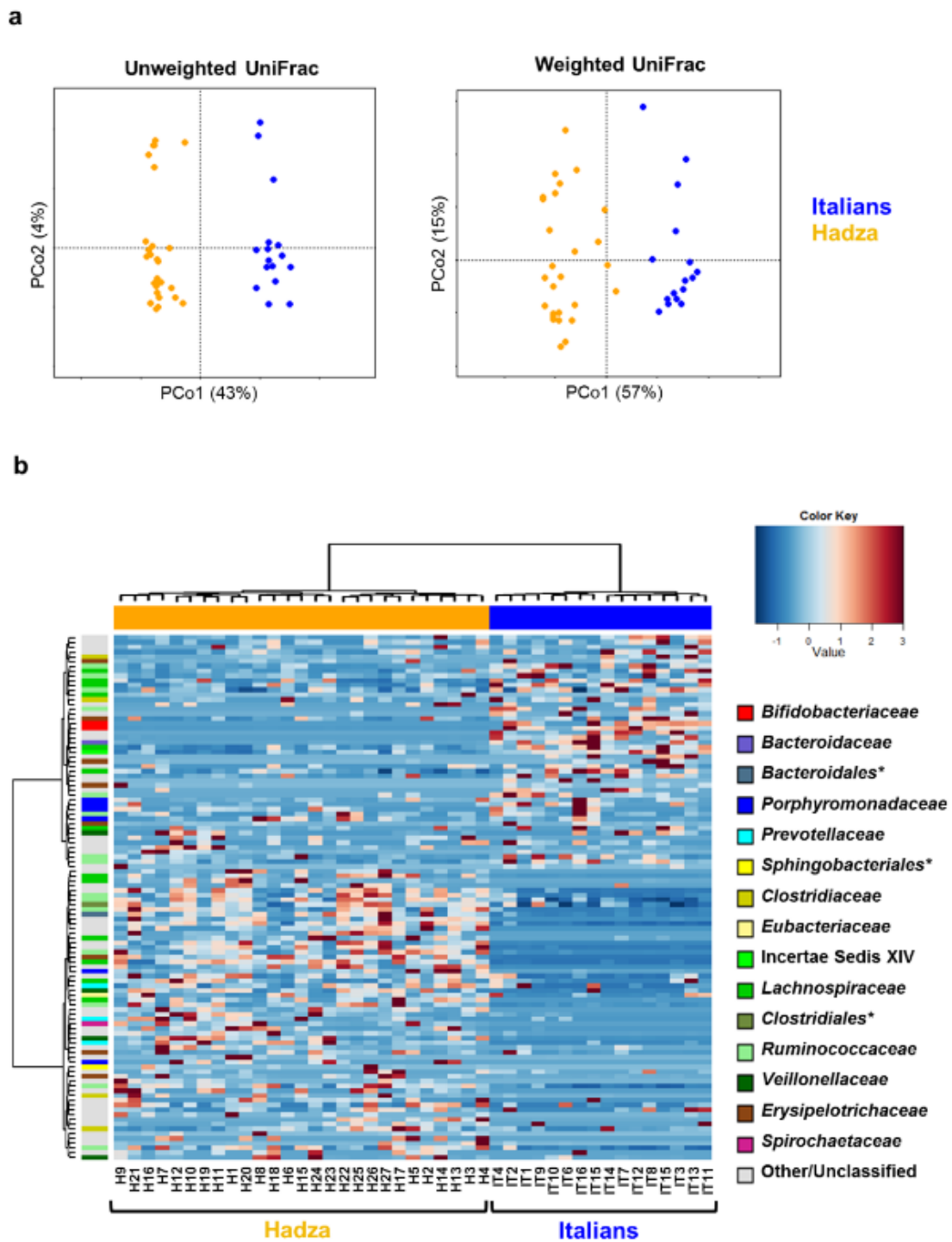


Fig 7. GM Phylogenetic difference between Hadza and Italian subjects. **(a)** Unweighted and weighted UniFrac distance PCoA of the fecal microbiota from 27 Hadza (orange dots) and 16 Italians (blue dots). **(b)** Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of genus proportion. Genera were filtered for subject prevalence of at least 30% within a population. Subjects are clustered on top of the panel and color-coded as in a. 110 genera clustered by the vertical tree are color-coded by family assignment. *denotes unclassified OTU reported at higher taxonomic level.

