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Age, water source, and sex do not significantly affect the microbiome of the Hadza people of Tanzania

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SUMMARY Very little is currently known about the factors affecting the gut and skin microbiomes of the Hadza people of Tanzania. Most of the current microbiome research has been conducted on industrialized populations, with age, sex, water, and diet identified as some of the factors influencing microbiome composition and variation. Societies like the Hadzabe are increasingly being studied, as their hunter-gatherer lifestyle may give insight into the pre-industrial microbiome. Since industrialized microbiomes show lower diversity and a higher incidence of inflammatory conditions compared to pre-industrial microbiomes, comparing these microbiomes can provide valuable information related to human health and disease. The purpose of our analyses was to use knowledge of the Hadza lifestyle to determine whether age, sex, and water source, which are important determinants of industrialized gut and skin microbiomes, also influence the Hadza microbiome. We investigated the effects of age and sex on the volatility and composition of the Hadza gut microbiome, respectively. We also analyzed the composition of the gut microbiome of individuals who used water sources with distinct microbial profiles. Additionally, we looked at the effects of sex on the composition of the skin microbiome of Hadza individuals. From the results of these analyses, we observed no significant impact of age or sex on the volatility or composition of the Hadza gut microbiome. Similarly, water source and sex were not found to impact the composition of the gut and skin microbiomes, respectively. Our study provides further insight into the unique lifestyle of the Hadza people and its effect on their microbiomes, which contributes to bridging the knowledge gap between industrialized and non-industrialized populations.

INTRODUCTION

Almost 55% of the world's population lives in urban areas today, and according to an estimate by the United Nations Department of Economic and Social Affairs, this number is expected to rise to 68% by 2050 (1). Despite this increasing shift towards urbanization, there are still some societies remaining which subsist on a traditional hunter-gatherer lifestyle. These include the Khoisan hunter-gatherers of Namibia (2) and the Hadzabe of Tanzania, one of the largest hunter-gatherer societies remaining today (3). In 2010, there were about 1000 Hadza hunter-gatherers living in Northern Tanzania, occupying an area of approximately 4000 km^2 around Lake Eyasi (3).

The Hadza hunter-gatherers live in mobile groups called bush camps, which can be composed of 2-100 people (3). Individuals frequently wander between camps, and the composition of bush camps is quite fluid. Bush camps, while mobile, are also constrained in their location by their proximity to a water source (4). There are fewer water sources available in the dry season (June through November) than in the wet season (December through May), and thus a limited number of locations for bush camps in the dry season. The Hadza people obtain their food from a mixture of hunting and foraging activities. Moreover, there is a sex-

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based separation of duties related to food acquisition. Men usually hunt to feed their camps, while women and children collect plant-based food (5). Both sexes are accustomed to snacking while out hunting or foraging (5), resulting in significant sex-based differences in food intake throughout the day. The Hadza diet also changes according to the season, with the women foraging mainly for tubers and baobab in the dry season, and for berries and occasional tubers in the wet season (6). The men collect honey in addition to sporadic hunting during the wet season (6).

While the Hadza hunter-gatherers rely on hunting and foraging activities, there is an increasing influence of agricultural groups from surrounding regions. In particular, the Makao and Kipamba bush camps, which are located in areas more amenable to crop agriculture, have been heavily influenced by the Sukuma, Isanzu, and Iramba people (Peterson, D. Personal communication). As these groups rely solely on agricultural activities, the lifestyle of the Makao and Kipamba bush camps has been altered to a certain extent, and the individuals in these bush camps consume a large number of cultivated foods (Peterson, D. Personal communication). However, notwithstanding these few agricultural influences, the Hadza lifestyle for the most part is very traditional and representative of what our pre-agricultural ancestors might have engaged in.

Since the Hadza lifestyle is completely different from urban lifestyles, there is an increasing interest in how the microbiome of the Hadza people reflects this difference. Urban populations have a higher incidence of inflammatory disease compared to non-industrialized populations (7, 8). Since inflammatory diseases have previously been linked to the human gut and skin microbiomes (9–11), comparing industrialized microbiomes with those of non-industrialized populations like the Hadzabe can provide valuable insight into human health. In fact, previous studies have found that industrialized microbiomes show lower diversity compared to pre-industrialized or rural microbiomes (12, 13). As well, a recent study analyzed the composition of the Hadza gut microbiome and found that there is a seasonal cycling pattern evident in the gut microbiome (14). A seasonal cycling pattern is defined as the composition of the gut microbiome changing from the dry to the wet season, but appearing similar between dry seasons. The dataset collected during this analysis also included skin swab samples, samples from surrounding animals and water sources, and rich metadata including information about bush camps, water sources, age, and sex of Hadza individuals (14). A different study used this dataset to further extend our understanding of the Hadza people's microbiota and their environment (15). In particular, they found that different water sources in the Hadza area had unique taxonomic compositions, and that certain taxa found in gut microbiomes could also be identified in the water sources being used. They also found that the specific foraging or hunting activities that individuals engaged in changed their skin microbiomes significantly. Another finding was that the diversity of the gut microbiome increased with age (15). These preliminary results were able to shed light on certain characteristics of the Hadza microbiome, and in our analyses using the original dataset (14) we aimed to extend previous findings using our knowledge of the Hadza lifestyle and society.

