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Fungi in Paleomicrobiological Samples Reveal the Flora and Diets of Ancient Caribbean Cultures

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Storytelling

I am a nerd and I always have been. I started loving science in school as a little kid, I loved it so much that I would read ahead in the lessons and ruin my friends' chances of getting test delays. I even tutored my older cousin and helped her pass genetics even when she was three years older than me. Even my favorite TV show was about science, it was Dexter's Laboratory and what I loved about that show was when I sat in front of the TV and watched this little boy have a place he could go where he could use all these tools, do science, and get answers. I never realized that this was the path I wanted because I did not know there were jobs like that. Thus, when I went to college, I thought I would be a physician, but then I learned about research and now I am a Ph.D. candidate. It is not a secret lab, but I do have space where I can go and ask questions and use amazing tools.

Thesis abstract

The gut microbiome plays essential functions in human health. Environmental disruptions such as changes in diet or lifestyle can exert a significant effect on a population's microbiomes, resulting in several diseases. The study of the ancient microbiota preserved in archaeological samples (paleomicrobiology) is a window for characterizing these possible changes. Recent advances advocate for the consideration of the human microbiome while studying the evolution of humans. However, while more efforts have been made to incorporate microbiome in the evolution of humans, only bacterial communities have been evaluated, whilst fungal communities have been neglected. In addition to the fungal component, regional and temporal variations in dietary habits remain to be defined.

In this thesis, one of the missing pieces of the puzzle is considered: the mycobiome. Metagenomics approaches were applied for characterizing the fecal mycobiome in thousand-year-old coprolites from pre-Columbian Caribbean cultures, and to elucidate the diets and lifestyles of two pre-Columbian cultures, i.e., the Huecoid and Saladoid, prior to the arrival of Europeans. For this purpose, ancient DNA in coprolites retrieved from the pre-Columbian Huecoid and Saladoid deposits in Vieques, Puerto Rico were analyzed using shotgun metagenomic sequencing. In addition, ancient DNA sequences from the Huecoid and Saladoid coprolites were compared with those detected in coprolites from other ancient cultures, as well as extant feces from more modern cultures. To date, relatively little is known about the Huecoid and Saladoid ethnic groups and their cultural heritage.

The Saladoid gut mycobiome exhibited a higher alpha-diversity than that of the Huecoid. This result is further supported by the well-distributed relative abundance of fungal genera in the

Saladoid coprolites compared to the Huecoid coprolites. The gut mycobiome of the Huecoid and Saladoid coprolites was similar at the phylum level, with Ascomycota representing the most abundant phyla, followed by Basidiomycota and Mucoromycota. However, the gut mycobiome composition at the genus level was highly different between the Huecoid and Saladoid coprolites, and the former resembled the ancestral gut mycobiome of Mexico. The gut mycobiome's α-diversity, as well as the composition and structure, distinguished the ancient and extant populations, with the pre-Columbian cultures harboring a lower total diversity and higher relative abundance of *Aspergillus* spp., whereas the extant populations were enriched with *Mucor* spp. and *Malassezia* spp. Despite differences in diet and lifestyles, certain fungal genera were present in most of the samples. Overall, these results suggest that the gut mycobiome reflects changes related to modern lifestyles. DNA from plants and phytopathogenic fungi from coprolites also showed that the Huecoid and Saladoid exhibited preferences in food items. The diet of the Huecoid culture included sweet potato, chili peppers, peanuts, and maize, and the edible maize smut, *Ustilago* spp., was likely consumed as well. In contrast, the Saladoid culture consumed chili peppers and papaya, and likely chewed tobacco (or ingested it in some way), for its narcotic and hallucinogenic effects. However, the Huecoid and the Saladoid diets were significantly more similar to each other than to the diets of present-day cultures. These results suggest that present-day diets diverge from ancient diets due to different available nutritional flora, social environments, and historical periods.

Our work revealed the gut mycobiome and dietary practices of pre-Columbian cultures, uncovered an unprecedented link between human lifestyles and ethnicity, and the diversity and composition of the gut mycobiome and diet. Results further support differences in diet and lifestyles among pre-Columbian Caribbean cultures (the Huecoid and Saladoid) with similar

ecological conditions before the Spanish conquest, and these dietary differences were linked to shifts in the gut mycobiome. We demonstrate and emphasize that DNA sequence data from coprolites complement archaeological data and provide information otherwise impossible to obtain.

Dedication

I dedicate my dissertation work to my family and partner for their endless love and support. Special feelings of gratitude to my loving and caring parents, María García and Ney Reynoso, for being an inspiration and instilling in me the value of education. I could not have done this without you. To my beloved partner for creating a safe space for me, and for dealing with my emotions with patience and love. I am thankful for your support and encouragement during this long and emotionally demanding process.

Le dedico mi trabajo de disertación a mi familia y pareja por su infinito amor y apoyo. Sentimientos especiales de gratitud a mis amados y afectuosos padres, María García y Ney Reynoso, por ser una inspiración e inculcarme el valor de la educación. No podría haber hecho esto sin ustedes. A mi amado compañero por crear un espacio seguro y por lidiar con mis emociones con paciencia y amor. Agradezco tu apoyo y aliento durante este proceso largo y emocionalmente demandante.

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I would like to extend my sincere thanks to Dr. Eileen Seguinot and my supportive peer community for their much-needed emotional support. Thanks should also go to the staff and peers of the Yale *Ciencia* Academy Program for providing me with the tools for better professional development and for showing me that my cultural background adds value to my role as a scientist.

Lastly, I would like to recognize my beloved parents, siblings, nieces, and partner for their unconditional love, support, and encouragement during this process.

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List of abbreviations

SCFAs	Short chain fatty acids
aDNA	ancient DNA
WGA	Whole genome amplification
SRA	Sequence Read Archive
HMP	Human Microbiome Project
PERMANOVA	Permutational Multivariate Analysis of Variance
clr	Centered log ratio
PCoA	Principal Coordinate Analysis
ALDEx2	ANOVA-Like Differential Expression version 2
FDR	False discovery rate
T-RFLP	Terminal Restriction Fragment
ITS	Internal Transcriber Spacer
IDeA	Institutional Development Award
NIGMS	National Institute of General Medical Science
NIH	National Institutes of Health
Nr	Non-redundant
rglobi	Global biotic interactions

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Objectives and hypotheses

Objective 1: To describe and compare the fecal mycobiomes of the Huecoid and Saladoid ancient cultures from Vieques, Puerto Rico.

Hypothesis: Differences will be found in the diversity and composition of the mycobiome of Huecoid and Saladoid individuals.

Objective 2: To compare the fecal mycobiome of Huecoid and Saladoid ancient cultures to infer the diets by detecting key ancient phytopathogenic fungi and plant sequences.

Hypothesis: Differences in foodstuffs used by the Huecoid and Saladoid individuals will be observed by focusing on phytopathogenic fungi and plant sequences.

Objective 3: To compare the mycobiome of Huecoid and Saladoid ancient cultures to the mycobiome of extant indigenous cultures.

Hypothesis: Major differences will be observed in the mycobiome of Huecoid and Saladoid Pre-Columbian cultures compared to the mycobiome of modern cultures.

Chapter 1: Ancient Human Microbiomes and Extinct Populations

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Chapter 1: Ancient Human Microbiomes and Extinct Populations

Introduction:

The human gut contains a diversity of microorganisms, including bacteria, archaea, fungi, and viruses [1–6], whose genes (~ 3, 300,000) surpass human genes (~ 22,000) [7,8]. This collection of microorganisms, known as the human microbiome, is considered an additional organ due to its essential role in human health, including immunological response, digestion and metabolism, nutrient and vitamin production, and protection against pathogens [9]. Therefore, the study of the ancient gut microbiome and its evolution through human history has received increasing attention. Modern lifestyle deeply reformed our relationship with food and likely the environment, thus modern lifestyles may have an impact on the gut microbiome, possibly resulting in the so-called diseases of modern civilization. The "Missing Microbe" hypothesis argues that the modern lifestyle and healthcare decreased the prevalence of infectious diseases but with a cost, a lower gut microbiome diversity, resulting in an increase in immune and metabolic diseases [10]. These diseases that increased with the modern lifestyle are also associated with shifts in sanitation and dietary habits [11,12]. Before modern agriculture, the ancient human diet consisted of high dietary fibers and complex carbohydrates. In contrast, the western diet is high in fat and simple sugars [13–15]. Studies of human populations with an ancient lifestyle offer a first glimpse of the ancient microbiome and diets, while also providing a baseline for a better evolutionary understanding of the human microbiome.

Extant native populations

Indeed, the gut microbiome of diverse human populations has revealed that differences in the composition of the gut microbiome may reflect variations in diets and lifestyles [16–23]. For instance, the Yanomami from the Amazonas of Venezuela have a higher gut microbial diversity compared to urban people from the United States [24]. In addition, *Prevotella* was more abundant in the Yanomami gut microbiome, whereas *Bacteroides* was enriched in the gut microbiome of United States individuals [24]. These results could be attributed to the frequent meals and food seasonality in the Yanomami, which differs from the large and infrequent meals in urban people with western diets [24]. Children from rural Buriram, with a high-vegetable Thai diet, have more Clostridiales and fewer Bacteroidales and Selemomonadales than children from urban Bangkok, which consumed a high-fat diet that resulted in a decrease of short-chain fatty acids (SCFAs) [25]. Bacterial diversity was also more diverse in children from rural Buriram than in children from urban Bangkok [25]. Similarly, the children from urban Italy and Burkina Faso, and children from rural Burkina Faso present differences in the gut microbiome diversity and composition [14]. Rural children were dominated by *Prevotella*, *Treponema*, and *Succinivibrio*, which are fiber-degrading bacteria [14]. In contrast, urban children were characterized by Bacteroidaceae, Bifidobacteriaceae, Porphyromonadaceae, and Rikenellaceae, which are better suited to metabolize fats, sugars, and animal protein [14].

