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Potential associations between environmental conditions and the gut microbiome of the Hadza hunter-gatherers

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SUMMARY The gut microbiome has co-evolved with humans over time, adapting according to environmental changes imposed by the host. Rural communities, such as the Hadza hunter-gatherers of Tanzania are exposed to unique environmental conditions that differ from those of urban communities. Many of these environmental factors are thought to impact gut microbial composition, which has led to an increased interest surrounding the analysis of the gut microbiome of these individuals. Here, we aimed to uncover the impact of water sources, geographical locations, and wildlife exposure on the gut microbiome of the Hadza people. Following parsing of amplicon sequencing data, analysis of the Hadza gut microbiota did not reveal any strong associations with individual water sources nor bush camp locations. However, similarities between the gut microbiome of the Hadza and of vervet monkeys were identified. These results highlight the complexity of the interplay between environmental factors unique to rural communities and the human gut microbiome. Our analysis of the Hadza gut microbiome adds to the body of knowledge that aims to provide insight into features representative of the ancestral human gut microbiome composition.

INTRODUCTION

The human gut microbiome, which is estimated to be comprised of roughly 10^{13} -10^{14} microbial cells, has become a central topic of inquiry in human health and disease research over the last few decades (1, 2). This shift has mainly been due to advances in sequencing technology, which have allowed for more thorough investigation of microbial communities (2). Several studies have evaluated the inherent influence an individual's genetics may have on their gut microbiome (3–5). Recently, however, more research has centred around investigating the impact of external and environmental factors, which are shown to have an even greater impact on human gut microbiome composition than genetic determinants (6, 7). Several groups have investigated the impact of broad external factors such as environmental exposure to pathogens and toxins on the gut microbiome (8, 9), while others have chosen to focus on more individual factors such as diet, antibiotic use, alcohol use, and smoking (10, 11). Many of these external and environmental factors vary based on lifestyle and location, leading to a surge of interest in comparative studies analyzing microbial differences in urban versus rural participant cohorts (12–14).

The Hadza hunter-gatherers of Tanzania are one of the few remaining communities in the world that practice a hunter-gatherer lifestyle (15). A previous study by Smits et al. identified a seasonal cycling pattern in the Hadzas' gut microbiome, as well as compositional differences between fecal samples collected from the Hadza and industrialised communities (15). Gut-associated microbes have co-evolved and co-speciated with humans over time, adapting to environmental changes imposed by the host (16). Modern, high hygiene standards in industrialized countries have led to major changes in microbiome compositions, including complete depletion of entire species from the microbiota (17). However, the microbiomes of pre-industrialized populations, such as the Hadza, have remained more stable, due to the persistence of a rural lifestyle and increased exposure to environmental factors (15). In this study we aimed to investigate whether inherent external environmental factors specific to rural communities impact the gut microbial composition of the Hadza hunter-gatherers. To achieve this, we parsed 16S rRNA sequencing data and associated metadata generated by Smits et al. in QIIME2 (15, 18).

While diet remains an important environmental factor known to impact the gut microbiome, several studies have recently indicated that drinking water sources may also

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impact gut microbial diversity (19–21). In rural environments, natural water sources are often shared with wild animals, which leads to contamination of water with animals' feces (22). Biotic factors in water such as phages and microbial life, including enteric pathogens, can strongly affect the gut-associated community composition (16). This can happen through direct interactions with microorganisms in the host and due to the immune response built by the host (16). The Hadza people exclusively use untreated water sources, such as water from wells, streams and rivers. As such, analysis of Hadza drinking habits provides a unique avenue into determining the impact of rural water sources on the gut microbiota.

More broadly geographic location can influence many factors known to impact the human gut microbiome, including dietary habits and environmental exposure to pollutants or pathogens (10, 23). Previous studies have identified a significant correlation between geographical location and individual microbial composition, even when individuals belong to communities of relatively close geographical proximity and with similar development and culture (24, 25). The Hadza occupy the central rift valley of Tanzania, which centers around Lake Eyasi. The Hadza organize themselves into geographically distinct camps, each loosely made up of relatives, in-laws and friends. Food is shared between members of the same camp, and relocation driven by scarcity of food and water occurs periodically. The presence of distinct Hadza communities, each theoretically exposed to a unique set of environmental conditions, allows for the investigation of the effects of geographical location on the gut microbiota.

As a hunter-gatherer community, the Hadza are in close contact with a number of wild animals, while people in urban communities are not. There has been interest surrounding the investigation of microbial sharing between humans and the animals present in their environmental surroundings (26). Research examining animal gut microbiome composition has demonstrated that, similar to humans, many animal species including monkeys, rhinoceroses and cattle have complex gut microbiomes that serve important functions in regulating digestion and host health (27–29). The One Health model suggests there is an intrinsic link between human health and surrounding environmental factors, including the health of animal populations that live within close proximity (26). This link is due in part to the degree of microbial transfer between animals and humans (26). The data collected by Smits et al. provides the unique opportunity to explore the extent of gut microbiome sharing between the Hadza people and animals in their surroundings.