In particular, we investigated the effects of variables such as age, sex, and water source on the seasonal volatility and/or composition of the microbiota of the Hadza people, since these factors have previously been identified as influencing the microbiome in industrialized populations (16-18). The seasonal volatility of the Hadza gut microbiome has been analyzed previously, and has been defined as a change in the composition of the microbiome between different seasons (14). In accordance with previously reported methods (14), our analysis used samples from individuals greater than 3 years of age and belonging to two adjacent bush camps, Hukamako and Sengeli, which are less than 7 km apart. In order to extend previous findings, we compared the gut microbiome volatility between individuals in two different life stages: children (ages 3-17) and adults (ages 18-80). We used 17 years as the cut-off to distinguish between children and adults since the dataset we used (14) differentiated between the two life stages at that age. We compared the composition of the gut microbiome between A) 2013 and 2014 dry seasons, B) 2013 dry and 2014 wet seasons, and C) 2014 wet and 2014 dry seasons using a beta diversity metric for both children and adults. Microbiomes that displayed a significant variation between seasons were identified as being volatile or demonstrating seasonal cycling. We hypothesized, based on previous observations of the plasticity of children's gut microbiomes (18), that seasonal cycling of the gut microbiome

would be evident in both life stages, with those of children showing a greater degree of variation between seasons.

We also verified the previously reported unique taxonomic compositions of different water sources (15) and hypothesized that these unique compositions would translate to a significant difference in gut microbiome composition between individuals using these water sources. We also combined our knowledge of sex-based differences in diet and the influence of foraging activities on the skin microbiome to hypothesize that the gut and skin microbiomes would differ significantly based on sex. Through these analyses, we aimed to understand whether factors such as age, sex, and water source, which are important determinants of industrialized microbiomes, also affect the pre-industrial microbiomes of the Hadza people. This information could add to existing knowledge of the determinants of human health and disease, while also filling the knowledge gap in the differences between industrialized and non-industrialized populations.

METHODS AND MATERIALS

All analyses were conducted in QIIME2 (19), as detailed below. PCoA (Principal Coordinate Analysis) and taxa bar plots were recreated in R (20). Relevant commands can be found in the attached supplemental text files.

Sample statistics. A previously published dataset (14) collected from the Hadza people of Tanzania was used for all analyses. It contained 682 microbiome samples in total, 608 of which were human samples (131 hand and 477 fecal samples). The remaining samples were obtained from environmental water sources (9 samples) and animals (65 samples). All samples were collected between the 2013 early wet and 2014 late dry seasons. The dataset represented 16 different bush camps, namely Hukamako, Sengeli, Saidi, Benja, Gideru, Ezekili, Domanga, Gidamilanda, Onowas, Makao, Kipamba, Saitoti, Mwamudu, Msafiri, Barazone, and Dedauko. Some samples were also collected from children at Endamanga School. Apart from bush camps, additional information in the dataset included the age of individuals from whom samples were collected, the time of collection, type of water source, and the species of animals sampled. Different analyses used different subsets of this data, as discussed below.

Importing data and sequence quality control. In order to import the sequencing data from the dataset into QIIME2, fastq.gz files for all samples were consolidated into one demux.qza file containing demultiplexed sequences (21). The demultiplexed sequences were then fed through the DADA2 pipeline (22) for sequence quality control. Sequences were truncated at 6 nucleotides on the left, and at 150 nucleotides on the right. The corrected and truncated sequences were then saved as two rep-seqs.qza and table.qza files.

Metadata-based filtering. Following sequence quality control, the table.qza file contained demultiplexed and truncated sequences which had also been corrected for errors arising from Illumina sequencing. This file was filtered based on the metadata using the q2-feature-table plugin to obtain the required subsets of samples in the form of table.qza files for each analysis. Specifically, fecal samples from children and adults from the Hukamako and Sengeli bush camps were stratified for the longitudinal analysis of the gut microbiome. Another subset of the data containing all fecal samples was used for a beta diversity analysis of the gut microbiomes of the two sexes. In order to conduct a beta diversity analysis of the gut microbiome of individuals using different water sources, this dataset was subsetted again to only include fecal samples collected in the 2014 ED (early dry) season. This season was chosen since each bush camp used a unique water source during that time. Furthermore, the original data was subsetted to retain only water samples from the 2014 ED season in order to conduct a taxonomic analysis of water samples. Finally, the original data was filtered in order to only include skin swab samples. This enabled beta diversity analysis of the right-hand microbiomes with the samples sorted according to sex to evaluate sex-dependent differences in microbiome composition.

Phylogenetic analysis and alpha rarefaction. Following metadata-based filtering, rooted and unrooted phylogenetic trees were generated using the fasttree2 pipeline (23) and used in all diversity analyses. The rooted phylogenetic tree was used to plot alpha rarefaction curves for each separate filtered table.qza file generated from the filtering step above. The alpha rarefaction curves were then used to choose an appropriate sampling depth for each subset of samples.