4.3.4 Comparison to African agricultural societies

The Hadza GM shares some features with other African populations, namely enrichment in *Prevotella*, *Succinivibrio* and *Treponema* (De Filippo et al., 2010; Yatsunenکو et al., 2012; Ou et al., 2013). Therefore, in order to explore community-level relationships within the GM that may be unique to a foraging lifestyle, we sought associations among genera by including two previously published rural African groups with an agriculture-based subsistence and their respective Western controls: 11 Mossi children from the Boulpon village, Burkina Faso (BF) aged 5-6 years and 12 Italian children aged 3-6 years (De Filippo et al., 2010); 22 young adult members from four rural Malawian communities, Chamba, Makwhira, Mayaka and Mbiza aged 20-44 years and 17 US adults aged 24-40 years (Yatsunenکو et al., 2012). Clustering analysis shows a significant ($P < 0.001$, Fisher's test) separation among Hadza, Malawians, BF and Western controls (Figure 8a). PCoA based on Bray Curtis distances of genera relative abundance confirms this separation ($P < 0.001$, permutation test with pseudo F-ratio) (Figure 8b). Interestingly, PC1, which represents 30% of the total variability, shows a clear separation between the Western controls and the African populations, while PC2, which explains a lower fraction of the total variability (19%), indicates a separation among Hadza, Malawians and BF. Separation along PC2 is also visualized among Western populations, but to a much lesser degree and with large interspersions between the U.S. and Italian children. Our data demonstrate biologically meaningful variation between the Western and non-Western GM profiles, showing that African populations with different lifestyles possess an overall more similar GM to each other than to Western populations. Although these results indicate a certain degree of GM variation among different African groups, we cannot exclude that a study effect may outweigh separation owing to actual differences in GM composition within these communities. While we do see that the US controls intersperse with the Italian children (green and light blue color coding respectively), the Italian adults from this study remain distinct from the other Western control samples, indicating that there may be some methodological bias that could also affect the observed GM differences among the African populations. Therefore, we urge caution in interpreting the strength of GM variation based on the separation seen among Hadza, BF, and Malawian populations in this single cross study comparison. Further caution is needed since subjects from all six populations are not age-matched.