Extinct populations

The evolutionary history of the gut microbiome should also be addressed with the study of well-preserved DNA from ancient samples (paleomicrobiology). Coprolites are

desiccated or mineralized feces [26] that may contain ancient microbial DNA that remains preserved under extraordinary conditions, including cold, dry, and tropical environments [27–29] (**Figure 1.1**). In particular, these rare and precious samples can be recovered from archaeological layers, latrines, rock shelters, pits, and mummies [26,30–32]. Fecal samples from archaeological contexts can be preserved for thousands of years, making them the preferred source for studying the evolution of the gut microbiome and paleodiets of humans and animals [27,33–37].

In fact, coprolites from La Cueva de Los Muertos Chiquitos matched feces from rural communities more closely than westernized populations [13,38]. In addition, the gut microbiome of coprolites and rural communities harbored a higher abundance of *Prevotella* [13,38] and *Treponema* [38]. Similarly, a recent study showed that coprolites from the United States and Mexico resemble more closely non-industrialized than industrialized gut microbiomes [39]. Particularly, *Treponema succinifaciens, Ruminococcus callidus,* and *Butyrivibrio crossotus* were more abundant in coprolites and present-day non-industrial samples compared to industrial samples [39]. Conversely, *Bacteroides* and *Prevotella* were more abundant in industrial feces, compared to non-industrial samples and coprolites [39].

Microbial DNA from archaeological samples could also be used to infer ancient human diets. Therefore, ancient DNA (aDNA) from ancient samples not only provides information about the diversity and composition of the ancient gut microbiome but also dietary information. Coprolites from Puerto Rico showed that two ancient cultures (Huecoid and Saladoid) that migrated from South America maintained dietary and cultural differences that were reflected in their gut microbiomes [40]. Sequences of maize

and Basidiomycetes in the Huecoid coprolites suggest that maize was part of their diet, while sequences of fish parasite in the Saladoid coprolites suggest the consumption of fish [40]. Compared to feces from extant Amazonia indigenous cultures, coprolites harbored a lower abundance of Firmicutes and Bacteroidetes and higher levels of Proteobacteria and Actinobacteria [40]. Nonetheless, taphonomic conditions may have a role in the detection of microbial species. More recently, retroviral DNA from 1,500-year-old coprolites suggested that birds, amphibians, reptiles, and fish were components of the Huecoid and Saladoid diets [41]. Retrovirus infecting nematodes, flatworms and rodents were also identified [41]. Rodents and canids inhabiting the settlement likely transmitted zoonotic enteric parasites to these pre-Columbian Caribbean cultures [42].

Figure 1.1. Coprolite specimen from the archeological site of Sorcé. Saladoid coprolite sample from the Center for Archaeological Research collection at the University of Puerto Rico, Rio Piedras Campus (Image credits: Chanlatte and Narganes-Storde).

Combining modern methods with those of ancient DNA may provide insights into the gut microbiome and diets through human evolution. Many studies of ancient DNA from coprolites have used amplicon-based sequencing [38,43], which uses primers that targets conserved genes to identify sequences of adjacent hypervariable regions. Sequencing of a single region, however, discards many DNA sequences from the metagenome and could lead to taxonomic bias [7]. Shotgun metagenomic sequencing targets all DNA present in the samples (non-targeted sequencing) and is not compromised by short length reads typical of degraded and fragmented DNA [7]. However, few coprolite studies have used shotgun sequencing (**Table 1.1**), and even fewer focused on the fungal component of the gut microbiome (gut mycobiome). Here we analyzed coprolites from the pre-Columbian Huecoid (n=6) and Saladoid (n=4) cultures. We used shotgun metagenomic sequencing to gain insights into the gut mycobiome and diets of these ancient cultures from Vieques, Puerto Rico.

Table 1.1 Studies on the ancient gut microbiome. Only human coprolites analyzed using shotgun metagenomic sequencing were included.

Archaeological site	Geographical region	Dating	Reference
La Cueva de Los Muertos Chiquitos	Rio, Zape in Durango, Mexico	700 CE	[13]
La Cueva de Los Muertos Chiquitos	Rio, Zape in Durango, Mexico	600 - 700 CE	[29]
Namur	Belgium	14th-century	[44]
Namur	Belgium	14th-century	[45]

Cuzco	Peru	11th century CE	[46]
La Cueva de Los Muertos Chiquitos	Rio, Zape in Durango, Mexico	700 CE	[47]
Surrey	United Kingdom	Post medieval	[47]
La Cueva de Los Muertos Chiquitos	Rio, Zape in Durango, Mexico	700 CE	[48]
Bushman Rock Shelter	Limpopo Province, South Africa	1460 CE	[49]
La Hueca-Sorcé	Vieques, Puerto Rico	1500 BP	[41]
La Hueca-Sorcé	Vieques, Puerto Rico	1500 BP	[42]
La Hueca-Sorcé	Vieques, Puerto Rico	1500 BP	[50]

The Huecoid and Saladoid cultures

The pre-Columbian Caribbean was populated by several indigenous cultures that immigrated from South America, including the Huecoid culture and the Saladoid culture. The Saladoid are pottery-making agriculturalists that immigrated from present-day Venezuela and arrived in Vieques and mainland Puerto Rico by 160 B. C. and 430 B. C., respectively [51,52]. Red and white pottery and carved shell ornaments characterized the Saladoid culture [53,54] (**Figure 1.2A**). Nonetheless, it has been suggested that the Saladoid culture diverged into different cultural groups, resulting in distinct pottery over time and space [55]. In the 1970s, however, the archaeologists Chanlatte and Narganes conducted a series of excavations in La Hueca-Sorcé site and discovered a different and overlapping human occupation: the Huecoid. It is through that the Huecoid settled in the

Caribbean by at least an A. D. in an independent migration [27]. In contrast to the Saladoid culture, plain pottery and semiprecious stones distinguished the Huecoid culture [56,57] (**Figure 1.2B**). Previously, the absence of Huecoid bones hampered molecular analyses needed to compare Huecoid and Saladoid cultures, but recently microbiome evidence supported the archaeological findings of the Saladoid and Huecoid being different cultures [37,40–42]; however, the gut mycobiomes of these cultures had not been studied. By using shotgun metagenomics, we reconstructed the diet and gut mycobiome of these cultures and compared it to extant gut mycobiomes for a better understanding of the human holobiont.

A.

B.

Figure 2. Saladoid and Huecoid cultural materials in the archeological site of Sorce´, Vieques. The first row (**A**) is an example of the Saladoid pottery (left) and a

representation of a frog in semiprecious stone (right). The second row **(B)** is an example of the Huecoid pottery (left) and an amulet representing the Andean condor carved in jade (right). (Source: Chanlatte and Narganes-Sorde).

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Chapter 2: Mycobiome-Host Coexistence? The Mycobiome of Ancestral Human Populations Seems to Be Different and Less Diverse Than Those of Extant Native and Urban-Industrialized Populations

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Chapter 2: Mycobiome-Host Coexistence? The Mycobiome of Ancestral Human Populations Seems to Be Different and Less Diverse Than Those of Extant Native and Urban-Industrialized Populations

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Abstract

Few data exist on the human gut mycobiome in relation to lifestyle, ethnicity, and dietary habits. To understand the effect of these factors on the structure of the human gut mycobiome, we analyzed sequences belonging to two extinct pre-Columbian cultures inhabiting Puerto Rico (the Huecoid and Saladoid) and compared them to coprolite samples found in Mexico and Ötzi, the Iceman's large intestine. Stool mycobiome samples from extant populations in Peru and urban cultures from the United States were also included. The analyses involved Kaiju's protein-level classification of the metagenomes. The ancient Puerto Rican cultures exhibited a lower fungal diversity in comparison to the extant populations. Dissimilarity distances showed that the Huecoid gut mycobiome resembled that from ancient Mexico. Fungal genera including *Aspergillus* spp., *Penicillium* spp., *Rasamsonia* spp., *Byssochlamys* spp., *Talaromyces* spp., *Blastomyces* spp., *Monascus* spp., and *Penicilliopsis* spp. were differentially abundant in the ancient and extant populations. Despite cultural differences, certain fungal taxa were present in all samples. These results suggest that culture and diet may impact the gut mycobiome and emphasize that modern lifestyles could be associated with the alteration

of gut mycobiome diversity. The present study presents data on ancient and extant human gut mycobiomes in terms of lifestyle, ethnicity, and diet in the Americas.

Introduction

Humans have coevolved with their gut microbiome, which plays an essential role in human health and well-being. The human and other animal gut microbiomes may be affected by factors such as geography, lifestyle, genetics, environment, and diet [1–5]. In addition to bacteria, diversity in intestinal fungi (both transient as well as intrinsic, referred to as the mycobiome) is being revealed. Recent studies have shown that modern lifestyles may result in a decreased diversity of the bacteriome and may have an impact on metabolic and immune diseases [6–8]. However, there is an information gap on the impact modern lifestyles and ethnicity may have on the gut mycobiome composition.

The human gut mycobiome of Westernized cultures seems to be mainly composed of the genera *Saccharomyces*, *Malassezia*, and *Candida*, with the species *Saccharomyces cerevisiae, Malassezia restricta* and *Candida albicans* dominating the human stool samples tested [9]. These fungal species have also shown to be persistent across time, suggesting that they may not be transient. This study also showed that the gut mycobiome usually exhibits a high degree of inter- and intra-subject variability. Gut fungal communities are also known to help maintain homeostasis and can directly influence host metabolism, and indirectly via alterations to bacterial community composition [10–14]. To understand the composition and effect of modern lifestyles and ethnicity on the human gut mycobiome, it is important to understand it through part of human evolutionary history. However, studies on the ancient human gut mycobiome significantly lag when compared to those on the ancient bacterial and viral components [15–20].