In this study we will use 16S rRNA sequences from Smits et al. to test the impact of distinct environmental factors (water source, bush camp membership and indigenous animals) on the gut microbiome of the Hadza people. Our analysis of individual water sources and bush camp locations did not reveal any strong impact on the Hadza gut microbial composition. However, similarities were identified between gut microbial samples derived from humans and non-human primate samples.

METHODS AND MATERIALS

16S rRNA samples were obtained by Smits et al. (15). Sequences were imported into QIIME2 and quality control using DADA2 was carried out to truncate reads to 150 base pairs. Only fecal samples were included in downstream analysis. Samples were rarefied down to 14250 (for water source analysis), 11000 (for bush camp analysis) or 10435 (for the analysis including animal samples). Taxonomies were assigned to the 16S sequences using Greengenes and 99% identity. Additional filtering was run to remove ASVs with frequency below 10 and those belonging to mitochondria as well as chloroplasts. Samples belonging to individuals younger than three years old were removed from downstream analysis. A phylogenetic tree of the ASVs was generated using FastTree in QIIME2 and the taxonomic data, phylogenetic tree and filtered reads were exported to RStudio 4.0.3 using the *phyloseq* package (30). The *phyloseq* package was used to conduct principal coordinate analysis (PCoA), and PCoA plots were built with *ggplot2* (31). QIIME2 workflow and R scripts are available at https://github.com/karanvirsingh99/hadza_447.

Beta-diversity analysis of human fecal samples based on water source. Individuals with missing water source information were excluded from analysis.

Beta-diversity analysis of human fecal samples based on bush camp. Additional filtering was done to limit the analysis to individuals from the bush camps Hukamako, Sengeli and Kipamba, who used either a well or spring water source. The number of samples corresponding to each of the 17 bush camps can be found in Tables S1 and S2.

Simultaneous analysis of human and animal samples. Both human and animal samples were included in this analysis. A summary of the different animal species sampled with the corresponding number of samples can be found in Table S3. The analysis was limited to two bush camps, Hukamako and Sengeli.

RESULTS

Drinking water source does not drive diversity in the gut microbiome of the Hadza hunter-gatherers. In order to assess if drinking water sources impact the diversity of the Hadza gut microbiomes, core beta diversity metrics were calculated in RStudio for the human fecal samples (n=473) (32). In order to reflect the choices made by the original authors, Smits et al., only the PCoA of unweighted UniFrac was selected for analysis (15). No clear clustering pattern was observed in the unweighted UniFrac PCoA (Fig. 1), evidenced by the overlap of differently coloured data points. Overall, this trend suggests that the type of drinking water source of the Hadza people does not drive microbial diversity of their gut microbiomes.

Sex and season were then investigated as factors that could induce clustering of gut microbial samples based on water source. Information about sex or season of each sample were separately overlaid on the PCoA in Figure 1. The combination of sex with water source (Fig. 2A) and season with water source (Fig. 2B) did not uncover any underlying patterns. Overall, these results appear to confirm that water source does not drive diversity in the Hadza even when considering other biological and environmental factors such as sex and season.

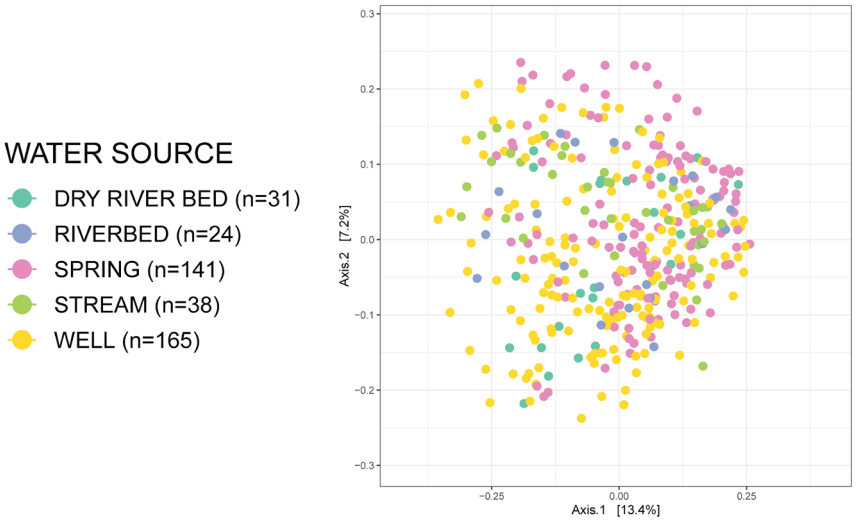

FIG. 1 Drinking water source does not appear to drive diversity in the Hadza gut microbiomes. Principal coordinate analysis (PCoA) of the drinking water sources used by the Hadza, plotted on unweighted UniFrac. Data points from children younger than 3 years old and whose water source was "NOT COLLECTED" were excluded. Individual water sources are colour-coded as shown in the legend. Drinking water sources of the Hadza do not appear to drive gut microbial diversity as all the data samples overlap, suggesting similar microbiota compositions.

Bush camp location does not appear to drive diversity in the gut microbiome of the Hadza. In order to investigate the potential impact of bush camp location on gut microbiome composition, a PCoA plotted unweighted UniFrac was generated. Individual samples were grouped by colour according to bush camp (Fig. 3). No clear clustering pattern was observed between samples from different bush camps (Fig. 3). The PCoA shows extensive overlap between the differently coloured points representing different bush camp locations. This trend suggests that bush camp location is not by itself a major driver of gut microbial diversity.

