Differences in gut microbiome composition among the Hadza population of Tanzania are driven by subsistence systems.

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SUMMARY The gut microbiome is a critical part of human health and is sensitive to various biotic and abiotic factors. Elucidating the interactions between these factors and the microbiome is essential in understanding how microbial species impact human health. Different subsistence systems have been shown to be a major factor that influences the gut microbiome composition. This study aims to build upon these findings in the context of the non-industrialized Hadza population in Tanzania, who exist in groups of bush camps that rely on hunting-gathering and agricultural practices. Of eight select bush camps, groups who relied on agriculture showed greater relative abundance of Bacteoridetes and Firmicutes in their gut microbiomes at a higher Firmicutes:Bacteroidetes ratio than their hunter-gatherer counterparts. While geographical distance was shown to have a weak correlative influence on differences between Hadza gut microbiomes, water source, age, and biological sex had no significant impact.

INTRODUCTION

The gut microbiome, composed of trillions of microorganisms in the gastrointestinal tract (1), plays an integral role in host health and disease, not only in the gut but in numerous far-reaching organs in the body. Consequently, the gut microbiome holds high therapeutic potential in developing novel treatments for disease. It has also become increasingly evident that gut microbiome composition differs significantly across environmental and biological factors, making it difficult to establish universal biomarkers and therapeutic targets (1).

Human evolution has progressed from hunter-gatherers, a traditional lifestyle consisting of hunting and foraging (2), to utilization of agricultural techniques and more modern methods of food acquisition. Farming allowed humans to have a consistent food supply and create permanent settlements (3) which has led to industrialized societies around the world.

This social evolution led to the formation of the Wealthy, Educated, Industrialized, Rich, and Developed (WEIRD) (4) countries present today, but something else has evolved along with the world's countries: the human gut microbiome. To date, studies investigating the effect of influential factors on microbiome diversity have been partial to WEIRD countries. Studies that do include populations leading traditional lifestyles have done so in the context of comparing between WEIRD and non-WEIRD populations. These studies have identified greater abundances of Actinobacteria and Firmicutes phyla in WEIRD populations, and greater abundances of the Proteobacteria phylum in non-WEIRD populations (5, 6). This distinction can be traced further back by comparing traditional populations with different means of obtaining food, also referred to as subsistence systems (7). It has been found that gut microbiomes of agriculturalists contain significantly greater abundances of Actinobacteria and Firmicutes than hunter-gatherers (8, 9), in parallel to the previous comparison between WEIRD and non-WEIRD populations. However, a knowledge gap still remains when it comes to elucidating the effects of environmental and biological factors solely in non-WEIRD populations. Currently, few studies investigate this subject, and further research is required to fully understand the impacts of human lifestyle transitions on the gut microflora. This not only deepens our knowledge of the differences between WEIRD and non-WEIRD populations, but also reveals potentially significant differences within non-WEIRD groups.

In addition to subsistence systems, a variety of environmental and biological factors are known to implicate gut microbial composition. Water harbours an abundant and diverse group of bacterial species (5). Previous studies have shown that water sources may drive bacterial composition within the gut microbiome (6–8). Given that industrialized water sources rely on filtration systems and sterile cleaners (6), looking into populations that obtain water from natural sources may be beneficial in identifying the extent to which the human gut microbiome may be influenced by microbes present in water. Geography has also been investigated as a potential driver of compositional variation in the gut microbiome (9, 10). However, it is difficult to discern the contributions of geography and its environmental factors from other variables such as culture, genetic similarity, and degree of industrialization that are also involved in large geographic distances. Therefore, it is worthwhile to determine the power of geographic distances to create variability on a small scale.

Aside from environmental factors, it has also been suggested that biological factors may influence the gut microbiome. While it is unclear how the gut microbiome is impacted by sex differences in humans (18, 19, 20), some studies have shown differences in microbiome alpha-diversity in male and female populations post-puberty (11). Further, age does seem to have an effect on gut microbial composition. Research has shown that the human microbiome transitions through distinct developmental phases, with microbiome stabilization occurring at around 2.5 years (12).