Coprolites are contributing valuable information on the gut microbiome of ancestral populations. In addition, coprolites provide insights to understand how humans and the gut microbiome coevolved in response to changes in environment, culture, and diet. Coprolites from two pre-Columbian cultures, the Huecoid and Saladoid, recovered in Puerto Rico have previously been characterized by our group to determine the gut microbiota [17,18], viral communities [19] and parasite composition [21], to further support archaeological evidence suggesting that two pre-Columbian populations, the Huecoid and Saladoid, were two different cultures. Prior the 1980s, most of the archaeological evidence suggested that the Huecoid and Saladoid were the same culture. One of the main standing hypotheses is that the Saladoid culture migrated from Venezuela during the last centuries of the pre-Christian era and the first of the Christian era, whereas the Huecoid culture were an earlier migration of pottery-making horticulturalists that originated from the Andean areas of Peru and Bolivia. Microbiome evidence by our group supported the archaeological findings of the Saladoid and Huecoid being different cultures [17–19,21]; however, the gut mycobiomes of these cultures have not been studied and compared to present and extant gut mycobiomes.

In the present study, we analyzed coprolites of the Huecoid and Saladoid cultures to determine their fecal gut mycobiome. To better understand how the gut mycobiome is impacted by modern lifestyles and human adaptation to different environments, the mycobiome from coprolites from the Huecoid and Saladoid cultures were compared to those obtained from Mexican coprolites, the large intestine content from Ötzi, the Iceman, as well as fecal samples from extant native populations of Peru (Tunapuco and Matses) and urban populations in the United States. The Matses are hunter-gatherers from

the Amazon with limited access to medical care and have a diet composed of food obtained from the environment. The Tunapuco, on the other hand, are agriculturalists from the Andes that practice small-scale agriculture, and animal domestication. In contrast, the United States (US) individuals have a Westernized lifestyle, with access to medical care and higher sanitation standards. Including these populations in the analysis allowed us to determine the possible impact(s) of modern lifestyles, ethnicity, diet, and geography on gut mycobiome composition and diversity. Therefore, the main aim of the present study was to determine the gut mycobiome composition of the Huecoid and Saladoid cultures in comparison to coprolites from Mexico, intestinal content from Ötzi, stool samples from extant native populations from Peru, and urbanized populations from the United States. We found that the α-diversity as well as the composition and structure distinguished the ancient from extant populations.

Materials and Methods

Study Site and Sample Collection

In the present study, we studied coprolites from the Huecoid (n = 4) and Saladoid (n = 5) cultures from La Hueca, Sorcé, an archeological settlement in Vieques, an island situated in the southeast of Puerto Rico (18°05′56″ Latitude North and 65°29′34″ Longitude West). The coprolites were recovered during an excavation by archeologists Chanlatte and Narganes and were stored in the Center for Archeological Research of the University of Puerto Rico. The geographical distance between the Huecoid and Saladoid archeological deposits were 15–20 km. Because excavations were conducted in a private property, no permissions were required except for the owner's authorization. The age of

the coprolites was determined using radiocarbon dating of shells and charcoal associated with the samples [22]. Coprolites were radiocarbon dated at Teledyne Isotopes (Westwood, NJ, USA) and BETA Analytic, Inc. (Miami, FL, USA) using standard protocols. The Huecoid coprolites were radiocarbon dated from 245 to 600 AD, whereas the Saladoid coprolites dated from 230 to 395 AD. The semi-arid climate in Sorcé, Vieques provided favorable conditions for the preservation of these coprolites and previous studies of coprolites from this archeological midden have shown that the samples have exhibited a well-preserved fecal microbiota [19,21].

Microbial DNA Isolation and Contamination Control

DNA extraction and sequencing were done previously [19]. In brief, nine coprolites belonging to Huecoid (n = 4) and Saladoid (n = 5) cultures were processed in a class II biosafety cabinet exclusive for ancient DNA using strict protocols and control required for ancient DNA studies (i.e., protective clothes and sterilized equipment). The class II biosafety cabinet was cleaned with 70% ethanol and UV-light decontaminated for 30 min prior and after use. To eliminate contamination with environmental DNA, the surface of the coprolites was removed using a sterile scalpel, thus only the core of the coprolites was used for analyses. The coprolites' cores were ground into a fine powder using a sterile mortar and pestle and moistened overnight in sterile C1 buffer at 4 °C. DNA was extracted from the Huecoid and Saladoid coprolites' cores using PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following manufacturer's recommendations with some modifications in the final membrane wash step. The samples were then pooled to one composite for each culture using a standard glycogen precipitation protocol due to low DNA yields.

Library Preparation and Shotgun Metagenomics Sequencing

DNA concentrations were measured by [19] using Qubit® dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, California, USA). Whole genome amplification (WGA) was performed using REPLI-g Midi kit (Qiagen, Valencia, CA, USA;). Amplified DNA was purified using PowerClean DNAClean-Up Kit (MO BIO Laboratories, Carlsbad, California, USA) and quantified on Qubit® dsDNA High Sensitivity Assay Kit (Life Technologies). Libraries were prepared with Nextera DNA Sample preparation kit (Illumina) according to the manufacturer's instructions. The concentration of the libraries was evaluated using Qubit® dsDNA High Sensitivity Assay Kit (Life Technologies) Then, libraries were pooled in equimolar amounts and shotgun sequenced on an Illumina MiSeq paired-end platform [19]. These libraries are available at MG-RAST (http://metagenomics.anl.gov) under the project name "Pre-Columbian Coprolite Metagenomes Merged Only" (MG-RAST library numbers mgl386790 and mgl386787).

Bioinformatics

Read Processing and Quality Control

Paired Illumina reads were trimmed and filtered with Trim-galore using default parameters (Phred score >20) as implemented in metaWRAP Read_qc module (v1.2.4) [23]. Then, reads were aligned to the *Homo sapiens* reference genome (build Hg38) to remove human DNA sequences from the metagenomics datasets using BMTagger as implemented in metaWRAP Read_qc module. The quality of the raw reads was visualized with FastQC [24] and the resulting pre-processed reads were used for subsequent analysis.

Comparison to Other Samples

Fastq files from published microbial metagenomics datasets were obtained from the NCBI Sequence Read Archive (SRA) database using the fasterq-dump command from the SRA Toolkit (v2.10.4). Apart from the coprolite sequences from the Huecoids and Saladoids, the public shotgun sequence datasets used in this work included: $n = 3$ coprolites from the Loma San Gabriel culture (Mexico) and an Iceman large intestine content sample downloaded from NCBI (BioProject ID PRJEB31971) [25]. As a means of comparison, a total of n = 24 extant stool from the Matses hunter-gatherers and n = 12 stool sequences from the agriculturalists Tunapuco (Peru), were downloaded from NCBI (BioProject ID PRJNA268964) [26]. Also included in the analyses were n = 28 stool sample sequences from the Human Microbiome Project (HMP) from extant US individuals (BioProject ID PRJNA48479) [27]. These datasets had been sequenced on Illumina platforms and were processed with data produced in this study using the parameters previously described.

The urbanized population is composed of US individuals living in metropolitan areas with a high number of individuals per geographic area. These individuals follow a western diet and have access to healthcare and sanitized environments. In contrast, the Matses hunter–gatherer population include individuals residing in isolated areas from the Peruvian Amazon. These hunter–gathers have limited access to medical care and their diet relies on food from the environment [26]. The Tunapuco agricultural population lives in the Andean highlands. Their diet consists of agricultural crops and domestic animals [26]. The pre-Columbian culture Loma San Gabriel inhabited the Rio Zape caves located in Durango, Mexico. These coprolites were found in the archeological site La Cueva de

Los Muertos Chiquitos (1300 ± 100 BP). The subsistence of Loma San Gabriel culture was based on agriculture and hunting-gathering that varied among seasons [28,29]. The Iceman, commonly known as Ötzi, is a European Copper Age mummy preserved in an Italian Alpine glacier for more than 5300 years [25]. The Iceman was an Early European farmer from the Eastern Italian Alps [30,31,32]. Occasionally, all the groups are referred as ethnic groups; however, the United States population is composed of individuals from multiple cultural backgrounds.

Taxonomic Profiling

Pre-processed fastq sequencing files (average read size ~250) were used for taxonomic classification with Kaiju (v1.5.0) [33] to assign reads to the lowest common ancestor [33,34] using the following parameters: -a greedy -E 0.05 to filter matches through e-value. Kaiju classification was performed against a subset of the NCBI BLAST non-redundant reference database (argument -nr_euk) that include proteins from bacteria, archaea, viruses, fungi, and microbial eukaryotes (accessed on 25 May 2020).

Data and Statistical Analysis

For general statistical analysis and data visualization, we used the R packages (v4.0.3): tidyverse (v1.3.1), cowplot (v1.1.1), picante (v1.8.2), vegan (v2.5.7), HMP (v 2.0.1), dendextend (v1.15.1), Microbiome (v1.12.0), ALDEx2 (1.22.0), ggplot2 (v3.3.5) and phyloseq (v1.34.0). At the genus level, the meta-taxonomic composition of the samples was done after removing taxa detected less than three times in at least 20% of the samples to remove possibly spurious results. The differences in relative abundance were assessed using the Xdc.sevsample function in the R HMP package to test for differences in the

overall composition between the groups [35]. Samples were rarified to the minimum number of sequences in the samples to avoid potential bias associated with variation in sampling depth. Observed richness, Shannon, and Simpson [36] indices were determined using the phyloseq package [37], and used to estimate the gut mycobiome alpha-diversity across the groups. Kruskal–Wallis and Wilcoxon statistical tests were applied to evaluate statistical difference in the alpha diversity values, and to compare the inter-group variation in gut mycobiome composition. For beta-diversity, Permutational Multivariate Analysis of Variance (PERMANOVA) was used to evaluate statistical differences in the gut mycobiome structure of ethnic groups based on Aitchison distances index dissimilarity measure using the adonis function in the R phyloseq package [38]. The Aitchison distances were centered log ratio (clr)-transformed using the microbiome package in R and visualized onto two-dimensions using Principal Coordinate Analysis (PCoA) plots [39]. The dendrograms were constructed using the R vegan package to examine hierarchical clustering of the samples. Bray–Curtis dissimilarity measures were computed for all the samples and then Ward's clustering algorithm was applied to assess sample clustering [40]. ANOVA-Like Differential Expression version 2 (ALDEx2) was applied to determine differentially abundant taxa across the groups. Significance of differences were evaluated using the nonparametric Wilcoxon rank-sum test and *p*-values were adjusted for multiple comparison using the Benjamini–Hochberg method [41]. False discovery rate (FDR) of <0.05 was used as cut-off. The core mycobiomes were defined as those fungal taxa with an abundance $>0.1\%$ in at least 90% of the samples and were determined using the R Microbiome package.

