Volume 7: 1-14

UJEMI+

The Undergraduate Journal of Experimental Microbiology & Immunology (+Peer Reviewed)

The effect of honey consumption on the gut microbiome of Hadza hunter-gatherers in Tanzania

Nicolas M. Gauthier, Andi Musa, Ayah Al-Ansari

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY The Hadza are a hunter-gatherer society who live in northern Tanzania and serve as an excellent model to study the dynamics of the human gut microbiome in a nonindustrial, nonurban setting. Females and males, in the Hadza society, gather different food to provide for their camp. During the wet season, their main food source is honey which is principally foraged by Hadza adult males and is therefore consumed in a higher proportion by men than by women and children. The Hadza microbiome is well studied, but little is known about how honey-associated microbial communities affect the Hadza gut microbiome. Our study investigated if there are differences between the microbial composition of honey in the local Tanzania area and Hadza fecal samples, and how they vary between sexes and life stages. Diversity and differential abundance analysis showed that humans have higher microbial diversity compared to honey samples, and that some components of honey microbial communities are more similar to adult samples than children or infant samples. Additionally, we found that adult males and females have similar gut microbiota, suggesting no honey consumption driven microbiota differences between sexes. We postulate that honey may have a greater effect on Hadza adult microbiomes compared to children and infants, however our results are inconclusive. Our research provides valuable insight into the dynamics of the Hadza gut microbiome with increasing age, as well as the effect of diet on the microbiome.

INTRODUCTION

Hadza hunter-gatherers as models for public health. Hadza hunter-gatherers live in a savanna-woodland habitat encompassing about 4000 km in Northern Tanzania [1] and live a lifestyle that follows ancestral dietary patterns, with activities largely centred around food acquisition [2]. In contrast to populations living in urban settings, the Hadza population does not own any domesticated livestock, does not grow food and does not store any food. They survive by hunting with hand-made bows and foraging for plants, making their diet primarily plant-based with occasional meat, fat and honey [3]. Hunter-gatherer populations, such as the Hadza hunter-gatherers, serve as excellent models in public health to study areas such as metabolic and cardiovascular health, as well as to gain insight into the roots and causes of certain non-communicable diseases [4]. Metabolic and cardiovascular diseases are rare in hunter-gatherer populations compared to industrial populations, and obesity prevalence (< 5%) is lower than the industrial counterpart [4]. This is, while maintaining adult survivorship levels near industrial levels, factoring out infant mortality [4]. Therefore, understanding hunter-gatherer lifestyle, diet and microbiota may provide insight into slowing the progression of these non-communicable diseases in public health [4].

Honey and the Hadza hunter-gatherer diet. The diet of Hadza hunter-gatherers can be categorized into five main food types, varying in nutritional value: honey, meat, berries,

Published Online: September 2021

Citation: Nicolas M. Gauthier, Andi Musa, Ayah Al-Ansari. 2021. The effect of honey consumption on the gut microbiome of Hadza hunter-gatherers in Tanzania. UJEMI+ 7:1-14

Editor: Daniela Morales, Stefanie Sternagel and Brianne Newman, University of British Columbia

Copyright: © 2021 Undergraduate Journal of Experimental Microbiology and Immunology.

All Rights Reserved.

Address correspondence to: https://jemi.microbiology.ubc.ca/

baobab and tubers [1]. The availability, acquisition and consumption of each food type is heavily influenced by the changes in conditions associated with each season. The wet season is mainly associated with berry foraging and honey consumption, whereas the dry season is largely centred around animal hunting [2]. Certain sources of food are available on a year-round basis, such as fiber-rich tubers, establishing the requirement for a gut microbiota well adapted for broad-spectrum carbohydrate metabolism [5]. Being one of the most energy dense foods in nature, honey is a crucial component of the diet of many human forager populations [6]. Honey is mainly composed of glucose, fructose and about 25 unique oligosaccharides, as well as small amounts of enzymes, proteins, amino acids, vitamins and trace elements [7]. In addition to honey's reported anti-inflammatory, antibacterial, antioxidant and anti-cariogenic effects, when consumed at 50 to 80 grams per intake, it has been noted to increase the relative abundance of lactobacilli and bifidobacteria in the gut microbiota, likely due to the prebiotic effects of the oligosaccharides [7].

Foraging and dietary patterns of Hadza hunter-gatherers. Although much of the food that is acquired during foraging is shared at camp between sexes, the food eaten while foraging is believed to result in differences in diet between sexes, since males and females forage and acquire different types of food [1]. Men typically forage alone, hunting for animals while carrying an ax to access honey, bringing meat and honey back to camp, along with baobab fruit. Women mostly stay at the camp along with children and infants, but also go foraging in groups, along with their older children, mainly collecting baobab, berries and tubers [1], in addition to honey [6]. Although both sexes collect honey, men are reported to collect much more of it than women [6]. Studies investigating the sex differences in Hadza eating frequency by food type have found that while women eat more frequently throughout the day than men do, men eat more harder to collect foods (meat and honey) as a proportion of their overall diet than women do [1]. Previous studies have reported differences in microbial composition between males and females, likely reflecting this sex-based division of labour, as Hadza men and women are believed to be differentially adapted to their pattern of food consumption, with children following the pattern of consumption of women [8]. It is unclear whether these differences in diet between sexes and age groups affect microbiome composition and diversity as a result of the direct exposure to the microbial communities associated with each diet, or solely through adaptive mechanisms of the microbiota to optimize metabolism.

Gut microbiome of Hadza hunter-gatherers. The microbiome profile of this population has served as a model in numerous studies for studying the dynamics of the human gut microbiome in a nonindustrial, nonurban setting devoid of modern influences [1-2] [4-6] [8-9]. Following the longitudinal collection of 350 stool samples, Smits *et al.* (2017) observed an annual cyclic reconfiguration of the Hadza microbiome, during which certain bacterial taxa became undetectable before reappearing in a subsequent season, such as the *Succinivibrionaceae*, *Paraprevotellaceae*, *Spirochaetaceae* and *Prevotellaceae* families [2]. They found that the microbial composition of Hadza fecal samples from subsequent dry seasons (generally spanning from May to October) were indistinguishable from one another but differed significantly from samples collected during the intervening wet season (November to April) [2]. Additionally, comparison of the Hadza microbiome with those of populations of varying lifestyles revealed unique features associated with modernization [2]. Previous and subsequent studies have made similar findings, discovering that microbiomes from Hadza and other traditional societies share bacterial families that are absent from those of industrialized populations [9], and that Hadza hunter-gatherers have higher levels of microbial richness and biodiversity than Italian urban controls [8]. These differences have mainly been attributed to lifestyle and environmental factors. The differential availability of food sources and nutrients during different seasons supports the notion of changes in microbiome taxonomic composition to help maintain metabolic homeostasis [10]. The Hadza gut microbiota has been shown to be well adapted for broad-spectrum carbohydrate metabolism, as well as for branched-chain amino acid degradation and aromatic amino acid biosynthesis, which is important as their diet is high in dietary proteins (particularly from meat and baobab fruit) [5].