In order to explore how the gut microbiome varies in response to these different environmental and biological factors, we analyzed the gut microbiomes of the Hadza hunter-gatherers of Tanzania. The Hadza population comprises bush camps leading a hunter-gatherer lifestyle, as well as others that have transitioned to a farming lifestyle. As one of the few remaining populations to lead a traditional lifestyle, the Hadza may provide insight on the variation of gut microbial communities within traditional populations and the effects of urbanization on the gut microbiome (13).


METHODS AND MATERIALS

Metadata source and pre-processing. The metadata used in this study was sourced from an earlier publication by Smits et al. in 2017 (14). To better suit the research aims of the current study, some variables contained within the metadata were modified in R (15) and then filtered in Qiime2 (16). Briefly, sex was recategorized into 4 classifications, as males that were either pre- or post-puberty, and similarly for females with puberty onset determined as 15 years of age (17). When redefining sex categories, subjects 3 years of age or below and 70 years of age or above were omitted (18, 19). The original age variable was reclassified to account for the 4 categories of gut microbiome development previously outlined by Stewart et al.: developmental (3-14 months), transitional (15-30 months), stable (31-46 months), and adult (46+ months) (12).

16S rRNA sequencing data were demultiplexed and quality filtered using packages within Qiime2 (16), with the first 13 bases of each sequence trimmed and the truncation length set to 193 to remove bases with a quality score less than 34. Trimmed reads were denoised into amplicon sequence variants (ASVs) and then rarefied to 13000.

Data analyses. Alpha diversity was assessed using Faith's Phylogenetic Diversity (PD) and Pielou's Evenness to evaluate community richness while considering phylogenetic distances and species evenness, respectively, as implemented in Qiime2 (16). Beta diversity was also analyzed with Qiime2 using weighted UniFrac (20). Statistical measures such as PERMANOVA, and pairwise Kruskal-Wallis tests (21) were similarly performed in Qiime2. Two-dimensional Principal Coordinate Analysis (PCoA) plots, box-and-whisker plots, and scatterplots were generated using R, along with visualization of relative abundance and differential abundance using the *tidyverse*, *vegan*, *phyloseq*, *DEseq*, and *microbiome* libraries.

Taxonomic Analysis. Taxonomic information for the ASVs were assigned according to Greengenes 16s reference database (22) at 99% identity. Taxonomy files were generated using the Qiime2 feature classifier.