Water source may drive differences in gut microbial composition between Hadza individuals belonging to different camp communities. Hukamako and Sengeli were the focus of the analysis conducted by the previous authors, Smits et al., and both use primarily spring water sources (15). To allow for comparison with a camp which uses a different primary water source, Kipamba was chosen as a representative subset of Hadza people who use well water. PCoA plotted on unweighted UniFrac (Fig. 4) revealed that samples from the

Kipamba camp, which use a well water source, form a distinct cluster separately from the Hukamako and Sengeli camps, which both use spring water. The presence of this clustering pattern suggests differences in gut microbial abundance and phylogenetic diversity between members of Kipamba and the other two camps.

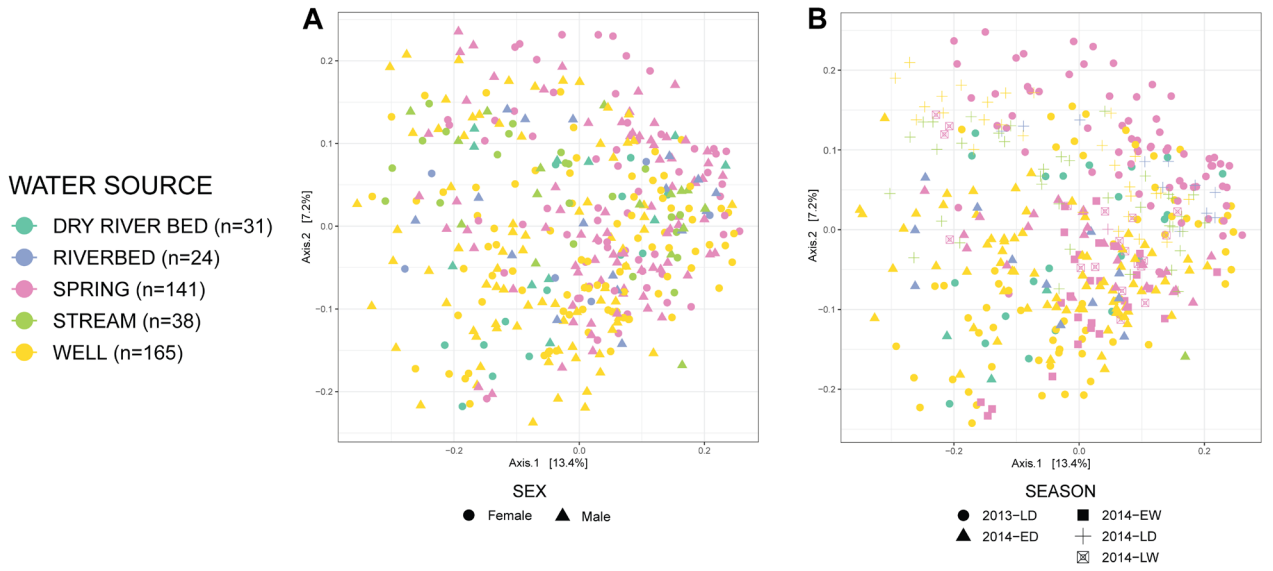

FIG. 2 Sex and season do not explain the trends in gut microbial sample clustering based on water source. Information about sex (A) and season (B) were overlaid to the filtered PCoA plotted in Figure 1. Individual water sources were colour-coded and the factors sex and season were independently distinuished based on shapes. Sex and season do not appear to explain the lack of clustering based on drinking sources, as shown by the high degree of overlaps of all the data points (A-B).