While it is known that the Hadza gut microbiome undergoes taxonomic reconfiguration between seasons to accommodate for the cyclic shift in available nutrients [2], little has been documented on the composition and diversity of the microbial communities associated with honey, and the extent to which exposure to these microbial communities directly affects the Hadza microbiome. Our study aims to explore if and how the microbial community of honey differs from that of the human gut as well as any association between honey consumption and microbiome composition of different sexes and life stages. Assuming the microbial communities associated with honey directly affect the microbiome composition and diversity, we hypothesize that 1) Hadza who consume more honey have gut microbiomes similar to honey microbial community, and 2) based on the reported higher quantity of honey consumption by adult males, similarity between honey and human samples is to be higher in males than in females. We also hypothesize that 3) the honey microbial communities are more similar to Hadza adult gut microbiomes than those of children and infants, since children and infants are reported to stay at camp with women, presumably following dietary patterns that more closely resembles that of their mothers.

Understanding the potential role a key food source has on gut microbiome composition can provide insight on the effect of diet on human microbiota. It could suggest that factors of our diet can influence our microbiome composition which is crucial for immunity and nutrition. Understanding the interplay between diet and the microbiome may help elucidate mechanisms involved in microbiome taxonomic shifts and may consequently lead to the understanding of non-communicable and microbiome-associated diseases [4,11].

METHODS AND MATERIALS

Dataset and metadata. We used a dataset generated by Smits *et al*. (2017), (Accession number: PRJEB27517), who collected 350 samples in 2013 and 2014 and which spanned five Hadza subseasons. Smits *et al*. sequenced the 350 Hadza hunter-gatherer fecal samples using Illumina sequencing and targeted the V4 region of the 16S rRNA gene using the 806R (5'-GGACTACHVHHHTWTCTAAT) and 515F (5'-GTGCCAGCMGCCGCGGT
AA) primers. Smits *et al.* used primers targeting the V4 constant region and amplified the variable regions in this gene via PCR amplification. The V4 constant regions are more conserved throughout the bacterial kingdom allowing for primer binding, and amplifying the variable regions allows for determination of unique amplicon sequence variants to be used downstream (ASVs) in our analysis. These unique variants are used to gain insight on the taxa present in the community and their relative abundances. Following PCR amplification, Smits *et al.* sequenced the amplified fragments by using Illumina sequencing. The output from each step of the QIIME2 pipeline, described below, serves as the input for subsequent steps. Quantitative Insights Into Microbial Ecology 2 (QIIME2) is a free, open source, community developed next-generation microbiome bioinformatics platform [12]. Smits *et al*. also collected other sample types such as Hadza skin swab samples, water samples and animal fecal and stomach swab samples, which were not included in this analysis and therefore filtered out [2]. Our study focused particularly on honey and Hadza fecal samples from a particular bush camp (Hukamako) as well as the metadata categories of sex and life stage [2]. This dataset and its processing methods have been made available for viewing on public databases and Smits *et al.* (2017) supplemental files by the study authors. The study metadata contains information about sample type, bush camp, treatment, sex, age, life stage (adult, infant, child), season collected, etc. Adult refers to individuals 18 years of age and over, child refers to individuals from 1-18 years of age and infant refers to individuals under one. However, it is noted that these records are estimates according to Hadza tribe members and sample takers due to a lack of documentation by the tribe [2].

Metadata filtering and grouping. Our investigation focused on comparison between honey samples and Hadza hunter-gatherer fecal samples from the Hukamako bush camp. All other sample types and sources were filtered out of the original dataset. The finalized filtered data consisted only of honey samples and human fecal samples from the Hukamako bush camp.

Samples from all seasons were also included in our analysis, as it is known honey is consumed by the Hadza year-round with higher consumption occurring during the wet season [8]. Furthermore, the dataset was filtered in QIIME2 to exclude mitochondrial sequences and low frequency amplicon sequence variants representing less than 0.005% of total sequencing reads.

QIIME2 pipeline for data processing. Using QIIME2, we imported 16S rRNA sequences from the original dataset, which had previously been demultiplexed with the QIIME 1.9.1 package [2]. The number of reads per sample had a range of 7,183 to 61,773, with a median and a mean of 25,525 and 25,809.8, respectively. We used Deblur [13] and Divisive Amplicon Denoising Algorithm 2 (DADA2) [14] to run sequence quality control and filter out the reads with a high number of PCR errors. We chose 246 nucleotides for the truncation length since more than 75% of our sequences contained at least 246 nucleotides, allowing us to maintain these sequences while minimizing excessive truncation and potential loss of ASVs. Additionally, the sequence quality was seen as relatively stable for the first 246 nucleotides, suggesting that sequences with true PCR errors are not likely to have been kept at this truncation length. For this study's dataset, the sequencing depth of 12,394 was chosen to adequately represent sample richness. Such as with determining truncation length, other rarefaction parameters are possible and could be supported. These steps are outlined in the associated Script 1 file.

Taxonomic classification. To determine the taxonomic composition of these samples, a feature classifier was constructed using pre-formatted Silva 138 SSU reference sequences and taxonomy files with 99% identity criteria, which was used to classify ASVs taxonomically [15]. Our taxonomic analysis was limited to bacterial class level data, due to the presence of chloroplasts in the honey samples that were not filtered out early enough in the data processing pipeline. Using class level data eliminated low abundance chloroplast reads from our taxonomic analyses.

Alpha and Beta diversity of honey and Hadza fecal samples. To investigate the difference in microbial diversity between honey and Hadza fecal samples collected by Smits *et al.*, we looked at commonly used within- (Alpha: Shannon's diversity index, Observed Features, Faith's phylogenetic diversity, Pielou's evenness) and between-sample (Beta: Jaccard distance, Bray-Curtis distance, Unweighted UniFrac distance, Weighted UniFrac distance) diversity metrics, along with Principal Coordinate Analysis (PCoA) visualizations for beta diversity. To undergo this diversity analysis, a phylogenetic tree was generated using the q2-fragment-insertion QIIME2 plugin and SILVA 128 SATé-enabled phylogenetic placement (SEPP) reference database [13], along with the list of each unique variant generated from the denoising step. Significance of the differences between groups was tested using Pairwise PERMANOVA, significance cut off of a q-value below 0.05 was used ($\alpha = 0.05$). Due to filtering based on feature counts and outliers, only a total of 8 honey samples were included in these calculations, in addition to 102 Hadza fecal samples. To explore the differences in microbial abundance and phylogenetic distances between honey samples, male fecal samples and female fecal samples, we performed a weighted UniFrac analysis. To investigate the difference in microbial diversity between both sexes, and between the three documented life stages of Hadza hunter-gatherers (infant, child, adult), we compared Faith's phylogenetic and Shannon's diversity metrics of both sexes and all three life stage groups.

Differential and relative abundance analysis. Tidyverse [16] and ggplot2 [17] packages (R [18] environment version 4.0.3) were used for statistical analysis and graphical visualization of differential abundance analysis and relative abundance analysis. Statistical significance was assessed using Kruskal-Wallis pairwise testing ($\alpha = 0.05$). Steps for this process are outlined in the associated Script 2 file.