Results

The Fecal Mycobiome of the Ancient Populations Is Less Diverse Than Those of Modern Populations

Coprolites and extant fecal samples from six ethnic groups (Huecoid, Saladoid, Mexican, Matses, Tunapuco, and US) distributed across four geographic areas (**Figure 2.1**) were analyzed using shotgun sequencing, resulting in 589,049 high quality sequences (including Ötzi the Iceman gut sample), with an average number of 8181 sequences per sample (ranging from 89 to 460,471).

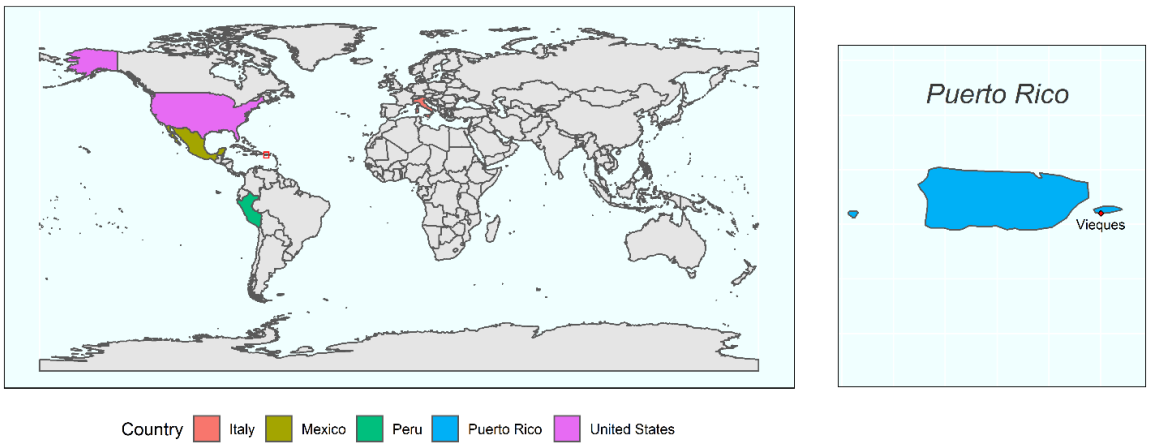

Figure 2.1. Geographic locations of the groups included in the present study. Puerto Rico is magnified on the right side to show the municipality of Vieques, where coprolite samples were recovered.

We characterized the fecal mycobiome of the Huecoid and Saladoid cultures and compared the composition with those from previous studies, namely coprolites from Loma San Gabriel culture (Mexico) and a gut sample from Ötzi The Iceman [203], as

well as extant stools from hunter–gatherers and agriculturalists from Peru [204] and urban individuals from US [205].

To elucidate the gut mycobiome's α-diversity of the ethnic groups, we measured the observed number of species, as well as Shannon and Simpson indices using the fungal genera according to ethnicity (**Figure 2.1**) and culture (Figure S2.1). All the diversity measures showed that the gut mycobiome of the Mexican group was significantly less diverse than that of the Matses (Kruskal–Wallis rank sum test, p-value < 0.05) and the Tunapuco (Kruskal–Wallis rank sum test, p-value < 0.05); and in turn, the gut mycobiome of the US individuals was significantly less diverse than the Matses (Kruskal–Wallis rank sum test, p-value < 0.05) (**Figure 2.2**). In addition, the observed richness of the United States gut mycobiome was significantly less diverse than that of the Tunapuco (Kruskal–Wallis rank sum test, p-value = 0.0241) (**Figure 2.2A**). No differences were detected between the gut mycobiome of the Matses and Tunapuco. The Huecoid and Mexican coprolites, and the Iceman gut sample had the lowest richness in the gut mycobiome. Nonetheless, the Iceman gut sample had a higher evenness than the Huecoid and Mexican coprolites, which indicates a better distribution (relative abundance) of fungal taxa in the former. The Saladoid coprolites had a higher α-diversity compared to the other pre-Columbian cultures. On the other hand, the Matses extant stools showed the highest gut mycobiome α-diversity followed by the Tunapuco and US extant stools, suggesting a greater richness and evenness of fungal genera in these samples. We also found that the ancient populations (Huecoid, Saladoid and Mexican and The Iceman) exhibited a lower α-diversity in comparison to extant populations (Matses, Tunapuco and US) (Mann–Whitney U-test, p-value < 0.001) (Figure S2.1). It has been

previously reported that urban populations have a higher gut fungal diversity when compared to rural populations [206,207]. In contrast, the gut bacteriome of hunter–gatherers and agriculturalists has as higher bacterial richness compared to urban populations [204,208–216]. In this regard, we found that the Saladoid culture had the highest α-diversity. Moreover, we found that the US individuals have a lower bacterial richness compared to Huecoids (Kruskal–Wallis; p-value = 0.0192), Saladoids (Kruskal–Wallis; p-value = 0.0209), Mexican (Kruskal–Wallis; p-value = 0.0040), Matses (Kruskal–Wallis; p-value < 0.001) and Tunapuco (Kruskal–Wallis p-value < 0.001) (Figure S2.2), which is consistent with previous studies.

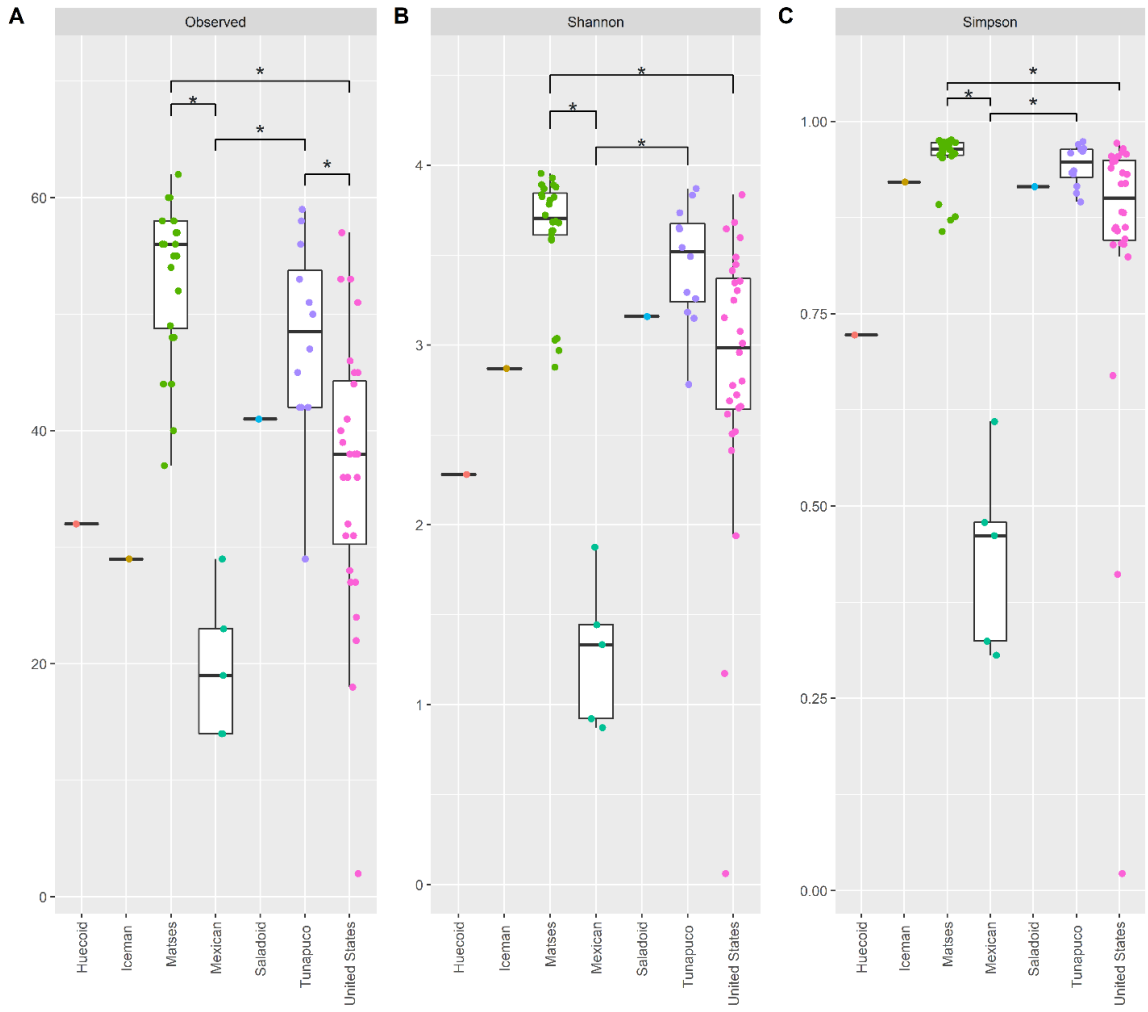

Figure 2.2. Alpha-diversity comparisons of the gut mycobiomes of each ethnic group. Analyses were performed at the genus-level. Boxplots show (**A**) observed richness, (**B**) Shannon, and (**C**) Simpson diversity of each ethnic group. Individual observations (dots) were colored according to ethnic group; *$p < 0.05$.

Hierarchical Clustering Revealed a Certain Degree of Clustering among the Ancient and Modern Populations

To evaluate the extent to which samples from the ethnic groups clustered together, we performed a hierarchical clustering using the Bray–Curtis dissimilarity measure, which

measures the difference in diversity between microbial communities. The Bray–Curtis dissimilarity is 0 when the samples have the same community composition, and 1 when the individuals share no fungal species. The hierarchical clustering of the gut mycobiome of the six ethnic groups showed some clustering of the samples according to ethnicity (**Figure 2.3**). However, several Tunapuco and US extant stools samples showed distinctiveness in the gut community structure. In addition, we observed that the coprolites were more similar to each other than to extant fecal samples. The compositional dissimilarity between the coprolites and the extant stool samples led to separation of the samples.