Beta diversity analyses. The sampling depths determined from the alpha rarefaction step were then used to conduct beta diversity analyses on the fecal, hand, and 2014 ED fecal sample subsets. Emperor plots (24) of the weighted UniFrac analysis (25) of fecal and hand samples were created, as done previously (15), and the samples were sorted according to the metadata category of sex in both cases. The resulting PCoA plots were recreated in R (20) using the ggplot2 package (26). The weighted UniFrac analysis of fecal samples from the 2014 ED season was also converted to an Emperor plot, and the samples were differentiated based on the variable of bush camp. The PERMANOVA (Permutational Multivariate Analysis Of Variance) statistical test was used to evaluate the variance between the multiple factors used to stratify the beta diversity using the group significance metric. Additionally, human fecal samples categorized into two host life stages (child: 3-17 years and adult:18-80 years) were merged and used for an analysis of the seasonal pattern using unweighted UniFrac (27), as done previously (14). The separated datasets were merged here since the previous seasonal cycling analysis did not separate samples by age, and this allowed us to directly compare our results to the ones previously reported (14).

Taxonomic analysis and taxa bar plot. In addition to the beta diversity analyses, the taxonomic composition of the gut and hand microbiomes was investigated. A taxonomic classifier was trained (28) using the Greengenes database (29) at 99% identity. The trained classifier was then applied to assign taxonomy to the sequences in the previously generated rep-seqs.qza file, and the resulting file was saved as a taxonomy.qza file.

The taxonomy.qza file was used to build taxa bar plots showing relative abundance for the three water samples collected in the 2014 ED season. The table.qza file containing sequences from the water samples was filtered to remove non-bacterial sequences, as well as sequences with an abundance < 1%, as previously described (15). The taxa bar plot generated with this input was recreated in R using the ggplot2 package. The bar plot was displayed at the family level, in accordance with a previous analysis (15). All ASVs (Amplicon Sequence Variant) with an abundance <2.5% were grouped together in order to simplify the plot.

Differential abundance analysis. The taxonomic information generated from the water samples was used to conduct differential abundance analyses using analysis of composition of microbes (ANCOM) (30). In order to do this, the three table.qza files containing all skin swab samples and fecal samples from the 2014 ED season were filtered to remove non-bacterial sequences and sequences with low abundance (<0.005% for skin samples and <0.05% for 2014 ED fecal samples). These differing cut-offs were based on sequence abundance information for the two sample types generated from the alpha rarefaction step described above. Differential abundance analyses were conducted on skin microbiome samples to detect differentially abundant taxa between males and females. A similar analysis was conducted on fecal samples from the 2014 ED season to detect differentially abundant taxa between individuals from distinct bush camps.

Longitudinal analysis of fecal samples across three seasons. Finally, we conducted a longitudinal analysis on the two human fecal sample sets separated by host life stage by using the QIIME2 q2-longitudinal library (31). The analysis was conducted to detect differences in the composition of the gut microbiome between A) 2013 and 2014 dry seasons, B) 2013 dry and 2014 wet seasons, and C) 2014 wet and 2014 dry seasons for both the ‘adult’ and ‘child’ age groups. Statistically significant differences between age groups were determined using the pairwise diversity distance comparison test using the unweighted UniFrac diversity parameter (27). This comparison determined whether the diversity distance changed significantly between adult and child age groups in the transition between sub-seasons.

RESULTS

Hadza adults and children do not show different changes in microbial composition as individuals progress through seasons. Contrary to previous findings, we did not observe a seasonal cycling pattern in the gut microbiome of the Hadza people (Fig.S1). In order to further explore the impact of season on the microbial composition of the Hadza people, we conducted a longitudinal analysis on human fecal samples from different age groups. This was done by calculating the unweighted UniFrac diversity metric for both adult and child age groups, allowing us to observe changes in the gut microbiome between each sub-season. The microbial diversity composition in each sub-season was compared between the two age groups using a pairwise diversity comparison (Fig. 1). The distance and the corresponding p-values in Figure 1 represent the differences in microbial diversity composition between the two age groups. We expected to see a significant difference between adults and children for each sub-season because previous studies had found that industrialized children showed a greater level of gut microbiome plasticity compared to adults in their response to changes in diet (32). However, Figure 1 shows that across all three seasons, the data for child and adult microbiomes overlaps, suggesting similar changes in the microbiomes of both age groups across seasons. It is important to consider the limited size of the collected samples while interpreting this observation, which makes it difficult to detect small differences between the groups. Overall, our findings suggest that in contrast to industrialized populations, adults and children in the Hadza community might have the same level of plasticity in their gut microbiomes across seasons.