RESULTS

Honey and Hadza fecal samples differ significantly in diversity and composition. To explore the nature of the difference in diversity between honey and Hadza fecal microbial communities, we performed beta diversity metrics (Jaccard, Bray-Curtis, Unweighted and Weighted UniFrac) and used PERMANOVA for pairwise comparisons to determine significance. Each assessed metric provides unique information on the nature of the difference between sample types, as different combinations of factors are used to evaluate compositional differences between 16S microbiome of samples, each comprising an individual data point (Jaccard: neither phylogenetic distance nor abundance, Bray Curtis: abundance only, Unweighted UniFrac: phylogenetic distance only, Weighted UniFrac: both phylogenetic distance and abundance). The clear clustering pattern between sample types on PCoA plots suggested a significant difference (q-value = 0.001) between them in each of the four assessed metrics, distinguishing honey samples from Hadza fecal samples and highlighting both phylogenetic distance and abundance as important driving factors for this difference (Fig. 1). PERMANOVA pairwise comparison confirmed the significance of these differences for each of the four assessed metrics, with a q-value of 0.001 (Fig. 1, Supplemental Table 1). To analyse the diversity within Hadza fecal samples and honey samples, we performed Shannon's and Faith's alpha diversity metrics (while Shannon's diversity only considers abundance, Faith's diversity only considers phylogenetic distance). In both assessed metrics, which compares the mean diversity between groups, Hadza fecal samples had significantly higher diversity than honey samples (Supplemental Fig. 1), confirmed by Kruskal-Wallis pairwise testing ($\alpha = 0.05$; Shannon's diversity q-value = 0.0004, Faith's phylogenetic diversity q-value = 0.002).

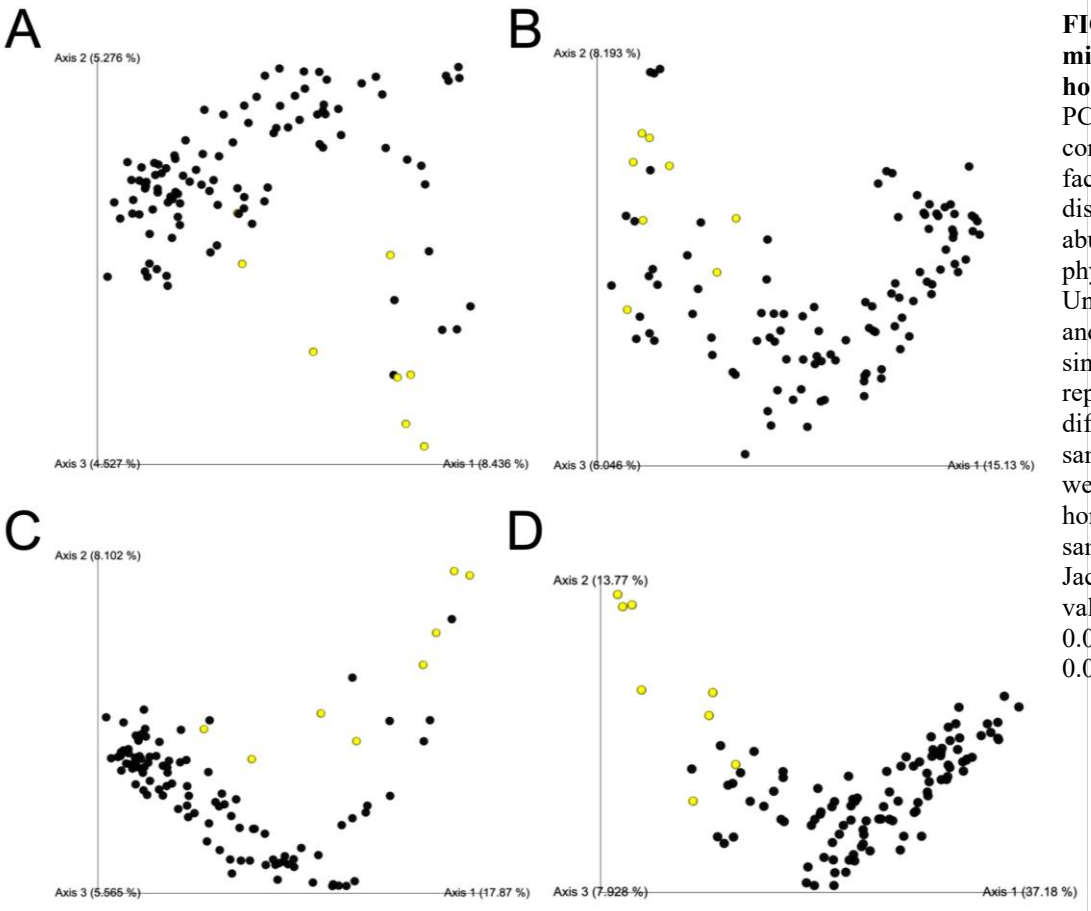

FIG. 1 PCoA plot shows distinct microbial communities between honey and Hadza fecal samples. PCoA plots are 3-dimensional; each considering a different combination of factors (Jaccard: neither phylogenetic distance nor abundance, Bray Curtis: abundance only, Unweighted UniFrac: phylogenetic distance only, Weighted UniFrac: both phylogenetic distance and abundance). Each point represents a single sample. Distance between points represents how compositionally different the 16S microbiomes of samples are from one another. There were significant differences between honey (yellow; n = 8) and Hadza fecal samples (black; n = 102) for (A) Jaccard (Pairwise PERMANOVA q-value = 0.01), (B) Bray-Curtis (q = 0.01), (C) unweighted UniFrac (q = 0.01) and weighted UniFrac (q = 0.01).

Additionally, we performed differential abundance analysis between honey and Hadza samples to determine microbial compositional differences. This analysis was performed at the Class level, compared the relative abundance of all bacterial classes between two groups,

and determined the classes that significantly differ in relative abundance between the groups. All other bacterial classes not mentioned do not differ significantly between groups in their relative abundance. We detected higher relative abundance of Bacteroidia and Negativicutes in Hadza fecal samples compared to honey samples (Supplemental Fig. 2), indicating that the microbial community of honey samples and Hadza fecal samples differ significantly in their microbial diversity and compositions.