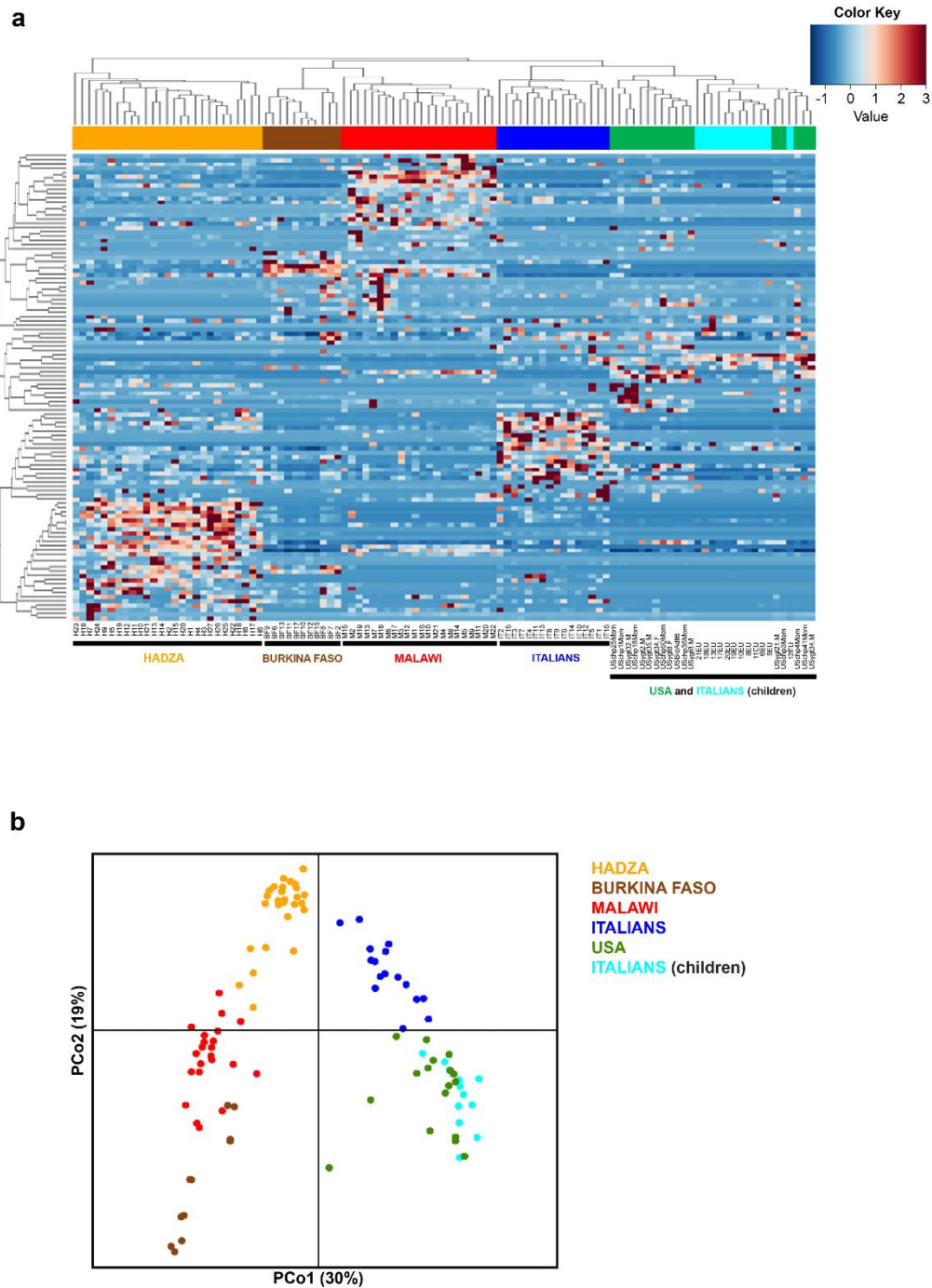


Fig 8. Comparison of GM relative abundance among populations reflects subsistence patterns. **(a)** Hierarchical clustering based on Eisen's formula of correlation similarity metric of bacterial genus proportion and average linkage clustering. Genera were filtered for subject prevalence of at least 30% of samples. Subjects are clustered in the top of panel and color-coded orange (Hadza), brown (Burkina Faso), red (Malawi), blue (Italian adult controls from this study), green (U.S. adults from Yatsunenko et al., 2012), and cyan (Italian children from De Filippo et al. 2010). 107 genera are visualized and clustered by the vertical tree. **(b)** PCoA based on Bray Curtis distances of the relative abundance of GM genera of each population.

To identify patterns of microbial community variation among Hadza, Malawian, BF and Western controls- Italian adults, Italian children, and US adults- we determined co-abundance associations between genera and then clustered them, resulting in six co-abundance groups (CAGs) (Supplementary Figure 2) (Claesson et al., 2012). In the context of this comparison, six CAGs define the microbial variation between populations ($P < 0.001$, permutational MANOVA). CAGs have been named according to the dominant genera in each group as follows: *Dialister*, *Faecalibacterium*, *Prevotella*, *Blautia*, *Clostridiales_unclassified* and *Ruminococcaceae_unclassified*. The Wiggum (Claesson et al., 2012) plot depicts the GM compositional relationship for each of the six populations and shows a correspondingly unique pattern of abundance of the six CAGs (Figure 9). Interestingly, African populations are characterized by the *Prevotella* CAG, while Western controls show a distinctive overall dominance of the *Faecalibacterium* CAG. With respect to Malawian and BF, Hadza show a peculiar combined enrichment of *Clostridiales_unclassified*, *Ruminococcaceae_unclassified* and *Blautia* CAGs. Given the dietary and lifestyle distinctions of each population, the CAG distribution in Hadza, Malawians, BF, and Western controls could represent predictable GM community specificity to three different modes of subsistence: foraging, rural farming, and industrial agriculture respectively. The unique CAG distribution of Hadza with respect to the other groups corresponds to the higher abundance of *Treponema* and unclassified *Bacteroidetes* and *Ruminococcaceae* co-residents in the Hadza microbiome. All Hadza we sampled share this configuration, therefore we must posit the possibility that these bacteria and their co-residents confer a structural and functional asset responding to the specific needs of the Hadza lifestyle. However, more forager and subsistence agriculture communities should be sampled to learn what aspects of subsistence drive microbe community assimilation and whether variability is a result of environment, host selection, or both.