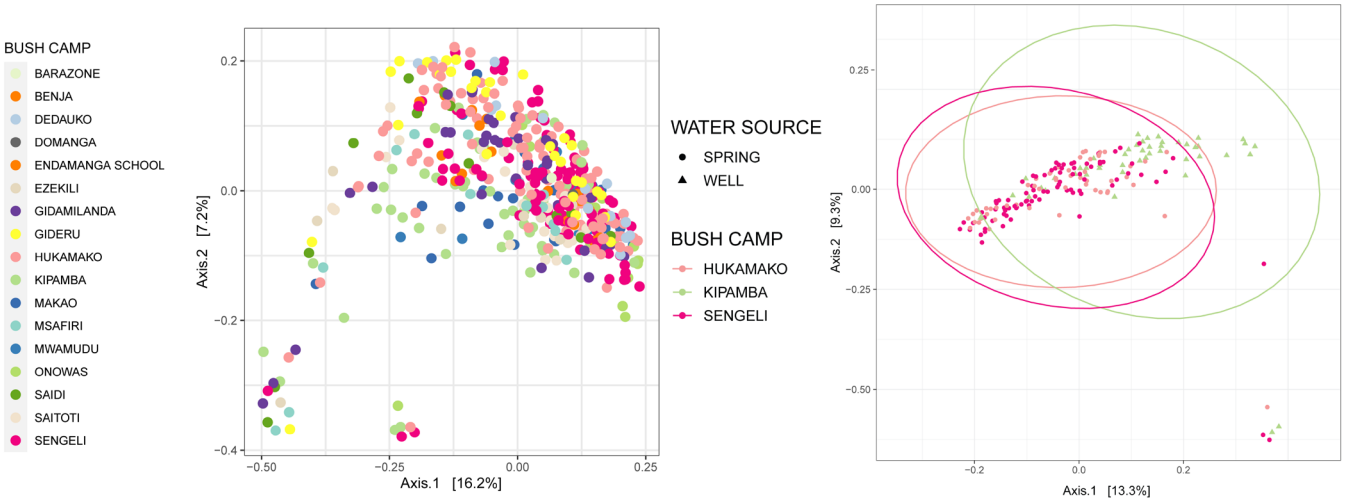

FIG. 3 Hadza camp location does not drive gut microbial composition based on Bray-Curtis or Unweighted UniFrac. PCoA of individual Hadza gut microbiota compositions plotted on Unweighted UniFrac distances. Includes all camp locations, grouped by colour as per legend. Filtered to exclude data points belonging to children aged less than 3 years old. No observable clustering based on bush camp location.

FIG. 4 Water source may drive differences in gut microbial composition between Hadza individuals belonging to different camp communities. PCoA of individual Hadza gut microbiota compositions plotted on Unweighted UniFrac distances. Bush camp locations are Hukamako (salmon), Sengeli (pink) and Kipamba (green). Water sources for each camp are either spring (circle) or well (triangle). Ellipses represent 95% confidence intervals. Kipamba camp which uses a well water source appears to cluster separately from Hukamako and Sengeli camps which use spring water sources.