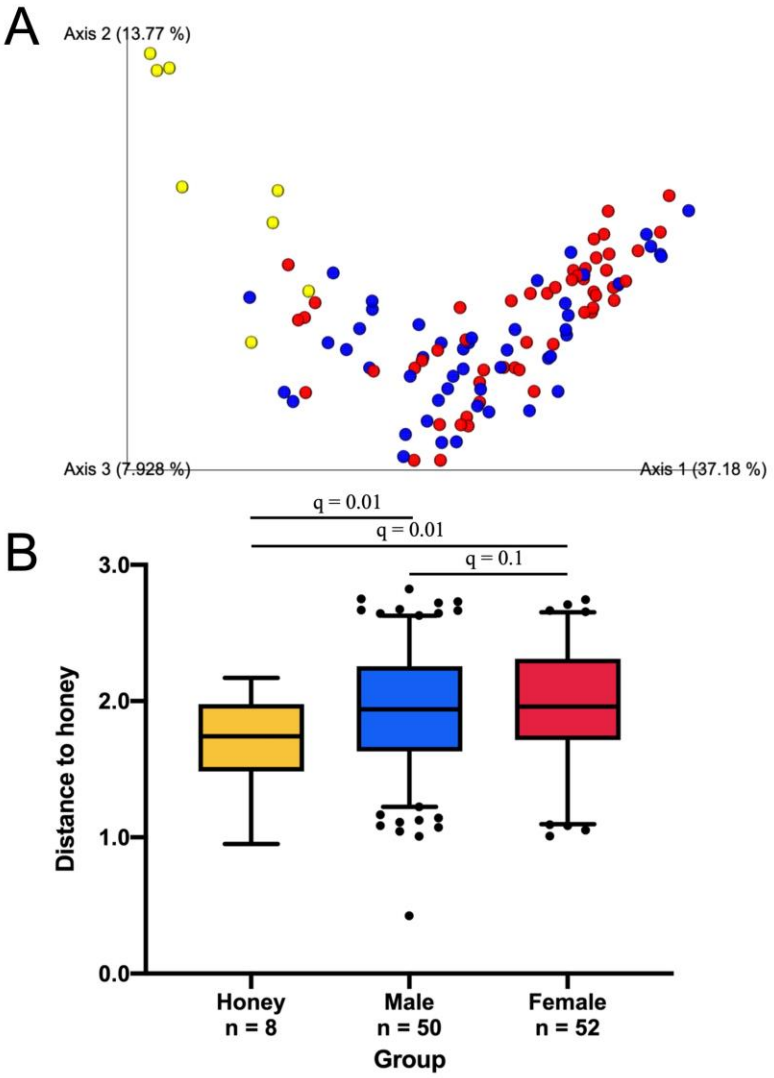

FIG. 2 Beta diversity between honey and Hadza fecal samples does not differ between sexes. (A) 3-dimensional PCoA plot of Weighted UniFrac Beta diversity, which considers both phylogenetic distance and abundance when plotting compositional differences between 16S microbiome of samples. Each colour represents 16S microbiome of honey samples (yellow; n = 8), Hadza male (blue; n = 50) or Hadza female (red; n = 52) fecal samples. Distance between points represents how compositionally different the 16S microbiomes of samples are from one another. Plot showed that honey has a distinct microbial community from humans but there is no distinct community between sexes, (B) illustrated by boxplot and confirmed by Pairwise PERMANOVA testing ($\alpha = 0.05$), using the same data as in PCoA plot. The box represents the interquartile range, and the middle line represents the median. Whiskers represent 95% confidence intervals.

Male and female Hadza fecal samples do not differ from each other or in their similarity to honey microbial samples. To determine whether male and female Hadza gut microbial communities differ, we similarly performed Shannon's and Faith's alpha diversity metrics and differential abundance analysis. Both Shannon's and Faith's box plots showed no significant sex-related differences in community diversity, confirmed by Kruskal-Wallis pairwise testing (Supplemental Fig. 3; Shannon's diversity q-value = 0.5, Faith's phylogenetic diversity q-value = 0.2*)*. Additionally, differential abundance analysis at the Class level between male and female samples revealed that there were no bacterial classes that differed in relative abundance between sexes.

In order to determine whether the microbial community of one sex group shares more similarity to that of honey's, we generated a PCoA plot (Fig. 2A) and a Weighted UniFrac box plot comparing "distance to honey" for the three groups (Hadza male, Hadza female, honey samples). Weighted UniFrac plots indicated a near identical order of magnitude for distance to honey between male and female groups (Figure 2B). This was confirmed by PERMANOVA pairwise comparison between sex groups, producing an q-value of 0.1, suggesting that neither male nor female fecal communities differ significantly in their similarity to the honey microbial communities (Fig. 2B). Further, Weighted UniFrac plot and PERMANOVA pairwise testing indicated significant differences between honey communities and both Hadza male and female samples (Fig. 2B; q = 0.01). Therefore, Hadza male and female fecal communities do not differ significantly in their composition and diversity. This was also observed by investigating the effect of sex within adult and child life stage groups, adjusting for life stage (Supplemental Fig. 4). Sex differences remained absent within both of these groups.

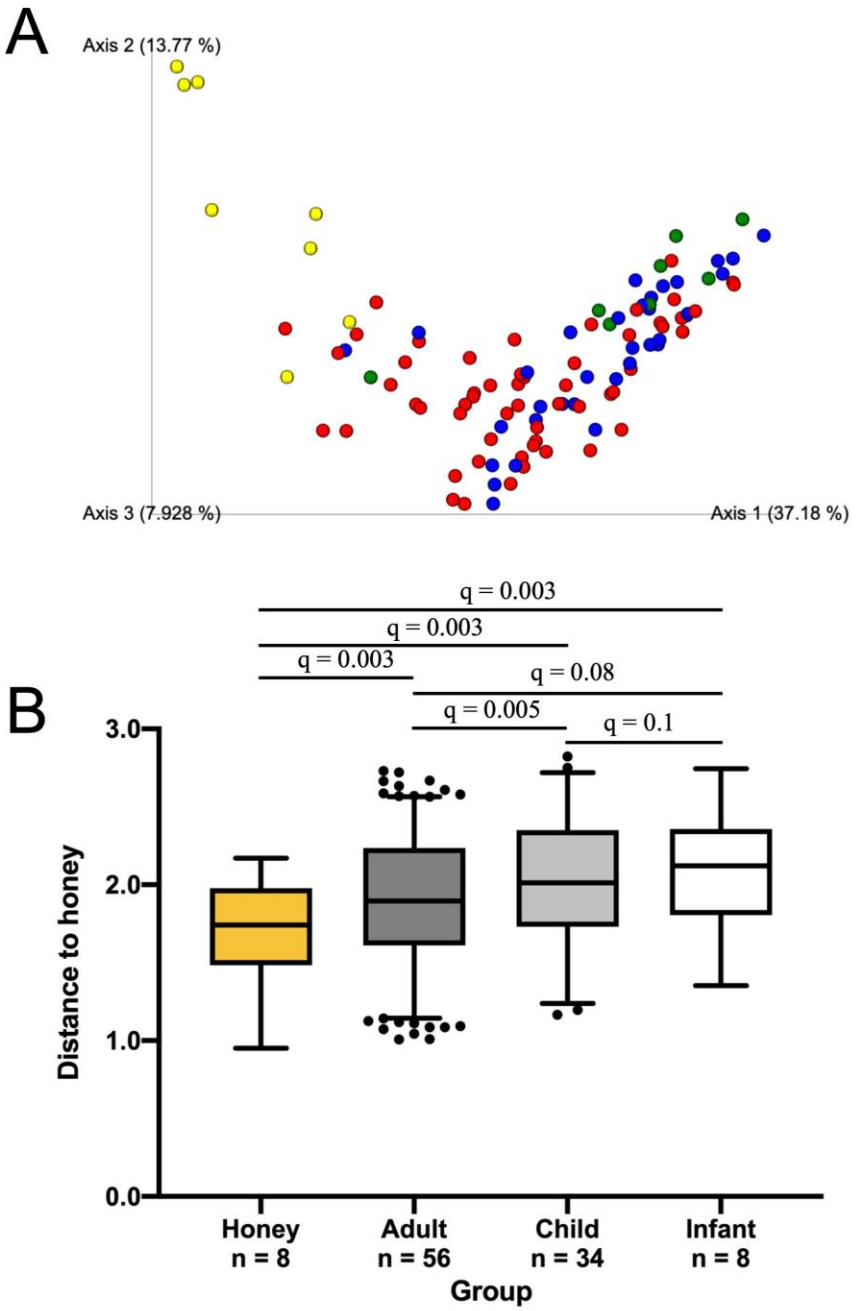

FIG. 3 Similarity between honey and Hadza fecal microbial communities is highest in adults. (A) 3-dimensional PCoA plot of Weighted UniFrac Beta diversity, which considers both phylogenetic distance and abundance when plotting compositional differences between 16S microbiome of samples. Each colour represents 16S microbiome of honey samples (yellow), Hadza adult (red), Hadza children (blue) and Hadza infant (green) fecal samples. Distance between points represents how compositionally different the 16S microbiomes of samples are from one another. PCoA plot shows that honey has a distinct microbial community from humans, but little distinction between life stage groups, illustrated by (B) boxplots and confirmed by Pairwise PERMANOVA testing ($\alpha = 0.05$), using the same data as in PCoA plot. The box represents the interquartile range, and the middle line represents the median. Whiskers represent 95% confidence intervals.