Mapping geographical coordinates and reference bush camp selection. Geographical coordinates of bush camps of interest (Dedauko, Gideru, Hukamako, Kipamba, Makao, Mwamudu, Onowas, and Sengeli) were obtained from Daudi Peterson of the Dorobo Fund and mapped using Google My Maps. Relative distances of bush camps from reference camp Hukamako were also determined using Google My Maps. Selection of Hukamako as the reference bush camp was determined on the basis of 2 factors: the largest proportion of filtered samples within the metadata were collected from Hukamako and mapping the bush camp coordinates revealed that Hukamako was centrally located amongst the remaining bush camps of interest.
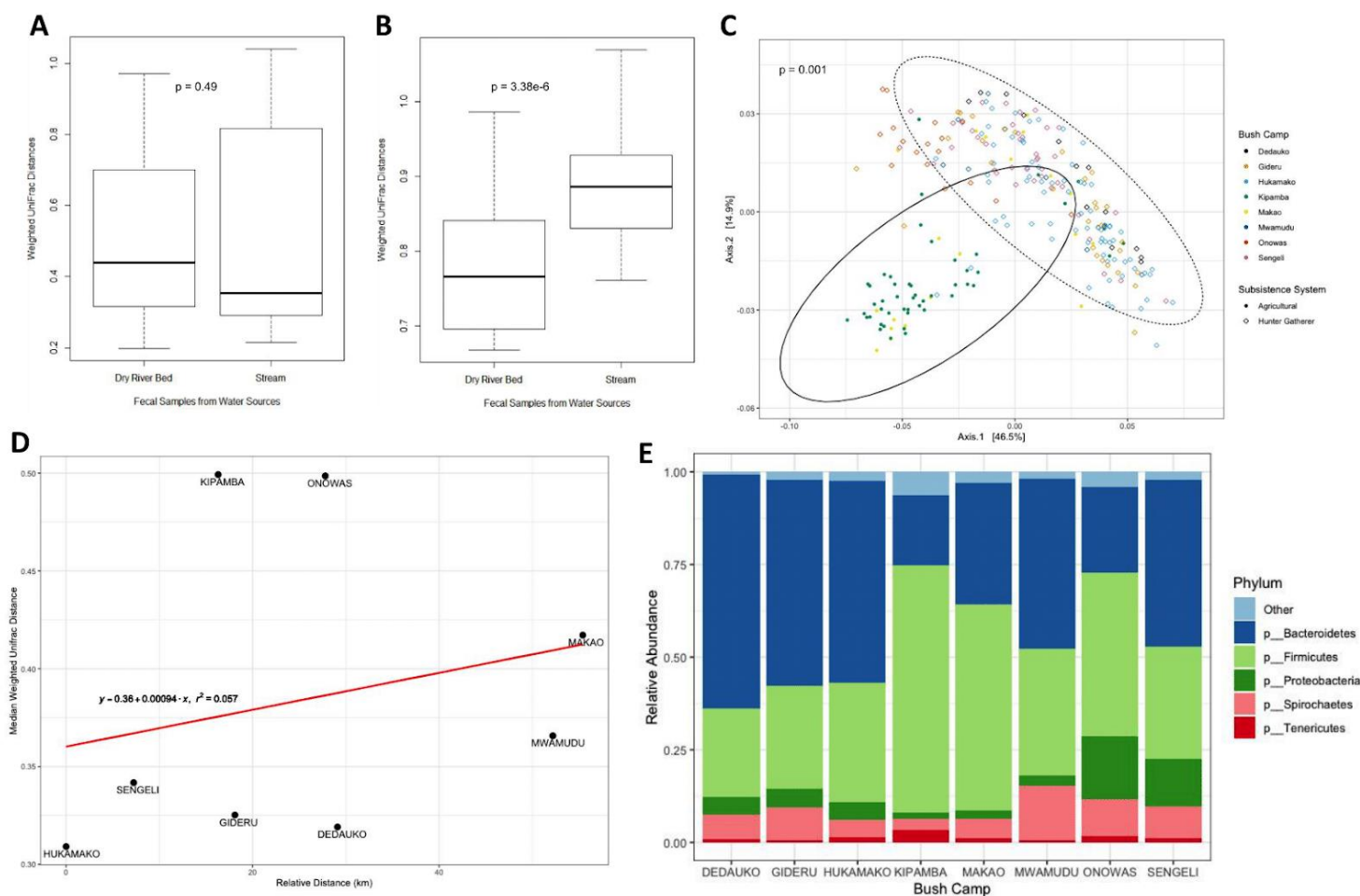
FIG. 1 Influences of water source, geographical location, and subsistence system on gut microbial composition. (A) Comparison of microbial differences between fecal samples collected from individuals utilizing dry river bed or stream as water source and water samples collected from stream (p-adjusted = 0.49; PERMANOVA). **(B)** Comparison of microbial differences between fecal samples collected from individuals utilizing dry river bed or stream as water source and water samples collected from dry river bed (p-adjusted = 3.38e-6; PERMANOVA). Boxplot whiskers represent the range of minimum and maximum distance values in both graphs. **(C)** Weighted UniFrac PCoA applied to gut microbial composition by bush camp and subsistence system. Ellipses borders represent 95% confidence intervals. **(D)** Median weighted UniFrac distance plotted against geographical distance relative to Hukamako (r^2 = 0.057). **(E)** Relative taxonomic abundance of fecal microbes summarized at the phylum level among bush camps.