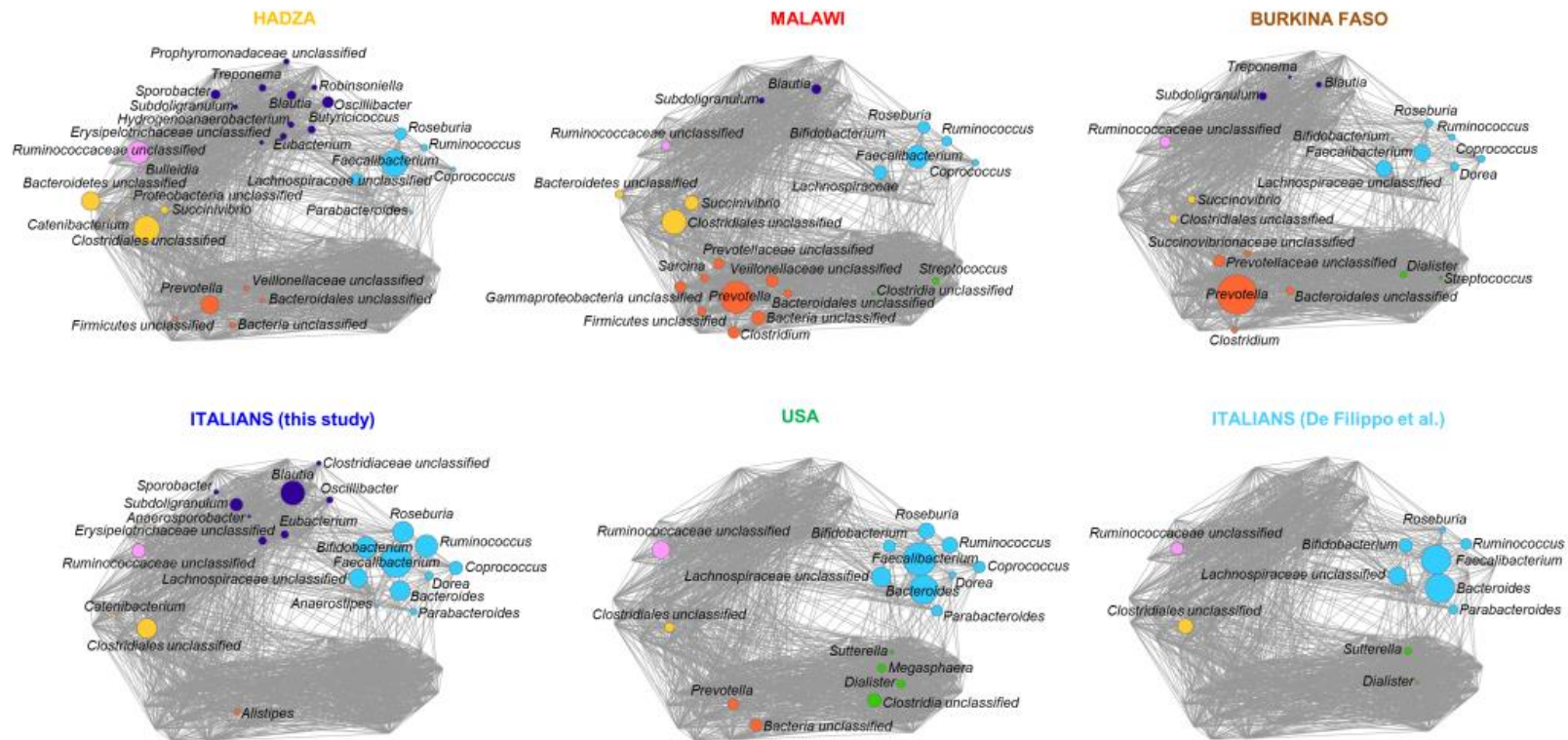


Fig 9. Distinct bacterial co-abundance groups define each population. Wiggum plots indicate pattern of variation of the six identified co-abundance groups (CAGs) in Hadza, Malawi, Burkina Faso, and Western controls. CAGs are named with the name of the most abundant genera and are color-coded as follows: Faecalibacterium (cyan), Dialister (green), Prevotella (orange), Clostridiales_unclassified (yellow), Ruminococcaceae_unclassified (pink), and Blautia (violet). Each node represents a bacterial genus and its dimension is proportional to the mean relative abundance within the population. Connections between nodes represent positive and significant Kendall correlation between genera (FDR < 0.05).