Adult Hadza fecal sample diversity differs from younger age groups but similarity to honey microbial diversity does not differ between age groups. In order to determine if Hadza fecal microbial communities vary between different age groups, we performed Faith's and Shannon's alpha diversity metrics and compared the microbial diversity between Hadza adult, Hadza child and Hadza infant fecal communities. For both alpha diversity metrics assessed, microbial diversity was significantly lower in infants than in adults or children (Supplemental Fig. 5; for Faith's phylogenetic diversity, q = 0.001 and 0.005, respectively, and for Shannon's diversity, q = 0.003 and 0.03, respectively). No significant differences in microbial richness were observed between adults and children for these alpha diversity metrics (Faith's phylogenetic diversity q-value = 0.08, Shannon's diversity q-value = 0.1). These observations were all confirmed by Kruskal-Wallis pairwise testing. This suggests that gut microbial communities between life stages differ, with infant Hadza communities having the lowest diversity within the life stage groups.

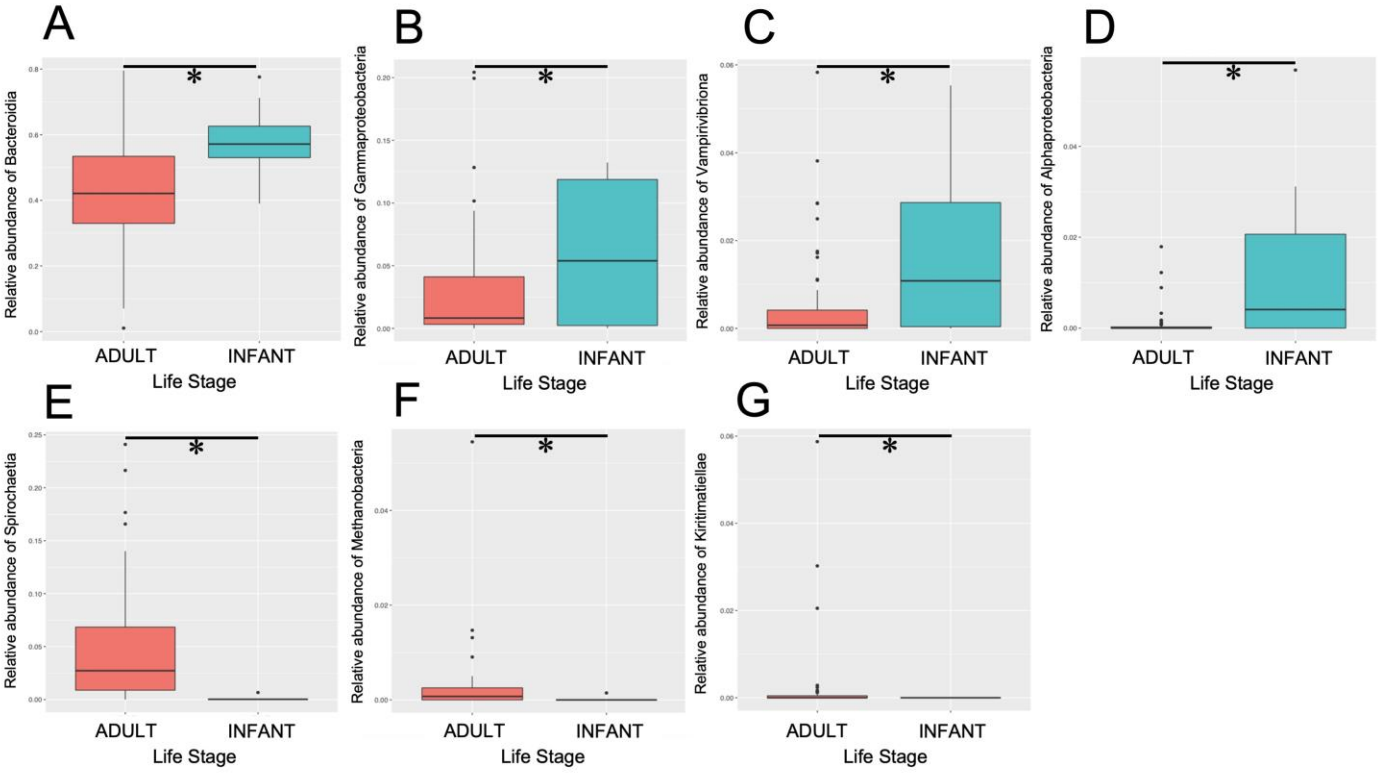

FIG. 4 Boxplots showing the relative abundances of seven bacterial classes between adult and infant Hadza fecal samples. Differential abundance analysis determines the bacterial Classes that differ significantly in relative abundance between adult and infant Hadza fecal samples. All bacterial Classes not mentioned do not differ significantly between groups in their relative abundance. Differential abundance analysis identified a relatively higher abundance of (A) Bacteroidia, (B) Gammaproteobacteria, (C) Vampirivibriona, (D) Alphaproteobacteria in infant samples, and of (E) Spirochaetia, (F) Methanobacteria and (G) Kiritimatiellae in adult samples. Differences are confirmed by differential abundance analysis with DESeq2 ($\alpha = 0.05$, asterisk indicates $q < 0.05$). The box represents the interquartile range (IQR) and the middle line represents the median. Upper and lower whiskers go to the highest or lowest measurement, or to the IQR multiplied by 1.5, whichever is higher or lower, respectively.

To further investigate whether life stage has an effect on the previously observed differences between microbial compositions of honey and Hadza fecal samples, we performed a weighted UniFrac analysis between honey samples, Hadza adult, Hadza child, and Hadza infant fecal samples (Fig. 3). As in previous PCoA plots, the resulting clustering pattern showed distinct microbial communities between honey and human samples, but no clear difference within human categories (Fig. 3A). The difference between these groups was

determined by PERMANOVA pairwise testing between life stage groups, which revealed no difference between the human life stage groups in their similarity to honey samples (Fig. 3B).

To identify bacterial classes that may contribute to differences between life stage groups, we performed differential abundance analysis between Hadza adult, Hadza child and Hadza infant fecal samples. This analysis revealed multiple bacterial classes that differ in relative abundance throughout life stages. Compared to infants, adults had a lower relative abundance of Alphaproteobacteria, Vampirivibriona, Gammaproteobacteria and Bacteroidia, and a higher relative abundance of Spirochaetia, Methanobacteria and Kiritimatiellae (Fig. 4). Compared to children, adults had a lower relative abundance of Bacteroidia and a higher relative abundance of Bacilli (Fig. 5). The only difference between children and infants is the higher relative abundance of Spirochaetia in children (Supplemental Fig. 6).