RESULTS

Water source and geographical distance do not drive differences in gut microbial composition in the Hadza population. Previous studies have shown that drinking water can have an impact on the gut microbiome of germ-free mice and humans (7, 8). To test whether the microbial composition of a bush camp population resembles that of its water source, the metadata was filtered for human fecal and environmental water samples from the Hukamako, Makao, and Mwamudu, who obtain water from stream, dry river bed, and dry river bed sources, respectively. While the microbial composition of groups accessing the dry river bed more closely resembled that of dry river bed samples (p-adjusted = 3.38e-6; PERMANOVA) (Fig. 1A), the microbial composition of groups accessing streams did not significantly differ from the other group in their resemblance to that of stream samples (p-adjusted = 0.49; PERMANOVA) (Fig. 1B). Weighted UniFrac was chosen to account for both abundance and phylogenetic relatedness, although comparisons made using other beta-diversity metrics produced similar results. Geographical location across fine spatial scales has also been shown to influence gut microbiome composition (23). The metadata was newly filtered for human fecal samples from the late-dry seasons from eight bush camps, and geographical data for the camps were obtained from personal communication with Daudi Peterson of the Dorobo Fund (Supplementary Table 1). Overall, species relatedness in microbial communities within camps did not differ significantly (Supplementary Fig. 1). Weighted UniFrac principal coordinate analysis did not show unique clustering for all bush camps (Fig. 1C), and specifying for sex or age groups within bush camps did not contribute to more significant clustering (Supplementary Fig. 2, 3) (see Methods for additonal filtering steps). Pairwise PERMANOVA tests revealed significant beta-diversity differences between the majority of camps (Supplementary Table 2). However, contrary to our hypothesis that the extent of compositional differences may be scalable by geographical distance, median weighted UniFrac distance only weakly increased with geographical distance relative to the Hukamako camp (see Methods for reference camp selection) (Fig. 1D). To investigate which species were contributing to the observed beta-diversity differences, we performed a taxonomic analysis then averaged relative abundances by bush camps at the phylum level, and found higher abundance of Firmicutes and lower abundance of Bacteroidetes in the Kipamba, Makao, and Onowas bush camps (Fig. 1E).
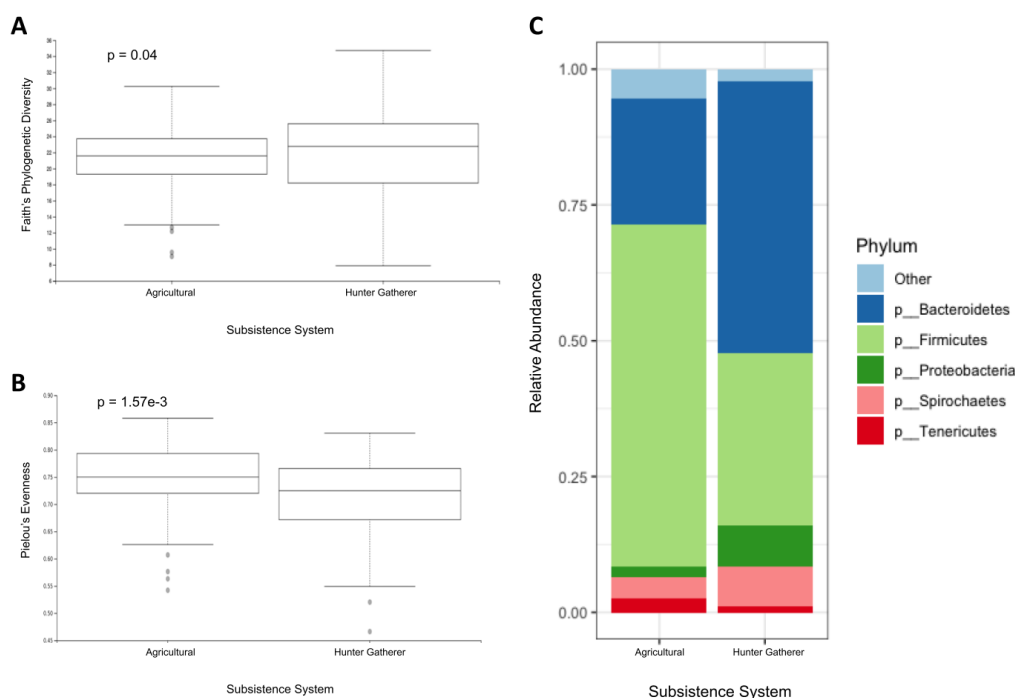
FIG. 2 Gut microbial differences between Hadza bush camps of Tanzania are associated with subsistence systems. **(A)** Faith's Phylogenetic Diversity and **(B)** Pielou's Evenness metrics applied to gut microbial composition of bush camps relying on agricultural or hunter gatherer subsistence systems (p=0.04 and p=1.57e-3, respectively; Kruskal-Wallis). The range of minimum and maximum alpha diversity values are represented as boxplot whiskers. Outliers are indicated as dots. **(C)** Relative taxonomic abundance of fecal microbes summarized at the phylum level among bush camps relying on either agricultural or hunter gatherer subsistence systems.