4.3.5 Short chain fatty acid profile of Hadza and Italians

End-products of bacterial fermentation are important for microbiota-host co-metabolism and evolution. Short chain fatty acids (SCFAs) are the dominant metabolites resulting from bacterial fermentation of plant derived substrates such as glycans and polysaccharides that pass undigested through the small intestine and into the colon. The SCFAs acetate, butyrate, and propionate are pivotal in several host physiological aspects such as nutrient acquisition, immune function, cell signaling, proliferation control, and pathogen protection (Tremaroli and Bäckhed, 2012).

Detected SCFA values for each sample are reported in Supplementary Table 2. PCA analysis of the SCFA relative abundance profiles show a segregation between Hadza and Italians ($P=0.02$, permutation test with pseudo F-ratio) (Figure 10). The Italian samples are characterized by a significantly ($P<0.01$, Mann-Whitney U test) greater relative abundance of butyrate, while Hadza samples are enriched in propionate ($P<0.01$). These differences may reflect dietary variation in both amount and type of fiber and carbohydrates consumed by Hadza and Italians, and the consequent relative depletion in butyrate producers belonging to the *Clostridium* cluster IV and XIVa in Hadza.

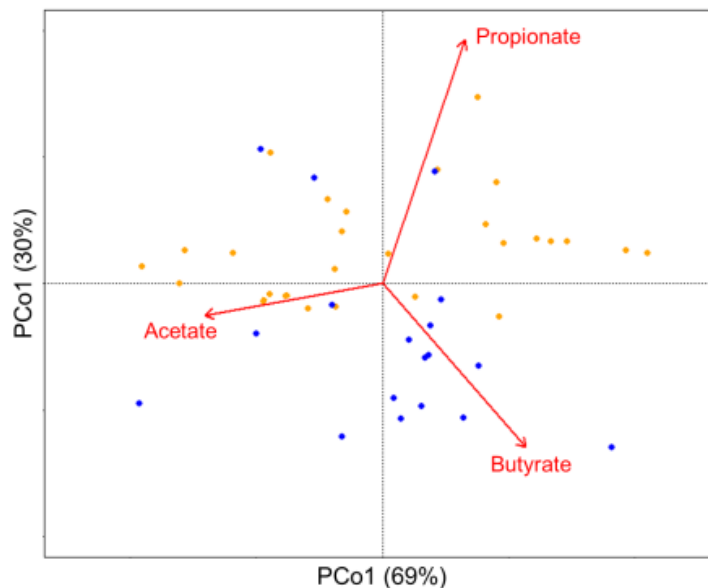


Fig 10. Comparison of metabolite production between Hadza and Italian samples. PCoA based on Euclidean distances of the profiles of short chain fatty acid relative abundance in Hadza (orange) and Italians (blue). Vectors show propionate, butyrate and acetate abundance.

However, because of the high degree of metabolic cross-feeding between members of the human gut microbial ecosystem (Flint et al., 2008), direct associative relations between bacteria presence/absence and SCFA production are not so simple. In order to investigate gut microbial networks on the basis of the observed differences in patterns of SCFA production in Hadza and Italians, we evaluated the GM

genera that correlate significantly with each SCFA (Supplementary Table 3). Among genera with greater than 5% relative abundance in at least one of the two populations, *Bifidobacterium*, *Bacteroides*, *Blautia*, *Faecalibacterium* and *Ruminococcus* are positively ($P < 0.05$, Kendall tau rank correlation coefficient) correlated with butyrate, showing Kendall correlation values of 0.30, 0.31, 0.32, 0.52 and 0.30, respectively. In contrast, *Bifidobacterium*, *Blautia* and *Lachnospiraceae* show a significant ($P < 0.05$, Kendall tau rank correlation) negative correlation with propionate of -0.36, -0.27 and -0.24, respectively, while *Prevotella* demonstrates a positive correlation of 0.41. The absence of *Bifidobacterium* and the lower relative abundance of *Blautia*, *Ruminococcus* and *Faecalibacterium* concurrent with greater relative abundance of *Prevotella* seen in the Hadza GM match a presence/absence scenario with SCFA concentrations that are enriched in propionate and reduced in butyrate with respect to Italians.

Though SCFAs are metabolic end-products for bacteria, they are important direct energy resources for the host. Butyrate is produced from dietary fiber, and when present in sufficient quantity, it becomes the major fuel source for colonic epithelial cells, reducing the need for energy allocation to these cells from the host (den Besten et al., 2013). Propionate is transferred to the liver where it serves as a precursor for hepatic gluconeogenesis (Nicholson et al., 2012; Tremaroli and Bäckhed, 2012; den Besten et al., 2013). The extra energy derived from these GM-produced SCFAs may provide nutritional support for the Hadza whose diet contains high amounts of fiber but is seasonally lean in lipids.