Additionally, we performed differential abundance analysis between honey samples and Hadza fecal samples of each life stage group at the Class level to determine bacterial classes that differ from honey samples in relative abundance throughout life stages. These differential abundance analyses revealed the following differences between honey and each life stage group: Compared to honey, Hadza infants fecal samples had a higher relative abundance of Bacteroidia and a lower relative abundance of Alphaproteobacteria and Spirochaetia (Supplemental Fig. 7). Hadza children fecal samples had a higher relative abundance of Bacteroidia and Negativicutes and a lower relative abundance of Alphaproteobacteria compared to honey (Supplemental Fig. 8). The only bacterial class with a significantly different relative abundance between Hadza adult fecal samples and honey samples was Actinobacteria, which was more highly abundant in honey samples (Supplemental Fig. 9).

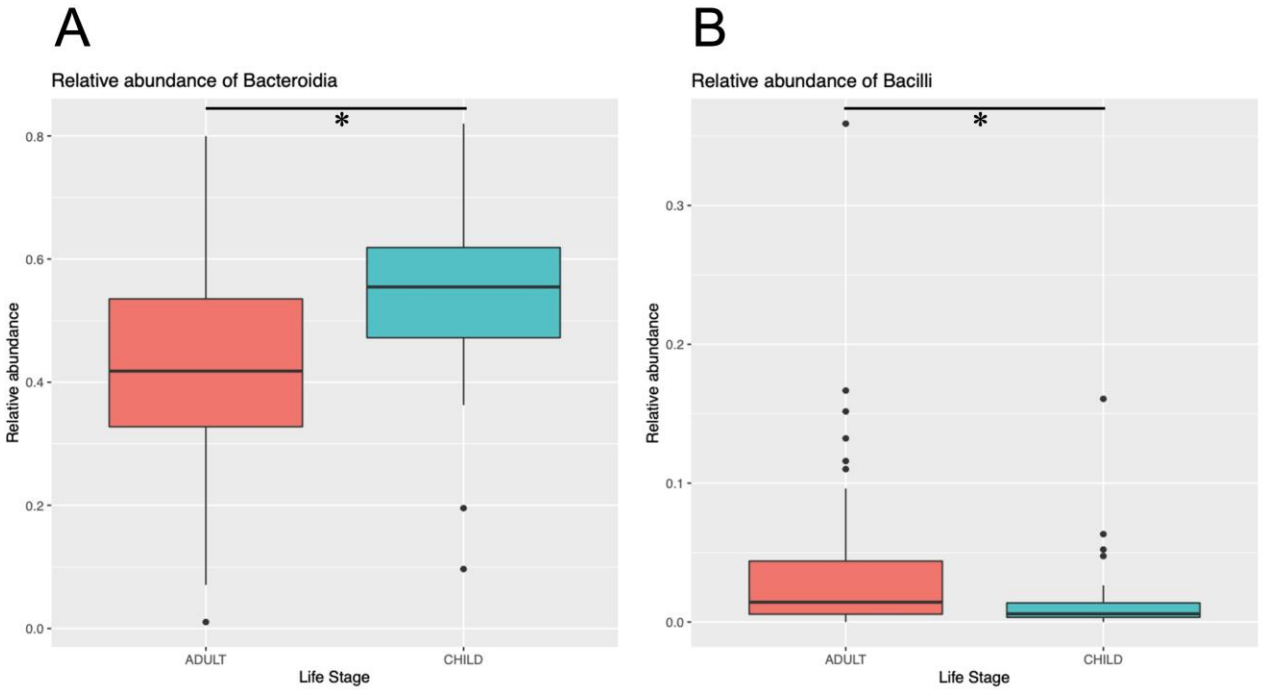

FIG. 5 Boxplots showing the relative abundance of Bacteroidia and Bacilli between adult and child Hadza fecal samples. Differential abundance analysis determines the bacterial Classes that differ significantly in relative abundance between Hadza adult and child Hadza fecal samples. All bacterial Classes not mentioned do not differ significantly between groups in their relative abundance. Differential abundance analysis identified a relatively higher abundance of (A) Bacteroidia in children, and a higher abundance of (B) Bacilli in adult samples. Differences are confirmed by differential abundance analysis with DESeq2 ($\alpha = 0.05$, asterisk indicates $q < 0.05$). The box represents the interquartile range (IQR) and the middle line represents the median. Upper and lower whiskers go to the highest or lowest measurement, or to the IQR multiplied by 1.5, whichever is higher or lower, respectively.

Therefore, these results indicate that Hadza fecal bacterial community diversity varies significantly between adults and children or infants, but that regarding similarity to honey microbial community diversity, there are no significant differences between life stage groups.

In comparing relative abundance of bacterial classes, Hadza adult fecal microbial communities had lower abundance of Bacteroidia and Bacilli compared to younger age groups and greater Spirochaetia abundance than younger age groups. Hadza child fecal communities also contained greater Spirochaetia abundance than Hadza infants. Hadza child and infant fecal communities carried greater abundance of Bacteriodia than honey communities and lesser abundance of Alphaproteobacteria. Hadza adult samples differed to honey samples only by a lower abundance of Actinobacteria.

DISCUSSION

We aimed to explore the effect of sex and life stage on the similarity of the human microbiome diversity and composition to honey microbial communities, with the objective of studying the effect of honey consumption on the gut microbiome of Hadza hunter-gatherers.

Honey and human microbial communities significantly differ in diversity and composition. Consistent with our first hypothesis, honey and Hadza fecal samples were shown to have significantly different beta diversity (Fig. 1). In addition, alpha diversity analysis and differential abundance analysis between honey samples and Hadza fecal samples revealed that the Hadza microbiome has higher diversity than honey (Supplemental Fig. 1), as well as higher relative abundance of Bacteroidia and Negativicutes, after analysing all bacterial classes between honey and Hadza groups (Supplementary Fig. 2). The high prevalence of these bacterial classes may suggest that they play an important role in the human microbiome that is not present in honey microbial communities. While little is known about the role of Negativicutes on the human microbiome other than its high abundance [19], Bacteroidia class microbes, have been known to be the most predominant anaerobic bacteria in the gut [20]. Bacteriodia generally maintain a beneficial relationship with their host as advantageous commensal bacteria. Bacteroidia class organisms adapt, survive and thrive in the gut due to their ability to expel toxins readily and influence the immune system, as well as their complex systems to adapt to nutrient availability [20]. This bacterial class generally benefits humans by providing its host with nutrient and energy sources via carbohydrate fermentation as well as activating T-cell-dependent immune responses [20]. Recent studies have suggested that Bacteroidia play a role in preventing *C. difficile* infection in humans [21]. The benefits this bacterial class provides to humans, along with its ability to adapt in the gut environment elucidate why they are found at a high relative abundance in humans. Furthermore, the decreased microbial diversity observed in honey may be due to honey's inherent antibacterial effect, mostly against gram positive bacteria [7]. Honey's low water content, low pH and production of hydrogen peroxides by glucose oxidase leads to a hostile environment for bacteria, leading to the lower observed diversity in the honey samples compared to fecal samples [7].