Subsistence system influences microbiome composition. The Kipamba and Makao bush camps differ from others in that their subsistence system has shifted from hunting-gathering to agriculture (information from personal correspondence with Daudi Peterson). Notably, it was apparent that the fecal samples from these two camps clustered significantly differently from those of other camps (Fig. 1C). Their microbial compositions, also differ from other camps (Fig. 1E), which prompted us to investigate further into how these contrasting subsistence systems may be contributing to the differences seen in microbial composition. A column for subsistence system was created in the filtered metadata file with samples from Kipamba and Makao bush camps categorized as "farmers" and the remaining as "hunter-gatherers". Interestingly, phylogenetic diversity was significantly higher in hunter-gatherer populations than in farmer populations ($p = 0.04$; Kruskal-Wallis) (Fig. 2A), while evenness was significantly lower ($p = 0.0016$; Kruskal-Wallis) (Fig. 2B). Weighted UniFrac analysis showed significantly different clustering between farmers and hunter-gatherers ($p = 0.001$) (Fig. 1C). Relative abundances averaged by subsistence systems at the phylum level found that Bacteroidetes and Firmicutes are much more dominant in the microbial composition of agricultural groups, at a higher Firmicutes:Bacteroidetes ratio than in hunter-gatherer groups. Hunter-gatherer groups also have higher abundances of Proteobacteria and Spirochaetes, and lower abundance of Tenericutes species (Fig. 2C). To identify phyla that were significantly different in abundance between farmer and hunter-gatherer groups, differential abundance analysis was performed ($\alpha = 0.05$). Six phyla were identified between the two subsistence systems, with Spirochaetes, Proteobacteria, and Bacteroidetes more abundant in hunter-gatherer populations and Firmicutes, Euryarchaeota, and Actinobacteria more abundant in agricultural populations (Fig. 3).
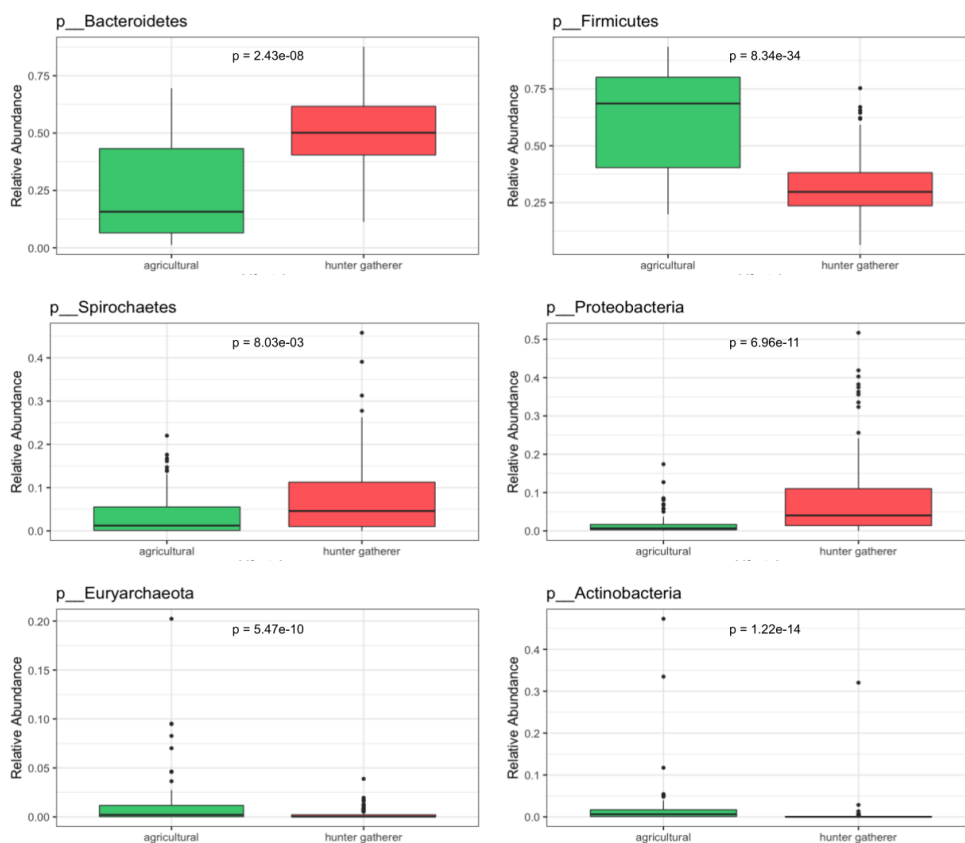
FIG. 3 Boxplots for taxa exhibiting significantly different relative abundances between agricultural and hunter gatherer bush camps. Assessed at the phylum level, relative abundances are displayed in a range from 0-1. Whiskers denote minimum and maximum values within bush camps relying on either subsistence system, while outliers are indicated as dots.