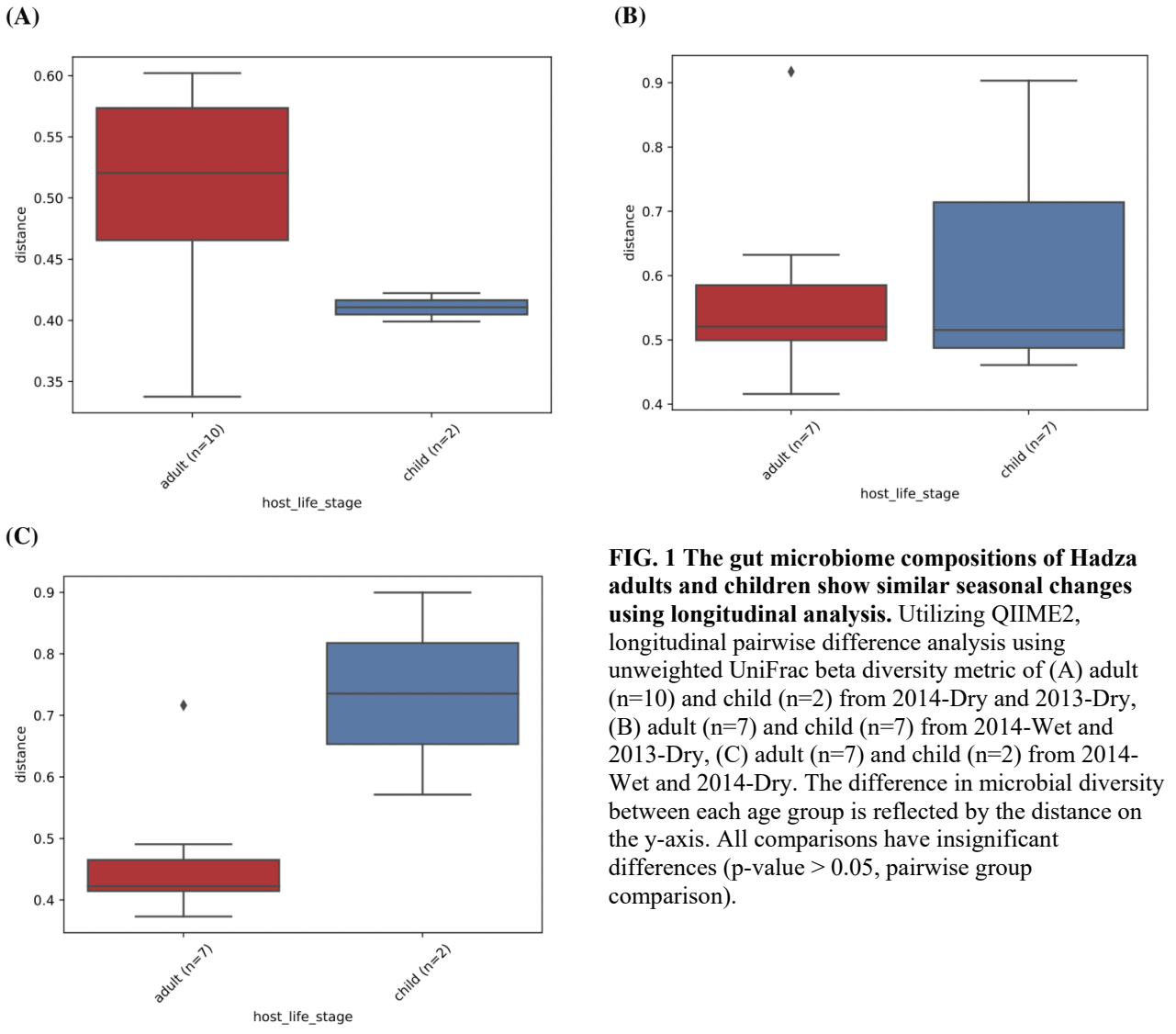

FIG. 1 The gut microbiome compositions of Hadza adults and children show similar seasonal changes using longitudinal analysis. Utilizing QIIME2, longitudinal pairwise difference analysis using unweighted UniFrac beta diversity metric of (A) adult (n=10) and child (n=2) from 2014-Dry and 2013-Dry, (B) adult (n=7) and child (n=7) from 2014-Wet and 2013-Dry, (C) adult (n=7) and child (n=2) from 2014-Wet and 2014-Dry. The difference in microbial diversity between each age group is reflected by the distance on the y-axis. All comparisons have insignificant differences (p-value > 0.05, pairwise group comparison).