Male and female microbiomes do not differ in their similarity to honey microbial communities. Alpha diversity analysis between male and female fecal samples revealed no sex-linked differences in microbial diversity (Supplemental Fig. 3), and differential abundance analysis showed no difference in relative abundance at the Class level between males and females. Our beta diversity analyses between sex groups revealed that there is no sex-linked effect on similarity of Hadza microbiome with honey microbial communities (Fig. 2). These results did not support our hypothesis concerning the differences between diversity and composition of Hadza male and female microbiomes. This may be due to the fact that sex differences are more commonly reported at lower taxonomic levels, such as the genera or family level [6], whereas potential significant relative abundance differences between males and females may have been masked in our study by our limitation to the Class level. Alternatively, it is possible that the variable consumption of honey, seen between male and female Hadza members, does not yield a discernible difference in diversity of the microbiota that would have more closely resembled honey in one of the groups [6]. It is also possible that the honey microbiome does not directly affect the human microbiota. Rather, honey

constituents, such as its ~25 unique oligosaccharides, produce changes in the microbiota that have not been highlighted in this analysis [7].

The adult microbiome is not significantly more similar to honey microbial communities than are the children and infant microbiomes. Alpha diversity analysis between Hadza fecal samples from each of the life stage groups confirmed the previously described increase in microbiome diversity with age (Supplementary Fig. 5). Furthermore, results from beta diversity analyses between honey and adult, child and infant gut samples, respectively, revealed no significant difference in microbial diversity between lifestage in comparison to the microbial composition of honey. Therefore, these results do not support our hypothesis that the honey microbial communities are more similar to Hadza adult gut microbiomes than those of children and infants, due to a diet following less honey consumption. This again may be due to the fact that the constituents in honey, primarily oligosaccharides, are responsible for changes in microbiota [7]. The prebiotic effects of these oligosaccharides have been documented to increase the abundance of lactobacilli and bifidobacterium in the human gut [7]. Therefore, we propose that adult life stages will have a higher abundance of lactobacilli and bifidobacteria compared to infant and child life stages.

Differential abundance analysis of all bacterial classes present across each life stage revealed a certain subset of bacterial classes that significantly differ in relative abundance between adults, children and infants (Fig. 4, 5, Supplementary Fig. 6). In comparison to fecal adult samples, both infant and children samples were shown to have significantly higher relative abundances of Bacteroidia (Fig. 4A, 5A, Supplementary Fig. 6). Similarly, in comparison to honey samples, both infant and children samples were shown to have significantly higher relative abundances of Bacteroidia (Supplementary Fig. 7A, 8A, 9), while adults did not. This aligns with previous findings that strains from Bacteroidia classes are vertically transmitted from mothers to infants during vaginal birth, leading to an initial and persistent colonization of the infant gut [22]. These maternally derived strains have been demonstrated to present a high stability during colonization [22]. A candidate explanation of this effect may involve maternal immunoglobulins derived from the gut microbiota and passed vertically through breast milk, providing an advantage to maternal bacterial strains [22].

Contrarily, Spirochaetia were found in higher relative abundance in both adults and children compared to infants (Fig. 4E, Supplementary Fig. 6). While relative abundance of Spirochaetia did not differ from honey samples in both child and adult samples, infants were shown to have a lower relative abundance of Spirochaetia compared to honey. These findings suggest that Spirochaetia are not highly abundant at birth, before increasing in relative abundance with age. Spirochaetia have been reported to be among the most abundant microbes in honeybee guts [25], providing support towards the notion that microbial communities associated with honey consumption may affect the gut microbiome. Furthermore, Spirochaetaceae, members of the Spirochaetia class, are reported to be enriched in traditional populations but absent in industrialized populations, therefore, little is known about their role in the gut [23]. This discrepancy may also be explained by the higher proportion of honey consumption in the Hadza diet compared to industrialized populations [1].

Additionally, children were shown to have a significantly lower relative abundance of Bacilli compared to adults (Fig. 5B); a distinction that was not made between adults and infants. Further investigation into the taxonomy of our study samples revealed that the main order comprising Bacilli in honey and human fecal samples was Lactobacillales. Bacterial strains from this order have been reported to be prevalent in beehives, constituting up to 90% of the honey microbial content [24]. Lactobacillales have been documented to transmit from food sources such as honey to the human gut and play a role in modulating the gut microbiota by producing lactic acid and interfering with pathogenic microbes [24]. Honey oligosaccharides have also been documented to increase the abundance of lactobacilli in the human gut [7]. A possible explanation for the higher relative abundance of Bacilli seen in adults compared to children may therefore be the increased honey consumption in adults compared to children. As adult males are known to acquire and consume a greater proportion of honey [1], this possibly results in greater colonization of the gut by Lactobacillales with

increasing age. However, it is important to note that bee and honey microbiota are affected by numerous environmental conditions, such as types of flowers, influencing the applicability of these findings [26].

Since the microbiome is known to alter drastically and increase in diversity with increasing age, resulting in shifts in composition and in relative abundance of bacterial strains, it is highly possible that the increase in similarity between honey and adult Hadza fecal samples occurs as a result of a multitude of factors unrelated to honey consumption. Although, honey serves as a pinnacle component of the Hadza diet, and it is known to be consumed at higher rates in adulthood than in infanthood and childhood [6], our results do not provide supporting evidence towards the notion that consumption of honey and its associated microbial communities have an impact on diversity of the Hadza microbiome. Although further investigations are required to better understand the specific role of diet-associated microbial communities on the gut microbiome, changes in differential abundance of certain taxa including the emergence of lactobacilli in adulthood, may suggest that microbial factors of the diet, such as honey-associated microbes, may influence our microbiome composition, providing new insights on an aspect of our diet that is not commonly thought about.

Limitations Several limitations exist in our study that should be considered. Firstly, the already small number of collected honey samples was narrowed down to 8 samples, following feature-based filtering and rarefaction. Although this is enough for statistical analysis, more samples would allow for higher statistical power. The low sample size of honey samples inevitably dampens the generalizability and reliability of our results. Secondly, we limited our samples to a specific bush camp of the Hadza population, the Hukamako bush camp, in order to decrease potential variance between camps. This might limit our results from being generalizable to the whole Hadza population. In addition, approximately half of the honey samples in the original dataset were collected from the Hukamako bush camp, while the other half were not assigned to a bush camp but were still used in this study. These honey samples may come from other bush camps, potentially interfering with the specificity of the study on the Hukamako bush camp. Lastly, our differential abundance analysis was conducted at the Class level, potentially limiting the observation of further differences between groups of interest. This was done because of the presence of chloroplasts in the honey samples that were not filtered out early enough in the data processing pipeline. Being restricted to the Class level likely masked potential differences between groups, such as between males and females, for example, that may have been present at higher taxonomic levels.

Conclusions Our study aimed to explore the effect of sex and life stage on microbiome diversity and composition of Hadza hunter-gatherers, as well as the effect of consumption of honey and its associated microbial communities on the Hadza microbiome. None of our hypotheses concerning sex-linked effects were supported by our results; we observed no sex-linked differences in microbiome diversity, or in composition at the Class taxonomic level. Additionally, we found that similarity between honey and human fecal samples is not affected by sex. Our analyses between life stage groups revealed no significant differences in microbiome diversity between adults, children and infants. As expected, the infant microbiome had the least diversity, and multiple bacterial classes were shown to differ in relative abundance between adults, children and infants. Differential abundance between honey samples and each of these life stage groups provided evidence towards the idea that honey-associated microbial communities may directly affect the human microbiome, but because the microbiome is so dynamic and gets altered as a result of a multitude of factors, it remains challenging to isolate the effect of a single factor, such as honey consumption. These results provide some insight into dietary factors affecting the human microbiota and may help explain changes in microbial composition in traditional populations.