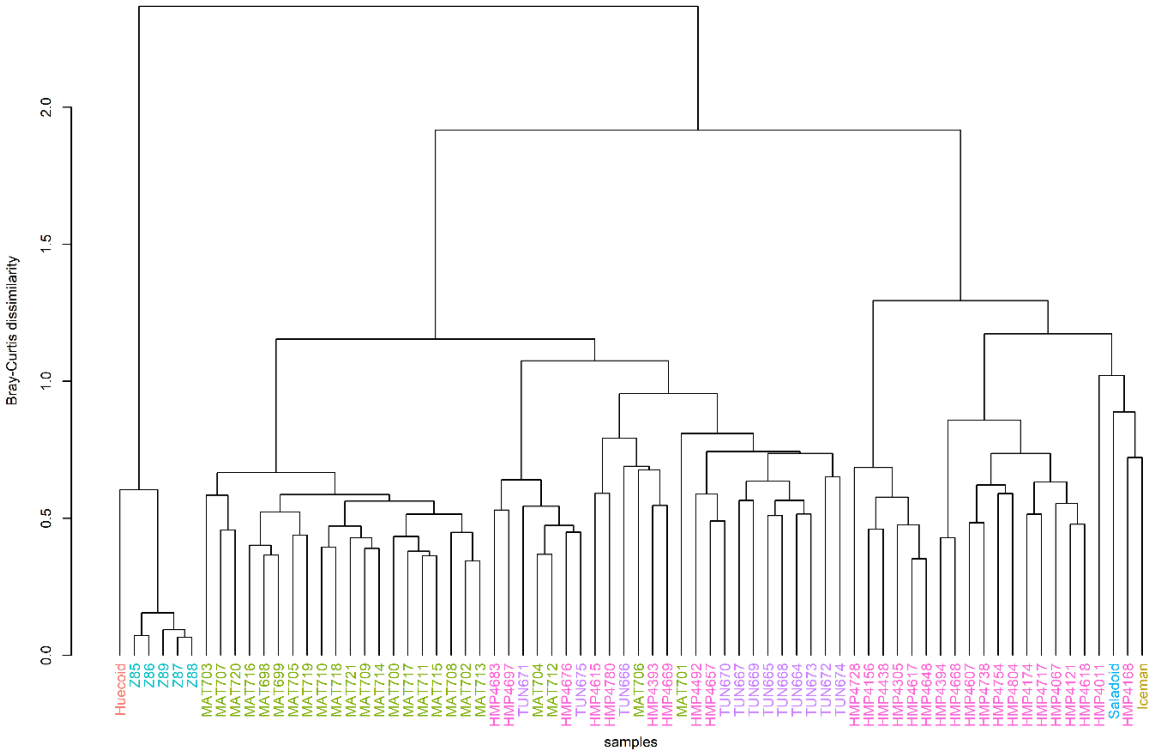

Figure 2.3. Hierarchical clustering. Hierarchical clustering of the Huecoid (coral/red), Saladoid (blue) and Mexican (turquoise) coprolites, and the Iceman gut sample (golden)

as well as the Matses (green), Tunapuco (purple), and US (pink) extant stools using the Bray–Curtis dissimilarity measure.

Fungal Communities of the Ancient Populations Differ from Those of Modern Populations

We calculated the microbial β-diversity, which are the differences in diversities across the samples, using the Aitchison distance (Euclidean distance of clr-transformed compositions). At the genus level, the PCoA ordination based on Aitchison distances showed a significant segregation between the ethnic groups (PERMANOVA, *p*-value = 0.001) (**Figure 2.4**), suggesting differences in the gut mycobiome composition and structure of these populations. However, the Huecoid and Saladoid were more similar to the Mexican coprolites than to the Matses and Tunapuco extant stool samples, which in turn were more similar to the US extant stool samples. To compare the heterogeneity (inter-individual mycobiome divergence) in community composition across the ethnic groups, we quantified the average sample dissimilarity from the group mean. We found that United States extant stools samples had a more heterogenous gut mycobiome composition compared to the Mexican coprolites (Kruskal–Wallis rank sum test, *p*-value = 0.0032) and the Matses extant stools (Kruskal–Wallis rank sum test, *p*-value < 0.001) (**Figure 2.5**). In agreement with the higher inter-group variation observed in the United States extant stools, we observed higher dispersion of samples in this population (**Figure 2.4**). Moreover, an increased overall heterogeneity in community composition of extant populations in comparison to ancient populations was found (Mann–Whitney U-test, *p*-value = 0.01675) (Figure S2.3).

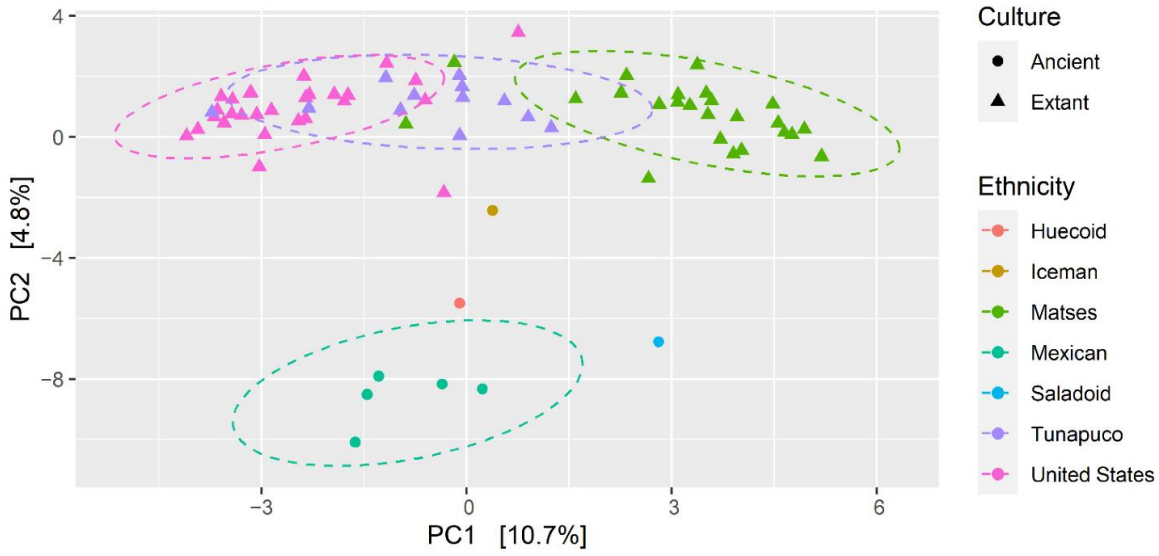

Figure 2.4. Beta-diversity comparisons of the gut mycobiomes of each ethnic group, principal coordinate analysis of Aitchison distances. The colors of the dots represent the different groups analyzed, whereas the symbols represent the ancient and extant cultures according to the legend. Symbols indicate whether cultures are ancient or extant.

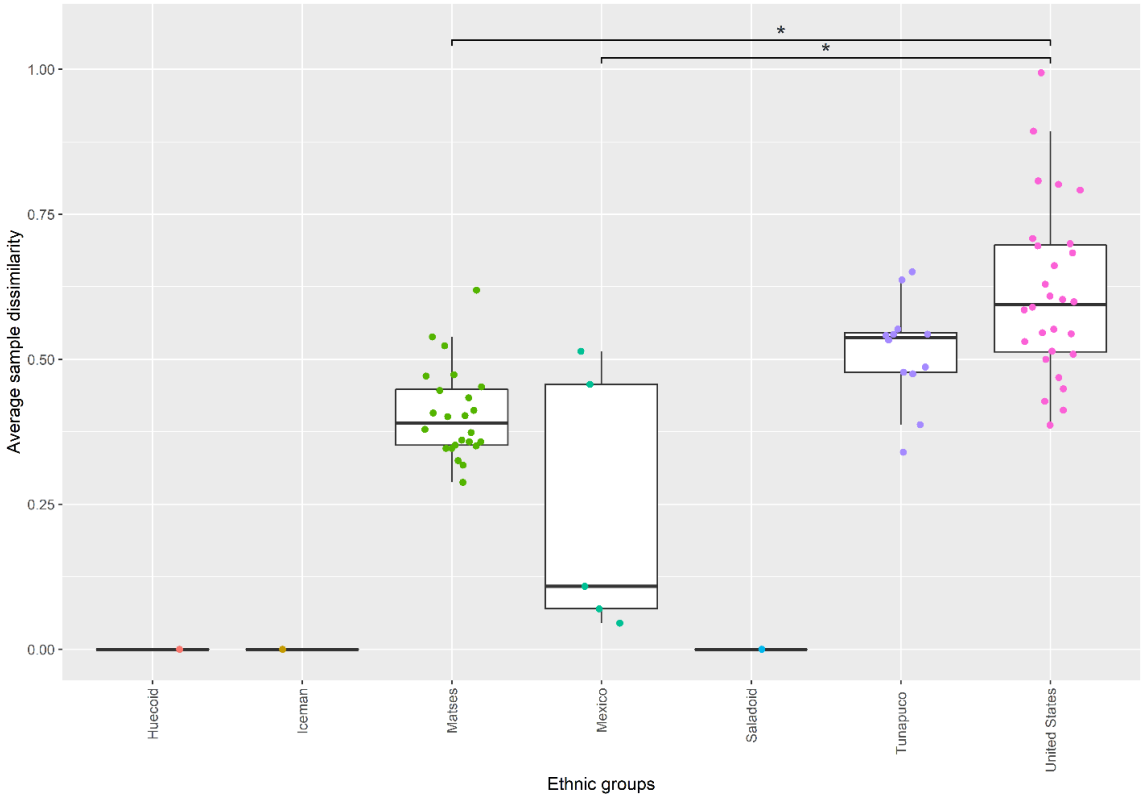

Figure 2.5. Mycobiome divergence across ethnic groups. Heterogeneity (inter-individual divergence) in community composition across the ethnic groups. Individual observations (dots) were colored according to ethnic group; *$p < 0.05$.