4.4 Discussion and conclusions

The Hadza represent a rare example of human subsistence through hunting and gathering that persists in the same East African region where early hominins lived. The Hadza maintain a direct interface with the natural environment, deriving their food, water, and shelter from a rich biosphere blanketed in the complexity of microbial communities and interactions. In our characterization of the Hadza GM, we report several findings that we feel support the conception of the microbiome as a diverse and responsive ecosystem adapting continuously as a commensal component of the host supra-organism. Keeping this framework in mind, we interpret the GM structure as an adaptation to the Hadza foraging lifestyle.



Image 12. Hadza woman stops to drink from a naturally occurring spring on the way to dig tubers.

The Hadza GM has characteristic features that are consistent with a heavily plant-based diet. Besides the presence of several well-known fiber-degrading Firmicutes that are also shared with Italians – e.g. members of *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, Clostridiales Incertae Sedis XIV and *Clostridiaceae*, the Hadza GM is enriched in *Prevotella*, *Treponema*, and unclassified members of Bacteroidetes, Clostridiales and *Ruminococcaceae*. These xylan-degrading *Prevotella* (Flint et al., 2008) and *Treponema* (Warnecke et al., 2007) and the abundance of still unclassified Bacteroidetes and Clostridiales, groups known for their fibrinolytic capabilities, may provide the Hadza GM with specific glycan-degrading abilities for Hadza to deal with a vast array of refractory and resistant organic materials introduced through diet.

Consistent with GM arrangements reported for other African groups (De Filippo et al., 2010; Yatsunenکو et al., 2012; Ou et al., 2013), the Hadza GM shows a higher relative abundance of *Prevotella*, but with a correspondent reduction of *Bacteroides* in the gut ecosystem compared to the Italian control cohort. Thus, similar to what has been proposed for rural Africans consuming grain-based high-fiber diets (De Filippo et al., 2010), it is tempting to speculate that this microbe community within the Bacteroidetes phylum could harbor the necessary GM functions to deal with their especially unique, but also highly fibrous, plant food dietary constituents (Hehemann et al., 2010).

During our visit in January, 2013, between two rainy periods, there were a variety of foods available and acquired, the majority of which were derived from plants. These included at least four species of tuber, small and large game, honey from stinging and stingless bees, leafy green foliage, baobab fruit, and one species of berry. Tubers are an incredibly important food in the Hadza diet because they are consistently available and exploited year round, despite being the lowest ranked food

resource (Marlowe and Berbesque, 2009). Hadza tubers are uncultivated wild species belonging to the plant families Fabaceae (legumes), Convolvulaceae (morning glories and herbaceous vines), and Cucurbitaceae (squashes, melons, and gourds). Only the underground root is harvested and consumed either raw or briefly roasted. It is noteworthy that most of the tubers consumed by Hadza contain high moisture and tough indigestible fibers that must be expelled as a quid during chewing (Figure 11). The digestible fraction is thus incredibly variable but composed largely of water, simple sugars, starch and soluble fiber.



Fig 11. Insoluble fiber found in most Hadza tubers. Pictured above are two slices of one mak'alitako tuber (*Emminia entennulifa*). The left slice is pre-chewed and the right slice is post-chewing to show the resiliency of the insoluble fiber. These fibers are not swallowed by the Hadza.

Several publications have outlined the basics of Hadza diet (Vincent, 1985; Murray et al., 2001; Schoeninger et al., 2001; Marlowe, 2010; Crittenden, 2011; Peterson et al., 2012) and have converged on the following general characteristics. The majority of the annual Hadza diet (approximately 70% of kilocalories) comes from plant foods (Marlowe, 2010). Birds, small, medium, and large sized game meat comprise approximately 30% of the annual diet (Marlowe, 2010). See Marlowe (2010) and Peterson et al. (2012) for an exhaustive list of all species targeted. Small variation exists between published sources depending on whether kilogram wet weight or kilocalories per gram were used to calculate percent contribution to diet. Resource availability – both plant and animal – is highly correlated with rainfall patterns; therefore diet varies year to year as well as season to season. A general dietary pattern does emerge, however, and indicates that more meat is consumed during the dry season when people and game animals converge to target the same watering holes (Woodburn, 1968). Foods like baobab, tubers, and honey are targeted year round. Based on these data, the resulting picture is a diet rich in simple sugars, starch and protein while lean in fat. It would be of great interest to learn whether the shift from a largely plant-based diet to one that includes more meat, such as during the dry season, might show a concurrent change in GM structure amongst Hadza.