Future Directions Further analysis of the Hadza dataset can be directed toward studying the Honey microbial dynamics according to season, since Hadza diet is highly dependent on seasonal availability of nutrition [2]. Relating these findings to the gut microbiota of the Hadza people may help further elucidate the role of honey in the change of the Hadza gut microbiome. If the microbial communities associated with honey really do affect the

microbiome as we have suggested it to, we may expect similarity between human and honey samples to increase in the wet season, during which honey consumption is at its highest. This could potentially provide further evidence towards the conclusions of this study. In addition, differential abundance between honey and the Hadza gut can be performed at a higher taxonomic level, which may provide insight into the colonization mechanisms of specific bacterial strains due to diet. This may also help uncover the role of some specific taxa unique to the Hadza microbiome. Investigation of the taxa unique to the Hadza microbiome may provide understanding of the microbial dynamics of traditional populations and may even lead to the understanding of microbiome shifts associated with the urbanization of humans.

ACKNOWLEDGEMENTS

We would like to thank Dr. Evelyn Sun and Dr. Stephan Koenig (course instructors), Dr. David Oliver (course director), Emily Adamczyk (teaching assistant) and the rest of the MICB 421 teaching team for providing technical and advisory support throughout the development of our project. We would also like to thank the UBC Microbiology and Immunology Department for providing the facilities, funding, and resources allowing us to conduct this study. Additionally, we would also like to thank Smits *et al.* for providing the dataset used to conduct this study.

We would also like to thank two anonymous reviewers for constructive feedback on this manuscript.

CONTRIBUTIONS

All authors contributed to the analysis of the dataset, research question of the paper and the ideas presented in the paper. Ayah contributed in performing beta analyses and writing the discussion and study limitation sections of the paper. Andi contributed in performing taxonomic analyses and in the writing of the discussion, future directions sections and final editing of the manuscript. Nicolas performed alpha, beta and differential abundance analyses, generated and formatted figures, wrote figure captions and wrote the abstract, introduction, methods & materials, results and conclusion sections, collaborated with teammates to write the discussion section, compiled references and edited the draft manuscript.

REFERENCES

1. Berbesque JC, FW Marlowe, AN Crittenden. 2011. Sex Differences in Hadza Eating Frequency by Food Type. Am J Hum Biol. 23(3):339-45.
2. Smits SA, Leach J, Sonnenburg ED, Gonzalez CG, Lichtman JS, Reid G, Knight R, Manjurano A, Changalucha J, Elias JE, Dominguez-Bello MG, Sonnenburg JL. 2017. Seasonal Cycling in the Gut Microbiome of the Hadza Hunter-Gatherers of Tanzania. Science. 357(6353):802-806.
3. Society NG. 2019. Hadza. Natl Geogr Soc.
4. Pontzer H, BM Wood, DA Raichlen. 2018. Hunter-Gatherers as Models in Public Health. Obes Rev. 19 Suppl 1:24-35.
5. Rampelli S, Schnorr SL, Consolandi C, Turroni S, Severgnini M, Peano C, Brigidi P, Crittenden AN, Henry AG, Candela M. 2015. Metagenome Sequencing of the Hadza Hunter-Gatherer Gut Microbiota. Curr Biol. 25(13):1682-93.
6. Marlowe FW, Berbesque JC, Wood B, Crittenden A, Porter C, Mabulla A. 2014. Honey, Hadza, Hunter-Gatherers, and Human Evolution. J Hum Evol. 71:119-28.
7. Bogdanov S, Jurendic T, Sieber R, Gallmann P. 2008. Honey for nutrition and health: a review. J Am Coll Nutr. 27(6): p. 677-89.
8. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, Turroni S, Biagi E, Peano C, Severgnini M, Fiori J, Gotti R, De Bellis G, Luiselli D, Brigidi P, Mabulla A, Marlowe F, Henry AG, Crittenden AN. 2014. Gut Microbiome of the Hadza Hunter-Gatherers. Nat Commun. 5:3654.
9. Fragiadakis GK, Smits SA, Sonnenburg ED, Van Treuren W, Reid G, Knight R, Manjurano A, Changalucha J, Dominguez-Bello MG, Leach J, Sonnenburg JL. 2019. Links Between Environment, Diet, and the Hunter-Gatherer Microbiome. Gut Microbes. 10(2):216-227.
10. Turroni S, Fiori J, Rampelli S, Schnorr SL, Consolandi C, Barone M, Biagi E, Fanelli F, Mezzullo M, Crittenden AN, Henry AG, Brigidi P, Candela M. 2016. Fecal Metabolome of the Hadza Hunter-Gatherers: a Host-Microbiome Integrative View. Sci Rep. 6:32826.

11. Durack J, Lynch SV. 2019. The Gut Microbiome: Relationships with Disease and Opportunities for Therapy. J Exp Med, 2019. 216(1): p. 20-40.
12. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Xu ZZ, Kightley EP, Thompson LR, Hyde ER, Gonzalez A, Knight R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. mSystems. 2(2).
13. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS 2nd, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2019. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. Nat Biotechnol. 37(8):852-857.
14. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-resolution Sample Inference from Illumina Amplicon Data. Nat Methods. 13(7):581-3.
15. Bokulich N, Robeson M, Dillon M, Ziemski M, Kaehler B, O'Rourke. 2021. bokulich-lab/RESCRIPt: 2021.4.0.dev0 (Version 2021.4.0.dev0). Zenodo.
16. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen T, Miller E, Bache S, Müller K, Ooms J, Robinson D, Seidel D, Spinu V, Yutani H. 2019. Welcome to the Tidyverse. J Open Source Softw. 4:1686.
17. Wickham H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
18. R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
19. Davey L, SA Halperin, SF Lee. 2016. Thiol-Disulfide Exchange in Gram-Positive Firmicutes. Trends Microbiol. 24(11):902-915.
20. Wexler HM. 2007. Bacteroides: the Good, the Bad, and the Nitty-gritty. Clin Microbiol Rev. 20(4):593-621.
21. Hopkins MJ, GT Macfarlane. 2002. Changes in Predominant Bacterial Populations in Human Faeces with Age and with Clostridium difficile Infection. J Med Microbiol. 51(5):448-454.
22. Korpela K, Costea P, Coelho LP, Kandels-Lewis S, Willemsen G, Boomsma DI, Segata N, Bork P. 2018. Selective Maternal Seeding and Environment Shape the Human Gut Microbiome. Genome Res. 28(4):561-568.
23. Sonnenburg ED, JL Sonnenburg. 2019. The Ancestral and Industrialized Gut Microbiota and Implications for Human Health. Nat Rev Microbiol. 17(6):383-390.
24. Ramos OY, Basualdo M, Libonatti C, Vega MF. 2020. Current Status and Application of Lactic Acid Bacteria in Animal Production Systems with a Focus on Bacteria from Honey Bee Colonies. J Appl Microbiol. 128(5): p. 1248-1260.
25. Wang H, Liu C, Liu Z, Wang Y, Ma L, Xu B. 2020. The Different Dietary Sugars Modulate the Composition of the Gut Microbiota in Honeybee During Overwintering. BMC Microbiol. 2020. 20(1):61.
26. Jones JC, Fruciano C, Hildebrand F, Al Toufalilia H, Balfour NJ, Bork P, Engel P, Ratnieks FL, Hughes WO. 2018. Gut microbiota composition is associated with environmental landscape in honey bees. Ecol Evol. 8(1): p. 441-451.