DISCUSSION

Our analysis of water source influences on the gut microbiome was limited by the small number of environmental samples available to us. Water samples were only collected from four different bush camps with one camp containing only one fecal sample, and thus was omitted from our study. Therefore, we cannot generalize the relationships that we found in

our study to fit the larger population. Three samples were obtained from the stream water source, whereas only two samples were obtained from the dry river bed water source. Thus, we cannot be certain that the obtained water samples accurately reflect the microbial composition of the water source. Differences in beta-diversity were statistically significant between most bush camps; however, the degree of difference only weakly correlated with increasing geographical distance. This suggests that distance alone may not be a major geographical contributor to compositional variation in the gut microbiome. Other environmental differences that vary by geography, such as elevation, climate, and vegetation, may be more influential in shaping different microbial communities within the gut.

The factor which was observed to have the greatest impact on the diversity and composition of the gut microbiome was the subsistence system on which bush camp members relied. This corroborates the results of previous research that compared human gut microbiomes of rural hunter-gatherer communities to recently transitioned farming communities in Himalayan populations, which found significant differences in beta-diversity dependent on group subsistence systems (24). Interestingly, some studies did not observe significant differences in alpha-diversity based on subsistence systems (13). The conflicting results in alpha-diversity differences may suggest that subsistence systems do not consistently impact alpha-diversity on a global scale. Rather, these differences in gut microbiome composition may be attributable to the vastly different cultures, climates, and diets of rural Himalayan populations (24).