Metataxonomic Composition of the Samples Revealed Fungal Taxa That Differentiate Ancient and Modern Populations

To determine the fungal taxa that distinguished the ancient and modern populations, we compared the composition of the samples at the phylum and genus levels. In general, we identified five fungal phyla in the ancient and modern fecal mycobiomes (**Figure 2.6**). All the samples examined were dominated by Ascomycota, followed by Basidiomycota and Mucoromycota. The average relative abundance of Ascomycota was similar among the Huecoid (88%), Saladoid (80%), and Mexican (96%) coprolites and higher when

compared to the Matses (45%), Tunapuco (44%), and US (42%) extant stools, and the Iceman gut sample (25%). Nonetheless, the Basidiomycota phylum was enriched in the Matses (18%), Tunapuco (14%), and US (27%) extant stools, and the Iceman gut samples (35%) in comparison with the Huecoid (6%), Saladoid (5%), and Mexican (2%) coprolites. In addition, the average relative abundance of Mucoromycota was higher in the Matses (19%), Tunapuco (23%), and US (19%) extant feces, and the Iceman gut sample (39%) while lower in the Huecoid (5%), Saladoid (14%), and Mexican (1%) coprolites. The taxonomic composition of the samples also revealed marked differences in the relative abundance of the Chytridiomycota phylum, which was higher in the Matses (15%), Tunapuco (16%), and US (10%) extant stools samples and rare in the Huecoid (0.5%), Saladoid (0.6%), and Mexican (1%) coprolites, and the Iceman gut sample (1%) (**Figure 2.6**).

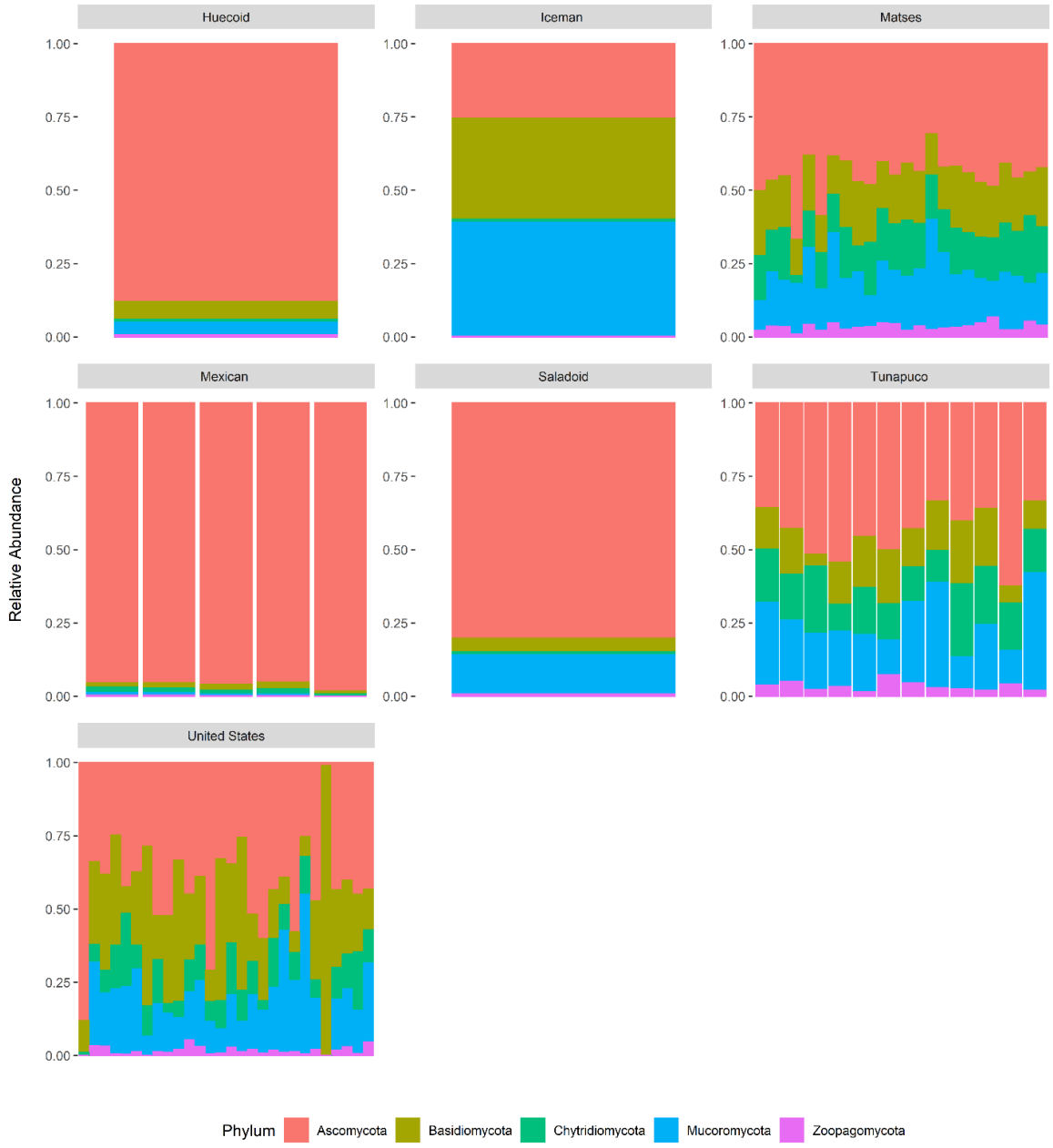

Figure 2.6. Gut mycobiome composition at the phylum level, relative abundance of all fungal phyla detected in the gut mycobiome of the ethnic groups.

The major genera detected in the fecal mycobiome of the Huecoid culture were *Aspergillus* spp. (73% mean relative abundance), *Malassezia* spp. (6%), *Penicillium* spp. (5%), *Mucor* spp. (3%), and *Pseudocercospora* spp. (2%) (**Figure 2.7**). While the fecal mycobiome of the Saladoid culture was enriched in *Rhizophagus* spp. (31%), *Aspergillus*

spp. (14%), *Diversispora* spp. (8%), *Glomus* spp. (5%), and *Penicillium* spp. (4%). In general, the most abundant fungal genera in the samples were *Aspergillus* spp., *Mucor* spp., *Rhizophagus* spp., *Malassezia* spp., and *Lichtheimia* spp. The overall mycobiome composition significantly differed among the group of samples ($X_{\text{several sample test}}$, *p*-value <0.001) (**Figure 2.7**). The mean relative abundance of the genus *Aspergillus* was higher in the Huecoid (74%), Mexican (65%), and Saladoid (6%) coprolites and the Iceman gut sample (18%) in comparison to the Matses (5%), Tunapuco (5%), and US (5%) extant feces. In contrast, *Mucor* spp. were more abundant in the Matses (16%), Tunapuco (18%) and US (15%) extant stools, and the Iceman gut sample compared to the Huecoid (3%), Saladoid (0.4%) and Mexican (0.3%) coprolites. Similarly, *Rhizophagus* spp. were more abundant in the extant stools of the Matses (5%), Tunapuco (7%) and US (4%), but also in Saladoid coprolites (31%) whereas almost absent in the Huecoid (2%) and Mexican (0.1%) coprolites, and the Iceman gut sample (0.3%). Interestingly, extant stools from United States showed high proportions of *Malassezia* spp. (25%), whereas lower proportions were detected in the Huecoid (6%), Saladoid (2%) and Mexican (0.1%) coprolites, and the Iceman gut sample as well as the Matses (2%) and Tunapuco (1%) extant feces (**Figure 2.7**). The distribution of the sequences of each fungal taxa suggests that the Huecoid and Mexican pre-Columbian cultures have a low diversity in the gut fungal communities as these coprolites were dominated by *Aspergillus* spp. On the other hand, the relative abundance of the fungal genera detected in the Saladoid coprolites, and the Iceman gut sample as well as the extant stools from the Matses, Tunapuco, and the United States were well distributed, suggesting a more diverse gut ecosystem.