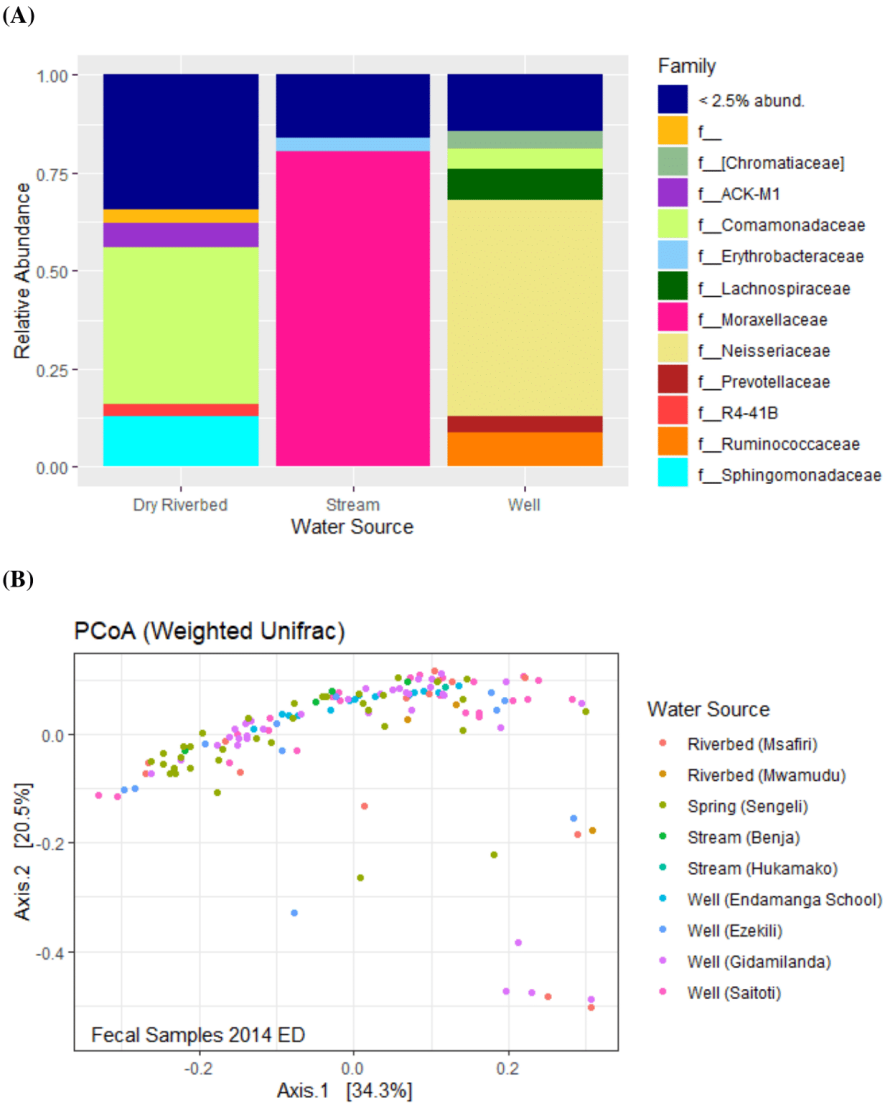

FIG. 2 Taxa bar plots of relative abundance from three water sources show unique taxonomic compositions which are not reflected in Weighted UniFrac analysis of corresponding gut microbiomes. (A) Environmental water samples collected in the 2014 ED season were used to create taxa bar plots representing relative microbial abundance in R. Sequences that were >1% abundant were retained, and ASVs with an abundance of < 2.5% were grouped together. Unique taxonomic compositions are observed for all three samples. The most abundant families in the dry riverbed, stream, and well samples are *Comamonadaceae*, *Moraxellaceae*, and *Neisseriaceae* respectively. n = 3. (B) Fecal samples collected in the 2014 ED season were used to construct a PCoA plot of weighted UniFrac beta diversity analysis in R. Samples were sorted according to water source. No significant clustering is observed. Variance explained by axis 1 is 34.3% and that explained by axis 2 is 20.5%. n = 142.

Differences in the microbial composition of water sources are not reflected in the gut microbiomes of Hadza individuals. Environmental samples from three different water sources (stream, well, and dry riverbed) were included in the dataset (14) during the 2014 ED season. These samples were shown to have unique taxonomic compositions (15). To confirm this, taxa bar plots representing relative abundance were created at the family level using R (Fig. 2A). Figure 2A shows that the most abundant family in the dry riverbed sample was *Comamonadaceae*; the stream sample, *Moraxellaceae*; and the well sample, *Neisseriaceae*. This composition corresponded with what was found previously (15). Since the three water samples analysed had unique compositions, we hypothesized that the gut microbiomes of Hadza individuals using different water sources would also be significantly different. Beta diversity analyses were conducted on the fecal samples from the 2014 ED season and sorted according to the water source used by each bush camp. A PCoA plot representing weighted UniFrac analysis was recreated in R (Fig. 2B). We expected that the distinct taxonomic composition of the water sources would be reflected in the gut microbiome and would result in the fecal samples clustering according to different bush camps. However, Fig. 2B shows that fecal samples don't form distinct clusters, apart from one cluster composed of samples from Gidamilanda and Msafiri bush camps in the bottom right corner of the plot. Upon further investigation, these 6 samples were all found to be from infants or children. The accompanying PERMANOVA analysis indicated significant differences between some pairs of bush camps (p-value = 0.001 for all comparisons, data not shown) but this was not reflected in the PCoA plot representing weighted UniFrac since all the samples overlapped. The significant results from PERMANOVA were concluded to be an artifact of multiple pairwise

comparisons. Furthermore, differential abundance analysis of the fecal samples at the family level showed that the families that differed between water sources had similar distributions in fecal samples from individuals using those water sources (Fig. S2). These results collectively led us to conclude that while water sources have unique microbial compositions, these differences do not translate to the composition of the gut microbiomes of individuals using distinct water sources.