A major benefit of agriculturally-based subsistence systems is that they allow greater food production and storage, and thus may contribute to more consistent diets compared to hunting-gathering (25). As diet greatly impacts the human gut microbiome, a variable diet may consequently induce changes to the gut microbial composition (26). Therefore, communities with agricultural subsistence systems may have more consistent and uniform gut microbiome compositions as a result of greater consistencies in diet. This is supported by comparisons between the Hadza hunter-gatherers and populations within WEIRD countries. Gut microbiomes of the Hadza people have been suggested to be more volatile and prone to seasonal shifts in bacterial taxa and diversity compared to the microbiomes of individuals inhabiting WEIRD countries (14), where commercial food production has largely decreased diet variability. This study extends beyond comparisons between WEIRD and non-WEIRD populations and depicts variability in the gut microbiome even within the Hadza population when comparing between hunting-gathering and farming bush camps. Hunter-gatherer bush camps had significantly lower Pielou's Evenness (**Fig. 2B**) than camps reliant on agriculture. Pielou's Evenness is a measure of relative abundance and compares the uniformity of samples, suggesting that Hadza hunter-gatherers have less evenly distributed gut microbiomes than individuals in Hadza agricultural bush camps. The consistent diets created by the agricultural subsistence systems in Hadza bush camps may aid in the establishment of specific microbes within the gut. This finding highlights the alpha-diversity differences that exist within the Hadza people and reveals the information that is lost when they are categorized inclusively as hunter-gatherers.

A parallel trend seems to occur between the progression of subsistence systems and the development of the human gut microbiome. As hunter-gatherers progress to agricultural subsistence systems, there appears to be a decrease in the abundance of Proteobacteria and an increase in the abundance of Actinobacteria and Firmicutes (24, 27). Hunter-gatherers of the Hadza have also been shown to have greater Spirochaetes than agriculturally-based communities (27), which is mirrored in our results (**Fig. 3**). This study shows Hadza hunter-gatherer bush camps possess a greater abundance of Spirochaetes and Proteobacteria while agricultural camps have more Actinobacteria and Firmicutes. Additionally, we reveal an interesting finding that has not previously been observed in the gut microbiome of the Hadza people when subsistence systems are taken into account, where hunter-gatherers appear to possess greater abundance of Bacteroidetes while farmers possess greater abundance of Euryarchaeota.

It is unknown when the human gut microbiome evolved during the industrialization of society or how long it took to transition. What is apparent is that there is a clear transformation in the human gut microbiome as human populations develop. This study

shows that this microbiome transition does not take long. Even on a small time scale, with the agricultural-based Hadza bush camps only recently transitioning from hunter-gatherers (28), a clear influence on the gut microbiome is seen. No mechanism has been identified to explain subsistence-based microbiome changes but possible explanations have been offered.

One explanation that is supported by this study is that rising obesity rates as hunter-gatherer communities develop into industrialized societies impacts the gut microbiome composition. The Firmicutes:Bacteroidetes ratio of the gut microbiome is a potential biomarker of obesity (29). It has been shown obese human subjects, when compared to healthy controls, have a higher Firmicutes:Bacteroidetes ratio (30). When subjects are put onto a calorie-restricted diet the decrease and normalization of the Firmicutes:Bacteroidetes ratio is proportional to weight loss (30). With hunter-gatherers having non-existent obesity levels they would have a very low Firmicutes-Bacteroidetes ratio which is supported by hunter-gatherer subsistence system bush camps having significantly less Firmicutes and more Bacteroidetes than farmers. While individuals in Hadza bush camps utilizing agriculture are not expected to be obese or drastically heavier than hunter-gatherers, the increased production and storage of food likely allows an increased caloric intake resulting in the increased Firmicutes-Bacteroidetes ratio observed.