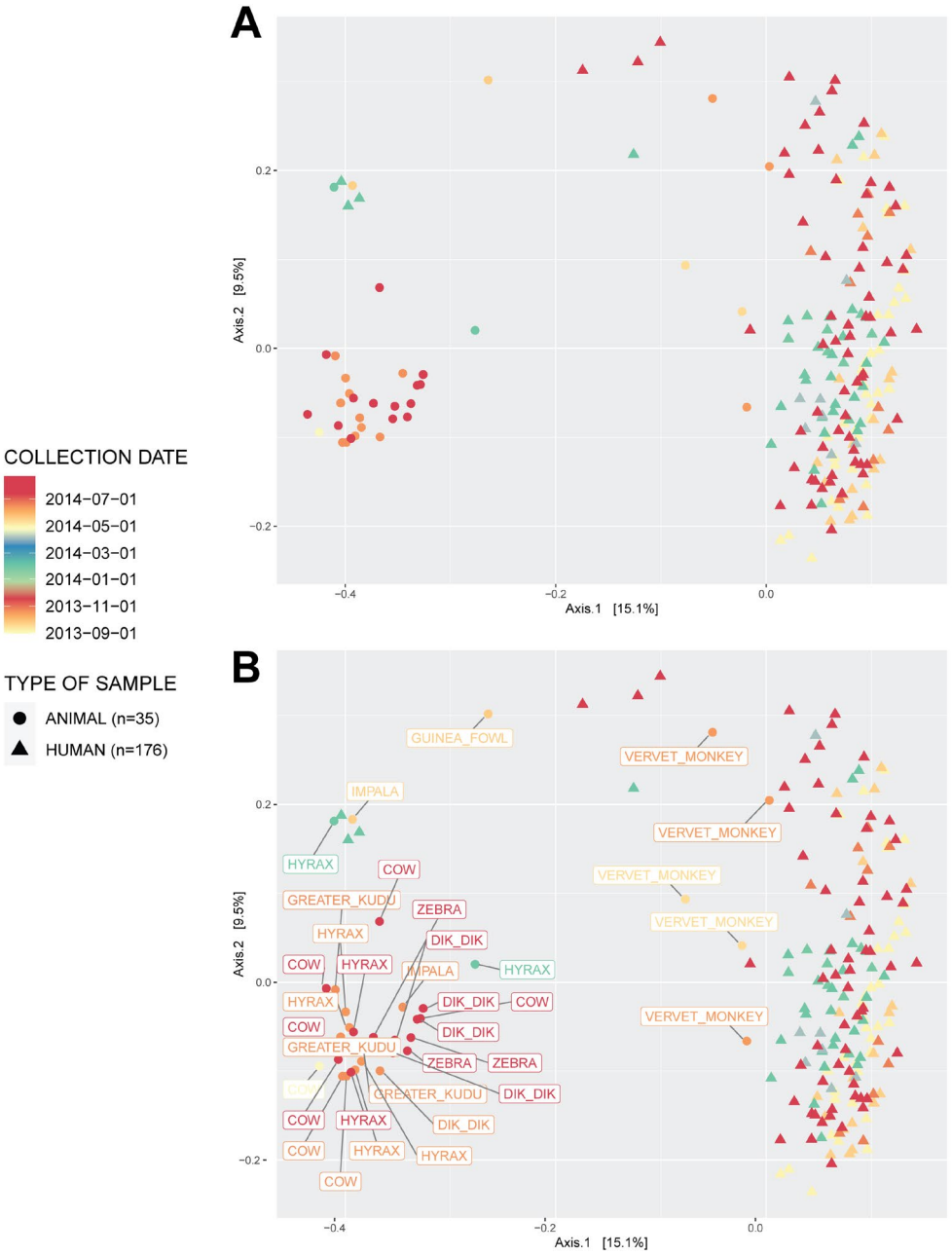

FIG. 5 The gut microbial composition of the Hadza people is more similar to that of vervet monkeys than to other animals. PCoA of unweighted UniFrac distances of animal samples and human samples. Human samples, triangles; animal samples, circles. Samples are coloured based on their collection date with a red-blue gradient for dry and wet seasons respectively. Most animal samples (circles) cluster separately from the human samples (triangles). Vervet monkey samples (highlighted in Panel B) cluster more closely to the human samples, suggesting similar microbial composition between the Hadza and vervet gut microbiome.

The gut microbial composition of the Hadza people is more similar to that of vervet monkeys than to other animals. As seen in the unweighted UniFrac PCoA plot (Fig. 5), the majority of animal samples clustered independently from human samples. This suggests differences in microbial community structure between animals and Hadza samples based on the presence of bacterial species and their phylogenetic distances. Interestingly, the vervet monkey samples clustered more closely with the human samples than the other animal samples, suggesting fewer differences between the microbial composition of the Hadza and vervet gut microbiomes (Fig. 5B). Further investigation into the taxonomic makeup of the vervet monkey samples showed that the predominant microbes are those from the Firmicutes and Bacteroidetes phyla, which are also found abundantly in the human samples (Fig. 6).

Seasonality of the Hadza gut microbiota does not contribute to increased similarity with the animal gut microbiota. Animal samples did not cluster more closely to human samples in either season (Fig. 5). Thus, season does not seem to be a strong driver between the differences in the gut microbiota of human and animal samples as much as the species of origin.

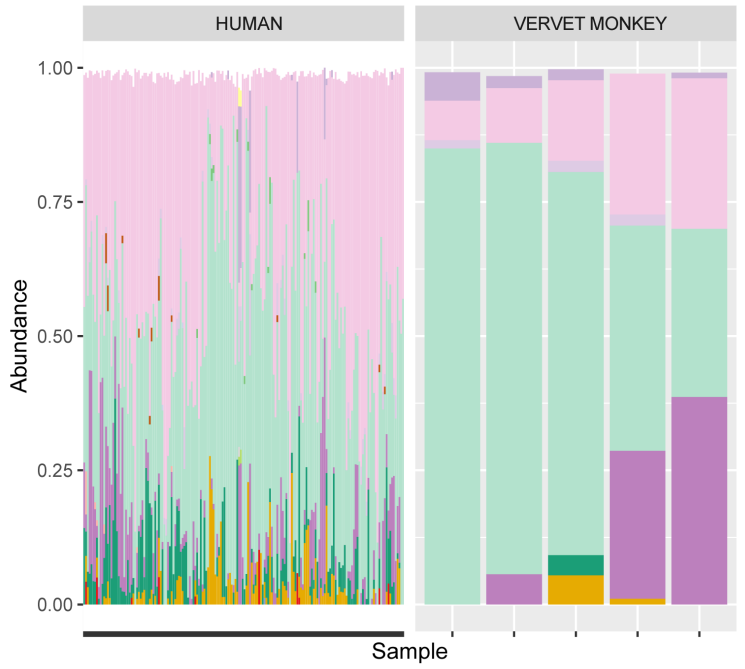

FIG. 6 Vervet monkeys and the Hadza people have similar taxonomic composition. Composition of microbiota from vervet monkey samples (n=5) and the human samples from the Hukamako and Sengeli bush camps (n=176), summarized at the phylum level. Only phyla with a relative abundance greater than 1% are plotted.