We find evidence of a sex-related divergence in Hadza GM structure, which is not documented in other human groups. This divergence corresponds to the Hadza sexual division of labor and sex

differences in diet composition. In the same environment with access to the same dietary resources, Hadza men and women are differentially adapted to their particular pattern of food consumption. The potential for Hadza women's GM to respond with significant structural differences to the increased consumption of plant foods represents a profound break with traditional thinking on the limited digestive capacity of the human gut and the constraint it imposes on nutritional provisioning for reproduction and brain growth (Aiello and Wheeler, 1995; Wrangham and Conklin-Brittain, 2003). Women's foraging must adequately provision for pregnancy and lactation, which is a strong adaptive pressure for the GM to derive the most energy from consistently available plant foods. In this regard, the GM aligns with the host nutrition acquisition strategy, thus potentially buffering women from resource "gaps" that may lead to nutritional deficiencies.

The reported presence of *Treponema* in now five geographically separate extant rural human communities from this and previous studies (Hadza, Burkinabe, Malawians, South Africans, and Venezuelan Amerindians) (Tito et al., 2012; Ou et al., 2013) supports an alternative functional role for this bacterial group whose expression in industrialized communities is normally attributed to pathogenic disease. De Filippo *et al.* hypothesize that the presence of *Treponema* in BF children enhance the host's ability to extract nutrients from the fibrous foods that comprise their traditional diet (De Filippo et al., 2010). While the Hadza do not eat agricultural or grain-based diets, they do rely heavily on fibrous tubers throughout the year, with women often consuming tubers for a greater percentage of daily calories than do men (Berbesque et al., 2011). These sources of fiber rich plant foods could similarly encourage a mutualistic *Treponema* population whose fibrinolytic specializations would be advantageous to Hadza nutritional acquisition, particularly in women.

Medical examinations conducted on Hadza found evidence of Treponematoses from serum samples at low rates (13 out of 215 sampled) with the highest prevalence in men of settled Hadza camps between 1966 and 1967 (Bennett et al., 1973). However, there was low but consistent prevalence for women in both settled and foraging Hadza groups with little clinical evidence of yaws, suggesting immunoregulation of *Treponema* pathogens.

Demographic reports of age structure, population density, growth and fertility indicate that the Hadza appear to be a healthy and stable savanna foraging population despite rapid encroachment of pastoralist groups in the same region (Blurton Jones et al., 1992). For a foraging population with little to no access to health care or medical facilities, the Hadza have relatively low rates of infectious disease, metabolic disease, and nutritional deficiencies in comparison to other settled groups in the northern Tanzania and southeastern Uganda region (Bennett et al., 1973; Work et al., 1973; Blurton Jones et al., 1992). However, these earlier assessments were more than 40 years ago, over two Hadza-generations, and many changes have since occurred to the land occupied by the Hadza. Re-evaluation of health and population metrics deserves renewed focus, especially now that research on the Hadza has garnered much attention.

The absence of Actinobacteria, particularly *Bifidobacterium*, in the Hadza GM is unexpected.

Bifidobacteria are associated with breastfeeding in infants and achieve large proportions of the GM in the first few months after birth (Turrone et al., 2009). Typically, in adults, bifidobacteria commonly make up 1-10% of the GM population. Complete absence of bifidobacteria, as observed in the Hadza, has never to our knowledge been reported for any other human group. We hypothesize that the lack of bifidobacteria in adult Hadza is a consequence of the post-weaning GM composition in the absence of agro-pastoral-derived foods. Support for this hypothesis comes from the observation that other populations in which meat and/or dairy consumption is low to absent, such as vegans and Koreans, also have very low representation of Actinobacteria and *Bifidobacterium* (Nam et al., 2011; Zimmer et al., 2012). The continued consumption of dairy into adulthood could be one reason most Western populations maintain a relatively large bifidobacterial presence. Aside from bifidobacterial species of human origin, the majority of *Bifidobacterium* have been isolated from livestock animals such as swine, cattle, and rabbit (Ventura et al., 2007; Turrone et al., 2009). The Hadza neither domesticate nor have direct contact with livestock animals. Thus, as they lack exposure to livestock bifidobacteria, this raises the question of whether the necessary conditions for interspecies transfer and colonization of bifidobacteria do not occur for the Hadza (Moeller et al., 2013). The Hadza retain a strong independent identity both in their native language and oral history, which says nothing about a previous pastoral or agricultural existence (Marlowe, 2010). Early Y chromosome and mitochondrial DNA analysis shows some of the highest genetic divergence between Hadza and members of the Khoisan/San language group, the Ju/'hoansi († Kung), evidence suggestive of a very ancient lineage (Knight et al., 2003). Given their penchant for social timidity during early attempts at first contact and resistance to assimilation in the second half of the twentieth century, it is very likely the Hadza persist with a very ancient traditional lifestyle into present times (Marlowe, 2010).