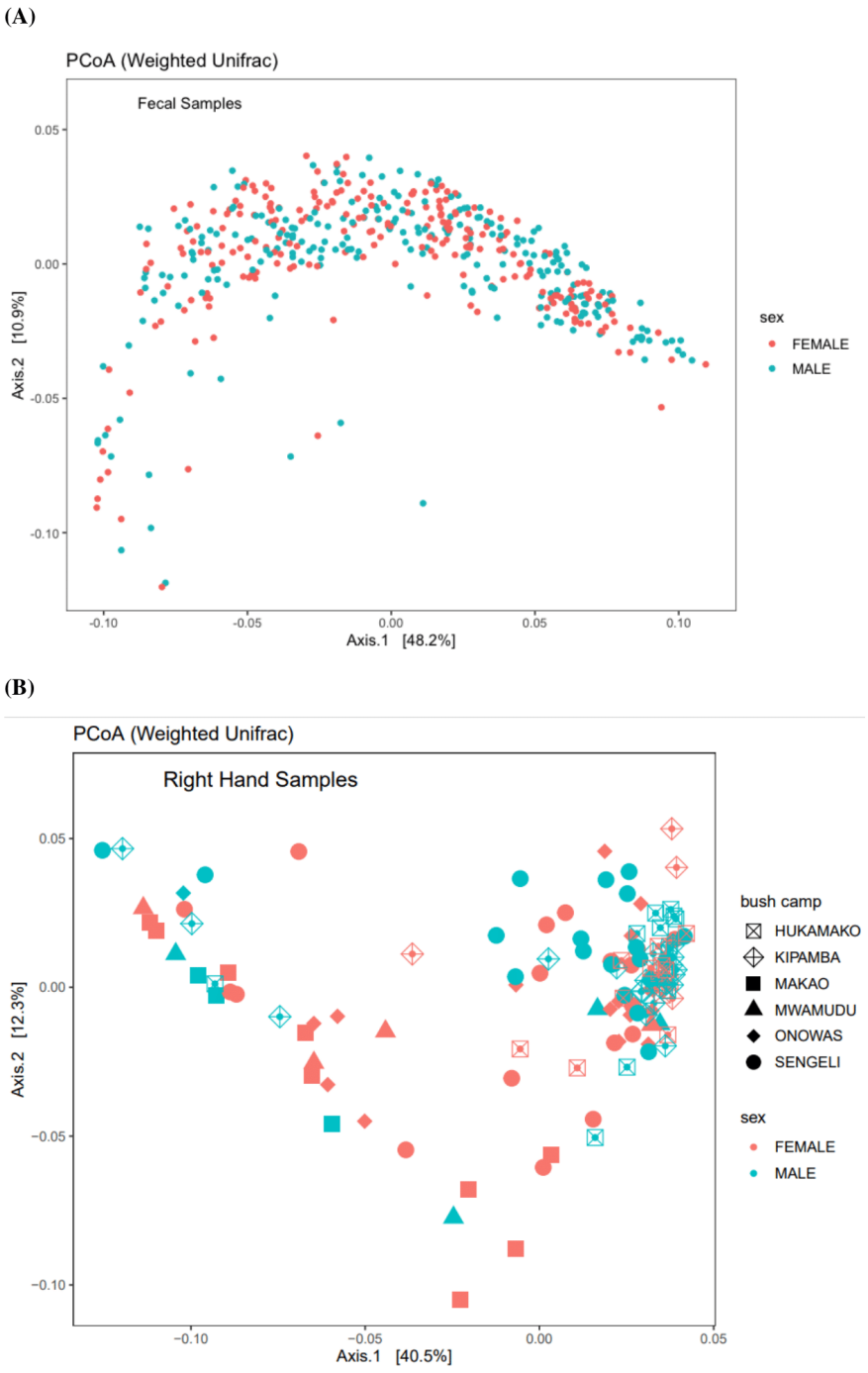

FIG. 3 Weighted UniFrac analysis of (A) Hadza gut and (B) Hadza right-hand microbiome samples display non-significant differences when categorized by sex . (A) Male and female fecal samples were filtered and analyzed using QIIME2, and a weighted UniFrac distance PCoA plot was created in R. Male (blue) and female (red) samples do not exhibit distinct clustering, indicating similar gut microbiomes. Percent variance explained on axis 1=48.2%, axis 2=10.9%. N=477. (B) Right-hand microbiome samples of Hadza hunter-gatherers were analyzed using both QIIME2 and R, and weighted UniFrac beta diversity analysis was visualized on a PCoA plot. Samples were classified based on sex (colour) and bush camps (shape). No distinct clustering can be seen between the sexes. Percent variance explained on axis 1: 40.13%. Percent variance explained on axis 2: 11.46%. n =127.

Hadza gut microbiome is influenced more strongly by bush camp than by sex. Previous studies have shown different gut microbiomes for males and females (33). We sought to determine if Hadza males and females show similar patterns. We conducted beta diversity analyses using QIIME2, and then used R to recreate the weighted UniFrac PCoA plot (Fig. 3A). The PCoA plot shows male and female samples clustering together with no clear distinction in their distributions. To confirm this, we performed pairwise PERMANOVA tests using QIIME2 and concluded that there was no significant difference between sexes

(q=0.095, data not shown). However, sex is not the only factor determining the dietary and environmental aspects that influence the gut microbiome. Geographical location, reflected by bush camp, is another factor that might indirectly influence the gut microbiome. We conducted a beta diversity analysis of fecal samples sorted according to bush camp. The weighted UniFrac PCoA plot showed that samples from individuals belonging to three geographically adjacent bush camps overlapped extensively, suggesting similar gut microbiome compositions (Fig. S3). Since clear qualitative patterns in gut microbiome composition were seen when samples were grouped by bush camp instead of sex, we investigated whether sex-based differences were evident within each bush camp. There were no clear sex differences in any bush camp (data not shown), and the analysis was further hampered by limited sample sizes. Overall, we concluded that differences in the gut microbiome could be ascribed to bush camp rather than sex.