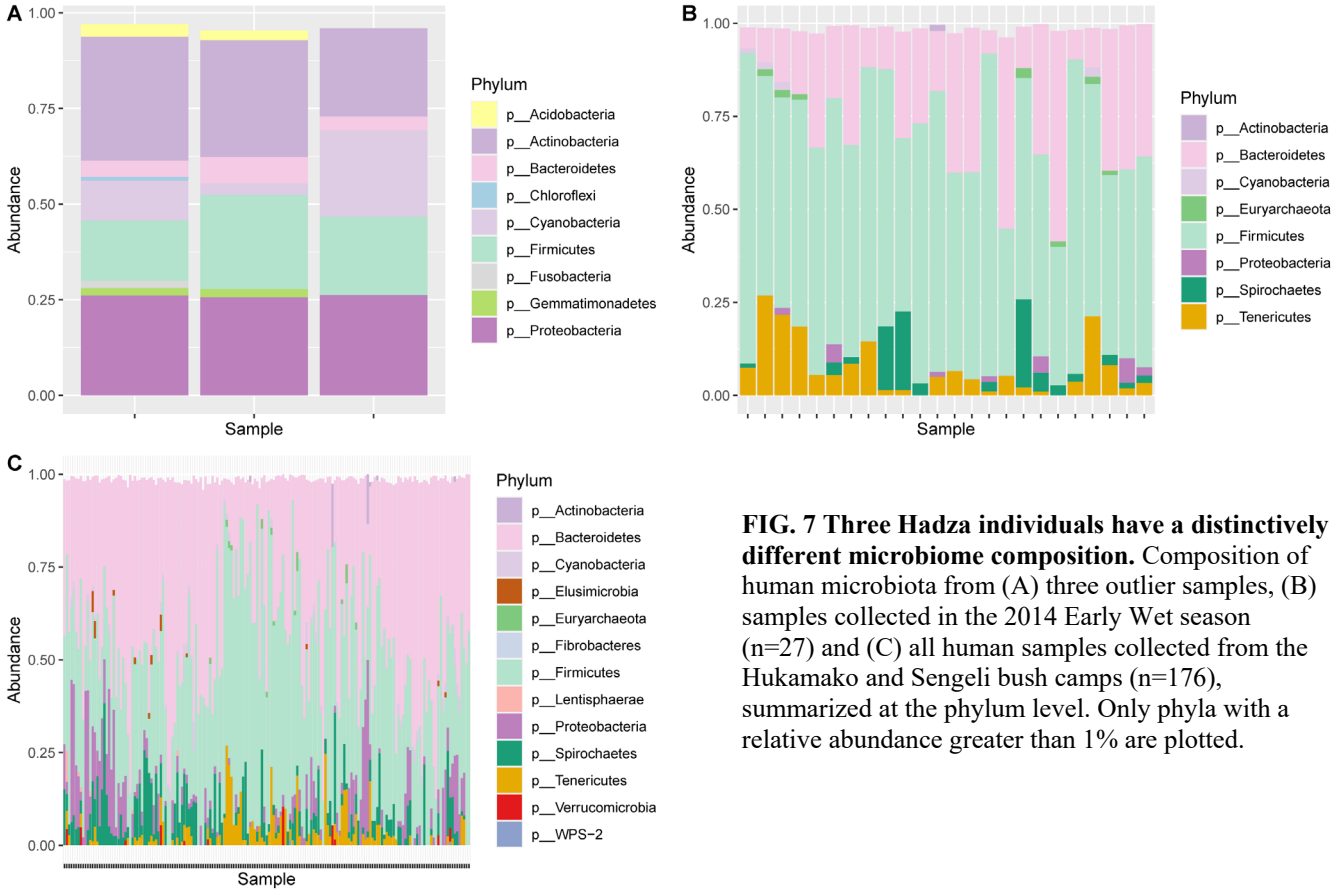

FIG. 7 Three Hadza individuals have a distinctively different microbiome composition. Composition of human microbiota from (A) three outlier samples, (B) samples collected in the 2014 Early Wet season (n=27) and (C) all human samples collected from the Hukamako and Sengeli bush camps (n=176), summarized at the phylum level. Only phyla with a relative abundance greater than 1% are plotted.

Three Hadza individuals have a distinctively different microbiome composition compared to the other human samples. Three human samples formed a distinct cluster in the unweighted UniFrac PCoA (Fig. 6). These three samples were collected in the 2014 Early Wet (2014-EW) season. Two of the samples belong to individuals from the Sengeli bush camp, and one to Hukamako. No distinctive features were found in the metadata collected that could explain this clustering pattern, as other samples from the same bush camps and season were collected but did not cluster with the three outliers. The three outlier samples

clustered together with two specific animal samples, identified as a hyrax and an impala. Qualitative taxonomic analysis revealed higher abundance of the Actinobacteria phylum in the three outliers (Fig. 7A), compared to the other human samples collected in 2014-EW (Fig. 7B). This trend was also evident when comparing the outliers to the subset of human samples from the Hukamako and Sengeli camps (Fig. 7C). Similarly, a lower abundance of Firmicutes and Bacteroidetes differentiated the microbial composition of the three outlier samples from that of other human samples (Fig. 7). Side-by-side comparison of the major phyla found in the three outlier samples against those of the hyrax and impala samples revealed a similarity across all five samples: reduced abundance of Firmicutes and Bacteroidetes and increased abundance of Actinobacteria (Fig. 8).