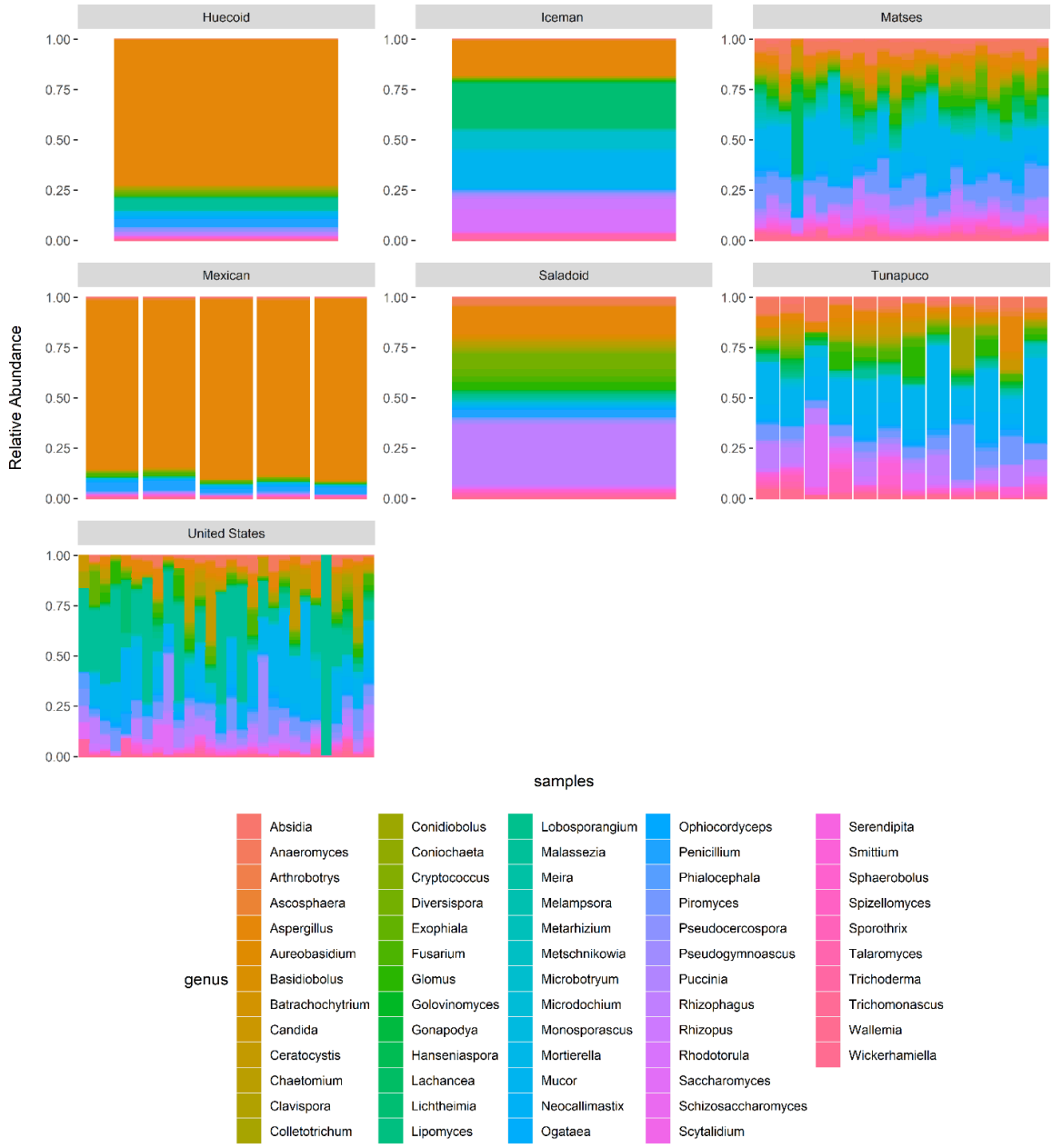

Figure 2.7. Gut mycobiome composition at the genus level, relative abundance of the fungal genera in the gut mycobiome of the ethnic groups (observed more than three times in at least 20% of the samples).

Differentially Abundant Fungal Genera in the Ascomycota phylum Were the Main Drivers of the Differences between the Ancient and Modern Mycobiomes

ALDEx2 was used to identify differentially abundant fungal genera associated to ancient and extant populations. The ALDEx2 method showed that *Aspergillus* spp., *Penicillium* spp., *Rasamsonia* spp., *Byssochlamys* spp., *Talaromyces* spp., *Blastomyces* spp., *Monascus* spp., and *Penicilliopsis* spp., distinguished the groups defined by culture (ancient and extant) (**Table 2.1**). All these fungal genera belong to the Ascomycota phylum, Pezizomycotina subphylum, Leotiomyceta clade, Eurotiomycetes class, and Eurotiomycetidae subclass. At the order level, we found genera belonging to the Eurotiales and Onygenales. The most abundant families were Aspergillaceae and Trichocomaceae followed by Thermoascaceae and Ajellomycetaceae.

Table 2.1. ANOVA-like differential expression (ALDEx2). Fungal genera differentially abundant in ancient and extant cultures.

	diff.btw	diff.win	Effect	wi.ep	wi.eBH	Genus
1	−6.68239	2.648387	−2.44229	1.11×10^{-5}	0.005524	*Aspergillus*
2	−4.12151	2.869157	−1.4792	2.81×10^{-5}	0.00606	*Penicillium*
3	−4.1763	2.971508	−1.4539	3.07×10^{-5}	0.006401	*Rasamsonia*
4	−4.44338	3.153649	−1.4055	3.57×10^{-5}	0.006584	*Byssochlamys*
5	−2.98222	2.36935	−1.26018	0.000116	0.013226	*Talaromyces*
6	−3.63104	3.269716	−1.14088	0.000141	0.014606	*Blastomyces*
7	−3.90086	3.668713	−1.13541	0.000667	0.037039	*Monascus*
8	−4.02314	3.927779	−1.02516	0.000609	0.033089	*Penicilliopsis*

The diff.btw represents the median difference among the groups on a log base2 scale; diff.win constitutes the largest mean variation within ancient and extant groups; effect designates the effect size of the difference (median of diff.btw/diff.win); wi.ep designates

the expected value of the Wilcoxon test *p*-value; wi.eBH represents the adjusted *p*-values for multiple comparison using Benjamini–Hochberg.

Core Mycobiome Results Show That the Most Abundant Fungal Genera Were Shared among the Ancient and Modern Populations

We considered the core mycobiome as the shared genera detected with >0.1% relative abundance in at least 90% of the samples [217]. The overall core mycobiome identified in all the ethnic groups were *Aspergillus* spp., *Fusarium* spp., *Malassezia* spp., *Mucor* spp., *Piromyces* spp., and *Rhizophagus* spp. (**Figure 2.8**). These genera were observed regardless of diet, culture, and lifestyle. Considering the ancient and extant populations, the core mycobiome of the Huecoid, Saladoid, and Mexican coprolites consisted of *Fusarium* spp., *Penicillium* spp., *Talaromyces* spp., *Mucor* spp., and *Aspergillus* spp., whereas *Anaeromyces* spp., *Neocallimastix* spp., *Fusarium* spp., *Rhizophagus* spp., *Malassezia* spp, *Mucor* spp., and *Aspergillus* spp. were the core fungi detected in the Matses, Tunapuco, and US extant stools. Within these fungi, *Fusarium* spp., *Aspergillus* spp., and *Mucor* spp. were detected in both ancient and extant populations (**Figure 2.8**).

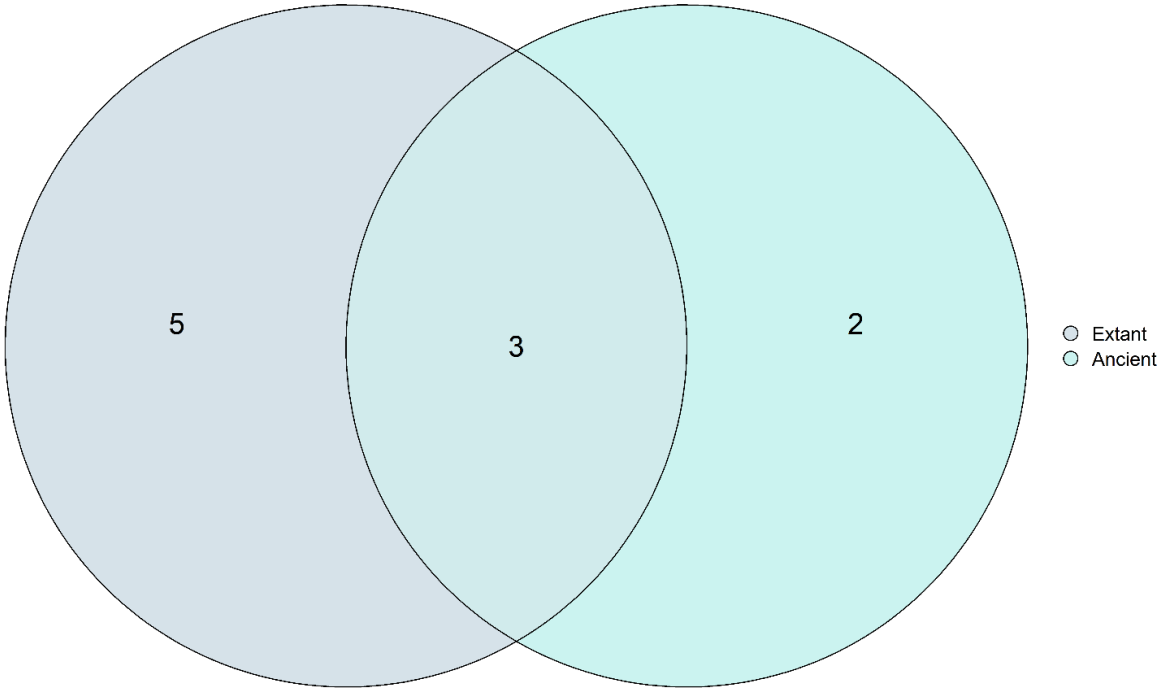

Figure 2.8. Core mycobiome. Total fungal genera detected with >0.1% relative abundance in at least 90% of the samples.

Discussion

The gut microbiome has immune and metabolic functions important to human health [53,54]. Modern lifestyles have the potential to impact the gut bacterial communities, resulting in a concomitant decrease in microbial diversity and an increase in diseases in the host. The study of the ancient microbiome preserved in archeological samples such as coprolites provide a window for characterizing these possible changes. Recent advances advocate for the consideration of the human microbiome while studying the evolution of humans. However, while more efforts have been made to incorporate the microbiome in the evolution of humans, most studies have focused on the bacterial communities, whilst

fungal communities have been neglected. In the present communication, we considered a missing piece of the puzzle: the mycobiome. We analyzed the gut mycobiome in coprolites from pre-Columbian cultures (Huecoid and Saladoid) from Puerto Rico and compared them with Mexican coprolites and an Iceman gut sample. In addition, we included stool samples from extant native populations from Peru (Tunapuco and Matses), as well as urban populations from the US. The information presented will contribute to a better understanding of the possible impacts of modern lifestyle (i.e., diet) and ethnicity on the gut mycobiome composition.

The gut mycobiome was used to successfully differentiate between extant and ancient cultures in the Americas and Europe (Ötzi the Iceman). These results are consistent with previous studies suggesting that the cultural traditions and dietary habits can exert a significant effect on a populations gut mycobiomes [9,18]. Overall, the Ascomycota was enriched in the Huecoid and Saladoid coprolites followed by Basidiomycota and Mucoromycota, consistent with previous studies showing that Ascomycota and Basidiomycota predominate as part of the human gut mycobiome [55,56]. The Ascomycota phylum has edible species as well as plant pathogens, and the presence of sequences suggests the consumption of Ascomycetes by these pre-Columbian cultures as well as the possible presence of phytopathogens in their diet [18]. Recently, Kabwe et al. reported differences in the gut mycobiomes of rural populations versus urban populations in Africa. The relative abundance of the phylum Ascomycota was higher in rural populations, whereas the phylum Basidiomycota was higher in urban populations [42]. Indeed, we found that the Huecoid, Saladoid, and Mexican coprolites had an increase in Ascomycota and a decrease in Basidiomycota compared to extant stools of the Matses,

Tunapuco, US, and the Iceman gut samples. The changes in the Ascomycota: Basidiomycota ratio throughout time suggests an adaptation of the human mycobiome in response to changes in our relationship with food, and, likely, the environment.