Bush camps, not sex, explain differences in the Hadza right-hand microbiome. Due to the differences in foraging activities between Hadza males and females, we explored whether there were variations in their right-hand microbiomes. Using QIIME2, we conducted beta diversity analysis on all skin swab samples. The PCoA plot for weighted UniFrac analysis was then recreated in R (Fig. 3B). The right-hand microbiome samples for both males and females show no clear trend in their distribution, and instead cluster together. Thus, we concluded that there are similarities in the right-hand microbial composition of males and females. To further confirm this result, a pairwise PERMANOVA test conducted by QIIME2 indicated non-significant differences between male and female right-hand samples (q-value=0.134, data not shown). Differential abundance analysis was also performed using ANCOM, which showed a lack of differentially abundant taxa between males and females (Fig. S4). Similar to the analysis of the gut microbiome, we predicted that geographical location, or bush camp, might better explain differences in the composition of the right-hand microbiome. This was analyzed qualitatively by sorting samples according to bush camp on the PCoA plot for weighted UniFrac (Fig. 3B). We observed that samples taken from camps such as Makao displayed a unique pattern compared to samples from camps like Hukamako (Fig. 3B). These two bush camps were analyzed in particular since they have significantly different environmental and geographical influences due to differing levels of agricultural encroachment. Through qualitative observation, the Makao samples were seen to cluster separately from the Hukamako samples. This observation indicates that bush camp identity might better explain changes in the composition of the right-hand microbiome, compared to sex. Although differences between bush camps are seen, male and female samples within each bush camp do not show unique clustering, which could be due to the low sample sizes within individual bush camps. This indicates that although the right-hand microbiome of the Hadza people can differ between individuals, these differences are better explained by variations in bush camps rather than sex.

DISCUSSION

Using data previously collected on the Hadza people of Tanzania (14), we set out to observe if factors such as age, water source, and sex had a significant impact on the Hadza microbiome.

Our analysis of the gut microbiome did not reveal a seasonal cycling pattern (Fig. S1), which contradicted the results obtained previously (14). Our analysis used fewer samples compared to the one conducted previously (14), resulting in lower powered statistics and possibly explaining the contradictory result. Another difference between the previously reported workflow and the one used here was that we used ASVs to assign taxonomy whereas the previous analysis used 97% OTUs. Higher OTU clustering is prone to random sequencing errors, while ASVs can assess diversity and allow higher resolution insight into low resolution gene markers such as 16s rRNA (34). However, some studies have reported that ASVs and 97% OTUs yield comparable taxonomic profiles and beta diversity values (35), which suggests that this difference might not be responsible for our contradictory findings. We concluded that smaller sample sizes probably best explained the disparate results.

We also wanted to address microbial plasticity between age groups of the Hadza people, as gut microbiome stability is an important factor in understanding microbiota-associated

diseases (36). By performing a longitudinal analysis on human fecal samples separated by host life stage between the 2013-dry, 2014-wet, and 2014-dry seasons, we determined that regardless of age, gut microbiomes did not differ across seasons (Fig. 1). These results were confirmed using the pairwise diversity difference comparison test for the unweighted UniFrac metric, which uncovered non-significant microbial changes for both adults and children in the Hadza community over time. This was unexpected, as previous studies involving industrialized populations have shown greater gut microbiome volatility in children compared to adults (18), suggesting that microbiota development in the Hadza community differs from that in industrialized populations, establishing a more stable microbiome earlier in life. However, these findings could also be attributed to the fact that very few individuals were sampled across multiple seasons, which was especially true for children (ages 3-17). As well, studies conducted on industrialized populations tend to classify 'children' (under 12 years) separately from 'teenagers' (12-18 years) (18), while our analysis used 17 years as the cut-off between children and adults. This cut-off might have resulted in the mixing of disparate groups and the resulting loss of information about the differences in the microbiomes of adults and children. Taxonomic analysis may be required to further understand microbial changes for different age groups, as previous studies have suggested that the microbiota may develop more slowly in children and might persist in an "intermediate microbiota state", making it more tractable to environmental influences (18). This hypothesis corresponds with the observation that *Bifidobacterium* spp. are more abundant in the gut microbiota of children than adults (18). Similar patterns may be present in the Hadza community on a smaller scale, which is worth considering for future studies.

In addition to looking for a seasonal cycling pattern, the gut microbiome of the Hadza people was further analyzed to ascertain whether using different water sources resulted in a measurable difference. The scope of this analysis was limited to the 2014 ED season since it was observed from the metadata included in the dataset (14) that individuals from different bush camps used different water sources in that season. The subset of samples collected in the 2014 ED season also included three water samples taken from three different bush camps. The taxonomic compositions of these water sources were found to be distinct, corroborating earlier results (15) (Fig. 2A). However, we did not find a significant difference in the beta diversity of fecal samples when they were sorted according to water source (Fig. 2B). From previous results, we know that water sources influence the Hadza gut microbiome to a certain extent, as certain taxa were identified both in fecal samples and in corresponding water sources (15). Another study similarly reported an influence of water source on the gut microbiome of Hadza individuals from the Hukamako bush camp (37). However, our analysis was limited by the fact that there were unequal numbers of samples collected from each bush camp. Of note, there were less than 5 fecal samples from three camps (Benja, Hukamako, and Mwamudu), so it was difficult to make conclusions about those communities. Additionally, there was a lot of variation between individuals from the same bush camp, as seen by the spread of the samples, which made it difficult to delineate sample clusters according to bush camp. As well, using bush camp as a proxy for a unique water source probably left out other important differences between bush camps, such as variations in environment and diet, which could influence the gut microbiome.

To gain further insight into the gut microbiome of the Hadza people, we examined if sex influenced microbial composition. Using weighted UniFrac analysis, we found that males and females displayed non-unique patterns in their gut microbiome (Fig. 3A). These results did not agree with a previous study done on the Hadza, which did find a significant difference between males and females (38). However, the authors of that paper recognized the need to repeat the study with a larger dataset, like the one used in our analyses. Additionally, we determined that differences in the bush camps of individuals were more indicative of gut microbial composition. This was evident when looking at the Sengeli, Hukamako, and Gideru bush camps; they all displayed similar patterns in gut microbiomes that were considerably different from other sampled communities (Fig. S3). This was interesting, as the Sengeli, Hukumako, and Gideru bush camps are all geographically near each other, with similar environments (Peterson, D. Personal communication). These results support the idea that geographical location (and its potential relationship with the available food source and environmental conditions) may play a strong role in influencing the hunter-gatherer

microbiome. Further understanding of the similarities between these camps is required to comprehend what factors are at play to unify the gut microbial composition of these individuals.