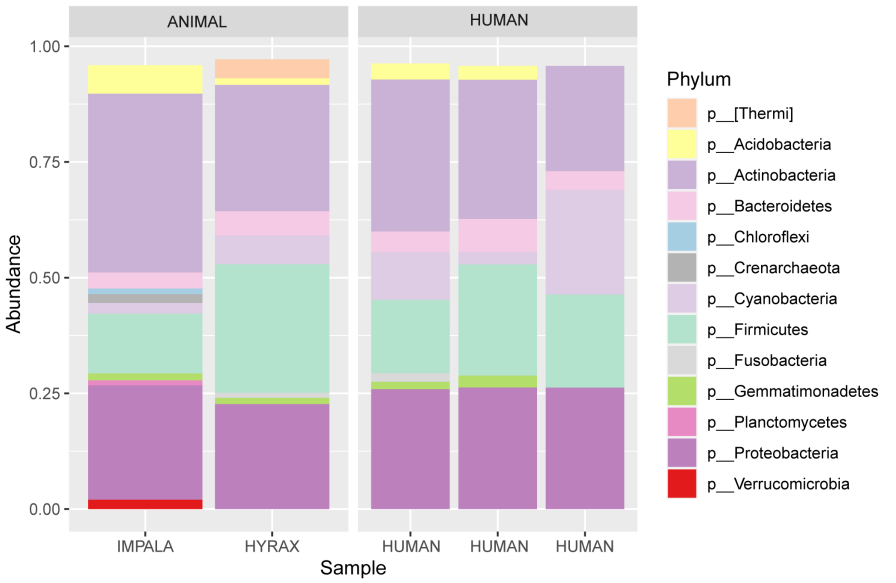

FIG. 8 The gut microbiota of the three outlier samples is similar to that of one impala and one hyrax sample. Composition of the microbiota from the three outlier human samples and one hyrax and one impala sample, summarized at the phylum level. Only phyla with a relative abundance greater than 1% are plotted.

DISCUSSION

Here, we investigated the impact of inherent environmental factors on the gut microbial composition and diversity of the Hadza hunter-gatherers. We calculated core-beta diversity metrics, focusing on unweighted UniFrac, to study how drinking water sources, bush camp locations, and exposure to wild animals are linked to the gut microbiota. Although analysis of the Hadza gut microbiota did not reveal any strong associations with individual water sources and bush camp locations, we found compositional similarities between human and animal samples.

The absence of clear clustering patterns of individuals' fecal microbiome based on the type of water they drink (Fig. 1 and Fig. 3) suggests that the water sources and bush camps of the Hadza do not appear to be major drivers of gut microbial diversity. These findings contradict previous research indicating that the different biotic and abiotic factors present in various untreated water sources impact the gut microbiome (16, 33). Similarly, we cannot replicate previous research showing that differences in community location impact individual gut microbial composition, even when these communities have cultural similarities and relatively small-scale geographic separation (24, 25). However, specific assumptions were made during our analysis that may limit the broader extrapolation of our findings. Firstly, we assumed that the water source recorded for each individual was their sole drinking water source. In practice, it is plausible to assume that Hadza individuals use more than one water source, and that this source may change throughout the year, mirroring the seasonal shift between dry and wet periods (15). Additionally, although the primary water source used by each Hadza was reported, the present analysis did not take into consideration the microbial composition of the individual water samples. It is possible that individual water sources or those in similar locations play a stronger role than type of water source, which may help explain why no clear clustering was observed. Furthermore, analysis considering water source in tandem with sex and season, two variables previously shown to impact the gut microbiota (6, 15, 24), did not elicit trends in gut microbial sample clustering (Fig. 2). We also operated under the assumption that each bush camp is linked to a location that is geographically

isolated and independent of the other camps. While Smits et al. did specify that the Hukamako and Sengeli camps are geographically and culturally related, the location and degree of interaction between the remaining 15 camps were not recorded (15). In the future, consideration of camp relatedness and location would allow for a more refined analysis of the relationship between bush camp locations and the gut microbiota. Nonetheless, our analysis revealed that the individuals belonging to the two camps that drink spring water, Hukamako and Sengeli, have different gut microbial composition than those in the Kipamba camp, who use well water. Because we only tested a single camp that uses well water, from this analysis we cannot conclude whether water source and bush camp location have independent effects on gut microbial composition.

Previous studies have revealed patterns of microbial sharing between human beings and the animals in their environment (34, 35). One particular study in Kenya investigated the degree of microbial sharing between cows and their owners (35). Although this study identified that the microbial sharing relationship between cows and humans was not as strong as the level of microbial sharing between members of the same household, we wanted to further investigate this phenomenon in a pre-industrialized community like the Hadza people. We were able to show that the Hadza gut microbiome is more similar to that of the vervet monkeys, compared to the gut microbiome of other animals in their environment (Fig. 5). No significant degree of similarity with other animals, including cows, zebras, hyrax and impalas, was observed on a large scale (Fig. 5). Taxonomic analysis at the phylum level revealed a relatively high abundance of Firmicutes in both vervets and humans, with other phyla shared between the two being Actinobacteria, Bacteroidetes, Proteobacteria, Spirochaetes and Tenericutes (Fig. 6). The similarity in microbial composition between vervets and the Hadza people has been previously shown by Amato et al. (36). The authors explored the changes in microbial composition in non-human primates induced by the adherence to a Western-style (36). A Western-diet is defined as being high in fat and protein, while a non-Western diet is low in animal fat and protein and high in fiber (36). In their findings, Amato et al. were able to show that regardless of the diet given to vervet monkeys, their gut microbiota resembled that of the Hadza people more than that of human samples from Italy and the US (36). The phylogenetic relatedness of humans and vervet monkeys is believed to explain this phenomenon (37). Host adaptations driven by the need to fit in a specific dietary niche have been shown to be major determinants in the microbial taxa that colonize the primate gut (37). Beyond anatomy and physiology, diet itself is a major driver of gut microbial composition (11). Non-human primates (NHPs), including vervet monkeys, depend on plant material as their main source of nutrients, similar to the Hadza people (15, 38). Together, taxonomic similarity and similar diets likely explain the observed similarity in gut microbial composition between the Hadza people and vervet monkeys. The similarity between the human gut microbiome and that of NHPs is of great significance as NHPs are the most biologically relevant research animal models for humans (39). Additionally, the gut microbiome has recently been linked with a number of health conditions including metabolic disease and obesity (40). Among other factors, the gut microbiome should be taken into consideration when modeling human diseases in NHPs, as a microbial composition that is divergent from that of human beings may lead to distorted findings (39).