Euryarchaeota is the only class of Archaea known to colonize the human body (31). A small number of their functional benefits in the gut have been identified. One being that methanogens, a major class of anaerobic methane producing Euryarchaeota (32), have a better ability to metabolize digested heavy metals compared to bacterial species (31). Heavy metals are prevalent in raw or contaminated meat (33) and if mining is prevalent in an area it can increase the amount of heavy metal toxins in soil which bioaccumulates into crops (34). With mining making up 50% of Tanzania's total exports it is highly prevalent throughout the country (35). Additionally, due to laxer mining regulations in non-WEIRD countries it has been shown they have drastically higher heavy metal levels in their soil and crops (36). The elevated levels of Euryarchaeota methanogens observed would benefit individual's of Hadza agricultural-based bush camps by increasing metabolism of the heavy metals being consumed from a diet based on crops.

Limitations The major limitation to this study is the limited sample size and sample diversity. With over 120 distinct bush camps throughout Tanzania (37), the microbiome data available covers a small subset of the Hadza people. The metadata coverage of the Hadza could also be improved with more participants, collection of more environmental samples from camps, and more samples in children under five years of age. With larger samples sizes, the subsistence system used can be linked to differences in the gut microbiome composition relative to distinct age groups (38). The lack of information on abiotic factors impacting the camps, beyond geographical coordinates and water source, also limits analysis. Factors such as elevation, local climate, soil health, suitable hunting and farming areas, as well as differences in tribe culture would help elucidate gut microbiome differences.

Conclusions In conclusion, our study showed a significant difference in the beta-diversity of gut microbiomes in Hadza people when differentiated by subsistence system, but not water source or geographical distance. Alpha diversity was significantly different between farmers and hunter-gatherers, with Faith's Phylogenetic Diversity greater in hunter-gatherer populations, and Pielou's evenness lower. Taxonomic analysis revealed differently abundant gut microbial composition at the phylum level between the two groups. Further, our analysis revealed greater abundances of Firmicutes, Euryarchaeota, and Actinobacteria in farmers, and greater abundances of Spirochaetes, Proteobacteria, and Bacteroidetes in hunter-gatherers, coinciding with previous literature studies.

Future Directions Differences in the gut microbiomes of industrialized and non-industrialized populations have been previously suggested in literature (24, 39, 40), and these differences may point to important health implications (30). Especially in the novel context of investigating lifestyle transitions on gut microbial composition (38, 41), it has become evident that the human evolution from hunter-gatherers, to usage of agricultural

techniques, leading to the development of WEIRD societies, has been a key driver in the shift of the human gut microbiome composition.

Although previous studies have highlighted the importance of conducting *in vitro* and *in vivo* studies to study the impact of this changing bacterial composition on the gut microbiome (42), we believe that further research needs to be conducted on the gut microbiomes of these traditional populations, such as the Hadza people, to refrain from generalizing the data from this population too quickly, as this threatens reliability and integrity of research (43). Additionally, future studies focusing on the transition of individuals from farming communities to more industrialized environments may also be relevant in supporting the differences we have seen in the Hadza gut microbiomes. Further insight into gut microbiome variation across traditional and industrialized populations would be beneficial in elucidating the drivers of gut microbial composition.

ACKNOWLEDGEMENTS

CONTRIBUTIONS

The participation was well balanced between all group members and communication was effective and reliable throughout the course work. HL completed the Qiime analysis, data generation, and figures based on sex differences. He wrote the discussion section of the report. TY completed the data analysis and Qiime figures based on differences seen in water sources as well as wrote the introduction, future directions, taxonomic analysis section of the methods, and conclusion for the report. CL completed the data analysis and Qiime figures for age related differences in the gut microbiomes and wrote the methods section of the paper. HZ generated the Qiime analysis for the geography based differences and wrote the results section of the paper. HZ and CL both generated the figures showing differences in subsistence systems. All members contributed research, writing support, and proof-reading to all sections of the paper.

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