Finally, we set out to determine if the right-hand microbiomes of the Hadza people varied based on sex. Interestingly, we found no clear differences in hand microbial composition between males and females using weighted UniFrac analysis (Fig. 3B). This was surprising, considering that previous studies identified sex as an important factor for influencing the hand microbiome (17, 39). It should be noted, however, that these previous studies were conducted using an industrialized population, meaning that their extrapolation to the Hadza people may not be valid. This sentiment is supported by the fact that hand microbiomes are known to differ based on geographical location. For example, differences in the hand microbiome were found between females living in the United States and Tanzania (40). Because of this, we investigated if the large variations seen in the hand microbiome were better explained by differences in bush camps. Indeed, individual bush camps displayed unique patterns in microbial composition. One clear example of this was the distinct clustering seen between the Hukamako and Makao right-hand microbiome samples. This observation could be due to the variations in lifestyle between different bush camps, where the Makao people have adopted farming and agricultural practices as a result of land sharing with the Sukuma people (Peterson, D. Personal communication). The Hukamako people, on the other hand, probably continue to live more traditional hunter-gatherer lifestyles. However, in a contradictory observation, individuals from the Kipamba bush camp, who have also adopted agricultural practices (Peterson, D. Personal communication), had similar right-hand microbiomes as the Hukamako people. This indicates that further knowledge of the activities that the Hadza people perform is required to understand this phenomenon.

Limitations Although our study provides insight into the Hadza hunter-gatherer microbiome, it has certain limitations. For many of our analyses, small sample sizes made it difficult to draw conclusions from our results. This was particularly true for the volatility of the gut microbiome across seasons, since the number of individuals sampled over the course of 3 seasons was limited. Additionally, uneven sampling between bush camps undermined the results acquired from the analysis of the effect of water source on the gut microbiome. This was particularly true for the Benja, Hukamako, and Mwamudu camps, where less than 5 fecal samples were acquired in the 2014 ED season. In addition, detailed information about the exact geographical locations of the bush camps, their proximity to water sources, and the activities that the individuals within these camps performed (especially on the day of sampling) was not available to us, which would have aided immensely in understanding how these factors may affect the Hadza microbiome.

Conclusions In conclusion, exploration of the effects of age, sex, and water source on the Hadza gut and hand microbiomes led to a set of observations unique to this hunter-gatherer society. We found that, upon using a slightly different workflow and a smaller number of samples than used previously (14), we could not replicate the seasonal cycling pattern of the gut microbiome obtained before (14). We also discovered that the Hadza gut microbiome may be less volatile than what is seen in industrialized populations, as seen by the longitudinal analysis comparing seasonal changes in the gut microbiome between children and adults. This lack of volatility was observed despite considerable changes in the Hadza diet across seasons. On top of this, we found that water source and sex did not significantly affect gut microbial composition, although other factors pertaining to differences in lifestyle and environment between bush camps may be important in this regard. The same conclusion was obtained from our analysis of the effects of sex on the right-hand microbiome, which showed a similar pattern for males and females, but displayed differences based on bush camps. Further insight into the Hadza lifestyle is required to extrapolate the importance of these findings, and to determine what factors may drive interpersonal microbial variation in this society. This knowledge will prove vital in understanding the pre-industrial microbiota, and will help illustrate the differences between the microbiomes of urban and hunter-gatherer populations.

Future Directions The Hadza people of Tanzania are known to have low rates of infectious and metabolic diseases (38) compared to industrialized populations. Furthermore, the human microbiome has been extensively linked to human health (41). This underscores the need to study the Hadza microbiome in depth in order to explore the factors that might affect the relationship between microbial communities and individual health. A greater understanding of how factors such as age, sex, and water source affect the unique microbiome of the Hadza, as explored here, may contribute to the link between health and the human microbiome, allowing for future medical applications concerning the manipulation of industrialized microbiomes. Various experimental and analytical approaches could be implemented in future studies to further understand the Hadza microbiome. First, a larger number of fecal samples collected across multiple seasons would increase the resolution of the impact of age on gut microbial volatility of the Hadza hunter-gatherers. Similarly, a larger sample size for each distinct bush camp would allow for a greater understanding of the impact of water source and sex on the microbiome, as analyses could be performed within each bush camp. Qualitative data involving the lifestyle of these individuals, such as what activities they were performing on the day of sampling, what they had been eating, and an understanding of their daily routine would help to determine the factors driving interpersonal variation of the microbiome between different bush camps. Apart from an increase in sample size and qualitative data, more computationally complex analyses such as a canonical correspondence analysis could be used to determine the relative contributions of factors like age, sex, and water source to the Hadza microbiome.

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CONTRIBUTIONS

A.C., M.D., M.G., and A.S. all contributed equally to both the research and writing of this manuscript. A.C. generated figures 1 and S1, M.D. generated figures 3B and S4, M.G. generated figures 3A and S3, and A.S. generated figures 2 and S2.

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