Future work must focus on the GM of breast-fed Hadza infants to determine the role of bifidobacteria in the kinetics of assembly and development of the Hadza GM, and to learn whether this bacterial group is completely absent in all Hadza, including infants, or if it is definitively lost from the gut ecosystem post-weaning. It is important to note that while bifidobacteria are considered a beneficial bacterial group in Western GM profiles, their absence in the Hadza GM, combined with the alternative enrichment in “opportunistic” bacteria from Proteobacteria and Spirochaetes, cannot be considered aberrant. On the contrary, the Hadza GM likely represents a new equilibrium that is beneficial and symbiotic to the Hadza living environment. Support for the advantage of such novel GM configurations comes from the finding that GM restructuring also occurs in centenarians (Biagi et al., 2010), who are extreme examples of organismal robusticity. Additionally, these findings illustrate a need to reevaluate the standards by which we consider gut microbiota “healthy” or “unhealthy”, as they are clearly context dependent.

GM diversity, as found in rural African populations and now in the Hadza, is almost certainly the ancestral state for humans. Adaptation to the post-industrialized Western lifestyle is coincident with a reduction in GM diversity, and as a result, a decline in GM stability. Diversity and stability are factors

with major health implications, particularly now that the human gastro-intestinal tract is increasingly recognized as the gateway to pathogenic, metabolic, and immunologic diseases (Blaser and Falkow, 2009). Co-speciation between host and microbiota over millions of years has shaped both sets of organisms into mutualistic supra-organisms. Dissolving that contact through sterilization and limited environmental exposure has had a drastic effect on health and immune function of modern Westernized human groups. The Hadza GM is likely an “old friend” and stable arrangement fitting their traditional hunter-gatherer lifestyle (Rook et al., 2004).

We are only just beginning to document GM diversity across populations. In our study, more than 33% of the total Hadza GM genera remain unidentified. Such taxonomic uncertainty holds exciting prospects for discovering yet-unknown microbial genetic arrangements. This finding also underscores the importance of increasing our reference phylogenies and resolving deep taxonomic relationships between bacteria by sampling a wider variety of environments and extreme ecological zones (Rinke et al., 2013).

In summary, the characterization of the Hadza GM presents a suite of unique features that suggest specific adaptation to a foraging lifestyle, which includes a large proportion of highly refractory plant foods. We expect that detailed study of the function of this GM community will expose a greater number of genetic specializations for degrading polysaccharides than what is currently found in other human populations. When viewed broadly, inconsistencies in associations among GM structure, diet, and disease belie interpretive confidence about GM phenotypes. The functional redundancy found in bacterial communities indicates that microbial activity, rather than composition, is conserved. However, the ability of novel genes to propagate through environmental transfer into common gut bacteria complicates the enterotype–function paradigm. Moreover, closely related human symbiont microorganisms have been demonstrated to differ widely in their glycan use phenotypes and corresponding genomic structures (Martens et al., 2011). Even if taxonomic similarities do exist between human populations, at finer scales their GM communities may exhibit dramatic metabolic differences tailored to suit disparate environmental constraints. With a microbiome functional assignment rate at 60% (Wu et al., 2011), these questions need to be resolved by testing GM activity using *in-vivo* techniques such as with gnotobiotic mice (Lecuit et al., 2007) or *in-vitro* techniques such as with computer controlled simulations of the large intestine (Minekus et al., 1999). Furthermore, comparative analysis between the human and great ape GM, especially with members of *Pan*, will highlight important distinctions that enabled early human ancestors to extend their dietary and ecological ranges without the need for technological buffering. Host-microbiome mutualism holds great relevance to the field of human evolution as it vastly propels the genetic landscape for adaptation well beyond somatic potential.

4.5 References

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