Overall, no evidence of microbial sharing between the majority of Hadza people and other animals in their environment was discovered. It is possible that despite their hunter-gatherer lifestyle, the lack of overlap between the Hadza and other animals was based on the assumption that all of the Hadza people interact equally with all animals sampled in their environment. This, however, cannot be tested without collecting more data. It is possible that a subset of the Hadza people are exposed to animals in their environment more than others. Identifying the level of exposure to wildlife across the human cohort would be beneficial in identifying stronger patterns of animal and human microbial sharing.

Interestingly, three Hadza people had an unusual microbial composition characterized by a high proportion of microbes from the Actinobacteria phylum (Fig. 7). Their microbial composition was similar to that of a hyrax and an impala sample (Fig. 8). Models of obesity in mice have reported an increase in the relative abundance of Actinobacteria in the gut microbiome, and in other human studies Actinobacteria have been associated with fat intake and negatively associated with fiber intake (41, 42). Collection of more in-depth

physiological information on these three individuals would help elucidate the cause of the differential abundance observed in their gut microbiota compared to the rest of the Hadza people.

Limitations Some Hadza people were sampled more than once in different seasons, which may potentially lead to overrepresentation of specific individuals in our analysis, especially when subsetting data based on bush camp association. In the original study done by Smits et al., replicates of the same individual were removed in order to keep only one sample per person in the downstream analysis (15). Repeating our analyses by further filtering the datasets to remove replicates from the same individual in different seasons may affect the trends observed in our study.

The patterns of clustering between the animal and human samples were not tested for statistical significance. A PERMANOVA test could be done to determine if the patterns of clustering between the human samples and the animal samples are statistically significant, in order to support the trends we observed.

Additionally, Hukamako and Sengeli were the only two bush camps from which samples were collected in the wet season as well as the dry season. Our analysis was restricted to comparing dry season samples from different bush camps to each other, which was inconclusive in identifying differences in the gut microbiota of Hadza people based on bush camp membership alone. It is possible that the seasonal cycling pattern observed by Smits et al. vary between different bush camps, which could only be confirmed by additional sampling during the wet season.

Although the primary water source used by each Hadza was reported, the present analysis did not take into consideration the microbial composition of the individual water samples. While an insufficient amount of environmental water samples were collected, integration of microbial data derived from these samples would allow for a more comprehensive overview of the association between water sources and the human microbiota.

Conclusions In conclusion, we aimed to identify associations between key environmental factors and the gut microbial composition of the Hadza hunter-gatherers. The factors we chose to focus on were drinking water sources, bush camp locations, and exposure to indigenous animals. We found that independent investigation of water sources and bush camp locations did not reveal any associations with gut microbial diversity. However, analysis encompassing the two factors in tandem indicated that usage of distinct water sources may play a role in driving differences in the gut microbial composition of Hadza camp communities. However, further analysis encompassing data from additional bush camps is necessary to reach this conclusion.

Analysis of the indigenous animal samples revealed that the Hadza gut microbiome shares similarities with that of wild vervet monkeys, however similarities in gut microbial composition between the majority of the Hadza people and other animals in their environment were not found. These results suggest that microbial sharing between the Hadza people and wild animals in their environment is not as relevant as we previously theorized. Although many interesting trends were identified, our study highlights the complexity of the interplay between environmental factors and the human gut microbiota.

Future Directions Water often harbors pathogens which can lead to waterborne diseases (43). Analyzing the water samples collected by Smits et al. for the presence of different enteric pathogens would allow to make associations between the gut microbiota of the Hadza who drink from specific water sources. Bacterial species of interest that are known to cause waterborne diseases include *Arcobacter* spp., *Campylobacter* spp., *Salmonella* spp., and enterohemorrhagic *E. coli* (43). Total coliform testing, or fecal coliform testing, of the different water sources used by the Hadza people could be performed to investigate the link between water quality and the gut microbiota. In a study conducted by Piperata et al. (2019), children that drank from water with high coliform concentrations had lower alpha-diversity and different gut microbial community structure than children who were less exposed to water pollutants (21).

Bush camp membership, as reported in the metadata, was used as a proxy for geographical location. Concrete information about the exact geographical location of each bush camp was not made available in the dataset provided by Smits et al. (15). This may act as a confounder in downstream analysis, as the geographical proximity between camps is unknown. Integration of the relative bush camp proximity would allow for comprehensive investigation of the impact of bush camp membership on the Hadza gut microbiome. Similarly, the location from which the majority of animal samples were collected was not reported. Gaining information on the relative locations of the human and animal samples in order to characterize which human and animal samples were more likely to interact than others would allow for a more granular analysis of this dataset.

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CONTRIBUTIONS

K.S. directed study progress and made significant contributions to the analysis. H.G. and A.G. contributed to designing and performing the research and analysing the data. All authors contributed equally in writing the manuscript.

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