We also observed differences in the gut mycobiome of the ethnic groups at the genus taxonomic level. A higher prevalence of *Aspergillus* spp. was detected in the coprolites and the Iceman gut compared to the extant stools. While it is known that the genus *Aspergillus* is ubiquitous in the environment [57,58], the genus has also been previously reported in the human gut mycobiome [59–61]. *Aspergillus* spp. are capable of surviving the transit through the gastrointestinal tract; however, these species are presumed to be transient (allochthonous) due to their abundance in the environment and their introduction through diet, meaning that they may be acquired by consuming certain food items [62]. In fact, studies have revealed a higher abundance of *Aspergillus* spp. in vegetarians when compared to people with a carnivorous diet [62,63]. Therefore, the higher relative abundance of *Aspergillus* spp. in Huecoid, Saladoid, and Mexican coprolites might be associated with the contamination of a wide variety of food included in the diet of these pre-Columbian cultures or the consumption of fermented foods [64]. Indeed, archaeological evidence suggests that Saladoids consumed fermentable carbohydrates from root crops [65,66]. Similarly, the prevalence of *Penicillium* spp. was much higher in coprolites compared to extant stools. *Penicillium* spp. also causes food spoilage and the detection of *Penicillium* spp. in this study might be related to the ingestion of contaminated foods. These results are compatible with previous paleomicrobiological studies based on Terminal Restriction Fragment (T-RFLP) Analyses [18]. On the other hand, low levels of *Mucor* spp. were detected in the coprolites

compared to the extant stools, and the Iceman gut sample. *Mucor* spp. are occasionally detected in feces of healthy humans [67,68]. Internal Transcriber Spacer (ITS)-based sequencing in obese and lean subjects has shown that *Mucor* spp. was more abundant in non-obese than in obese patients [68]. In addition, the low abundance of *Mucor* spp. in obese individuals was restored with loss weight, pointing to a possible association between diet and the gut mycobiome. The microbiota composition is sensitive to diverse perturbations, including dietary changes and the invasion of enteric pathogens [69].

Interestingly, the US extant stools showed an increased relative abundance in *Malassezia* spp. as compared to the fecal samples from extant native communities from Peru as well as coprolites and the Iceman gut sample. *Malassezia* spp. have been described as a commensal of the skin and oral mycobiome [70,71]. Additionally, *Malassezia* spp. are frequently reported in the gut of adults [9,56,58,67,72,73] and infants [74]. It is possible that *Malassezia* spp. are acquired in early life during breastfeeding [75]. Nash et al. reported a high prevalence of *Malassezia* (i.e., *M. restricta*) in fecal samples from healthy volunteers of the HMP. Such data are consistent with our results. However, this yeast was rarely detected in samples from individuals with a western diet compared to vegetarian counterparts [62,63]. Similarly, *Malassezia* spp. was not detected [76] or was detected less consistently [77] in other studies. These differences could be due to differences in cohorts (diet and location) or methodologies. Therefore, whether *Malassezia* spp. survives transiently in the human gut needs further study.

The extant populations showed a more diverse gut mycobiome than the ancient populations. Melanized fungi are well-preserved [78,79]; however, taphonomic conditions could have contributed to the decomposition of chitin in the cell wall of some

genera of fungi, which in turn could contribute to the decreased diversity observed in the coprolites and the Iceman gut sample. Nonetheless, our results are partially consistent with previous studies showing a higher gut fungal diversity in urban populations as compared to rural populations [42,43]. These results suggest that culture and dietary habits have the potential to impact the gut mycobiome diversity and emphasized that modern lifestyle could be associated with the alteration of the gut mycobiome α-diversity. Moreover, the effect of modern lifestyle on the gut mycobiome depends on ethnicity, which is in agreement with previous studies on the gut mycobiome from different cohorts [42,43,80].

The fungal community structure of the Huecoid and Saladoid coprolites was more similar to that of the Mexican coprolites than the Matses, Tunapuco, and US extant stools, and the Iceman gut sample. Moreover, the Matses and Tunapuco extant feces were more similar to that of the US. This suggests similarities in the relative abundance of fungal genera among the ancient and extant populations despite differences in traditional customs, geography, and genetics. One possible explanation is that the diet of the Huecoid and Saladoid cultures is more similar to that of ancient Mexican communities rather than extant native communities (Matses and Tunapuco) and urban-industrialized populations (US). The Huecoid and Saladoid cultures were agriculturalists whose diet was mainly composed of root-crops and fruits [81,82], similar to the Mexican group included in the present study [29,83]. Regarding the extant populations, it has been shown that the gut bacterial microbiome β-diversity of urban populations are different from those of traditional communities [46,48]. These differences have been associated with several factors including a diet rich in fiber and complex carbohydrate in traditional

populations and a diet rich in animal protein and sugars in urban populations. However, Jha et al. demonstrated that the gut bacterial microbiome of foraging populations that transitioned to farming were more similar to that of US individuals [84]. In addition, the Hadza hunter–gatherers have reflected seasonal gut bacterial microbiomes related to seasonal availability of food [69]. Between seasons, the populations reflected differences in their bacterial microbiota and some taxa disappeared although reappeared when the seasons turned. Interestingly, when the bacterial taxa disappeared, the hunter–gathers' bacterial microbiota were similar to those of industrialized microbiota [69]. These studies suggest that changes in dietary habits may shape the microbial community structure. The differences in the gut mycobiome of the Huecoid and Saladoid coprolites and the Tunapuco and Matses extant stools could be related to migratory events. The Huecoid and Saladoid cultures migrated from South America to the Caribbean regions, which suggests the possible role of environmental factors on the gut mycobiome. Differences in geography and climate may affect the fungi we are exposed, which in turn may impact the gut mycobiome composition [9,42]. In addition, industrialization also affects extant native societies as they are connected to the global commerce.

The Mexican coprolites and the Matses extant stools had the lowest inter-individual variation detected, whilst the US extant stools showed the highest heterogeneity in community structure. Some studies have found lower inter-individual variation in the traditional populations than in urban-industrial populations [26,85]. Western populations have a diverse genetics backgrounds, cultural traditions and diet compared to non-western populations [48]. Likely, this heterogeneity in western populations led to selective pressures that increase the inter-individual variation observed in extant fecal

samples from US. Modernization is associated with differences in food processing, and increased hygiene and sanitation standards that could limit microbial transition among the individuals and increase the mycobiome dissimilarity among individuals [43].

The gut mycobiome appears to be less stable over time than the gut bacterial microbiome and is dependent on environmental factors and dietary habits. [9,63]. However, we identified fungal genera that may constitute an ancestral core mycobiome. *Aspergillus* spp., *Fusarium* spp., *Malassezia* spp., *Mucor* spp., *Piromyces* spp., and *Rhizophagus* spp. were detected in all the ethnic groups despite differences in lifestyle and ethnicity. *Aspergillus* spp., *Fusarium* spp., *Malassezia* spp., and *Mucor* spp. are frequently detected in the human gut mycobiome and are associated with diet [56,86]. There is evidence of a core bacterial microbiome that have coevolved with humans and play an important role in the host's health [87,88]. However, information about the core mycobiome is scarce. The coprolite samples from the Huecoid, Saladoid and Mexican, and the Iceman gut sample shared a core mycobiome composed of *Fusarium* spp., *Penicillium* spp., *Talaromyces* spp., *Mucor* spp., and *Aspergillus* spp. Ancient cultures had a fiber-rich diet and complex carbohydrates, which could explain the detection of plant-associated fungi of the families Mucoraceae, Nectriaceae, and Aspergillaceae. For instance, *Fusarium* spp. are plant pathogens commonly detected in vegetarians [62,63]. On the other hand, the core fungi detected in the Matses, Tunapuco, and US extant fecal samples were *Anaeromyces* spp., *Neocallimastix* spp., *Fusarium* spp., *Rhizophagus* spp., *Malassezia* spp, *Mucor* spp., and *Aspergillus* spp. Previous studies have shown that gut fungi are usually transient and diet-associated suggesting that fungi might not colonize due to ecological niches or the human gut environment. However, the human gut has been considered the primary niche of few

Candida spp. The genus *Candida* is commonly identified in the human gut [76,86,89]. Nonetheless, we detected low abundance of *Candida* spp., especially in ancient populations. These results are consistent with the low prevalence of *Candida* found in stool samples from an indigenous population living in a remote region of French Guiana [90].

Despite the limited sample size in our study due to the nature of coprolite samples, which in turn may influence the diversity comparisons, we observed differences across the ethnic groups. Although there were no statistical differences in all the groups, we observed a decreased diversity in the ancient populations compared to the extant populations. Future work should focus on various stages of human evolution to understand how modern lifestyle contributes to changes in the human mycobiome.

Conclusions

Gut mycobiomes in relation to diet and culture have not been thoroughly studied as the human gut bacteriomes and viromes have. The study of the ancestral mycobiome is essential to understand the effect of modern lifestyles on the gut mycobiome composition. Here, it was revealed that coprolites from the Huecoid and Saladoid pre-Columbian cultures as well as Mexican coprolites had a different taxonomic composition when compared to fecal samples from extant native communities from Peru, the Matses and Tunapuco, and the United States individuals. These differences may be a reflection of modern lifestyles and human adaptation to different environments. Overall, the α-diversity as well as the composition and structure distinguished the ancient from extant populations, with the pre-Columbian cultures harboring a lower total diversity and higher

relative abundance of *Aspergillus* spp. whereas the extant populations were enriched for *Mucor* spp. and *Malassezia* spp. The gut mycobiome preserved in coprolites from pre-Columbian cultures may provide a baseline to better understand human holomicrobiome evolution.

Supplementary Materials: The following are available online at **https://www.mdpi.com/article/10.3390/microorganisms10020459/s1**, Figure S1: Alpha-diversity comparisons of the gut mycobiomes of ancient and extant cultures, Figure S2: Alpha-diversity comparisons of the gut bacterial microbiomes of each ethnic group, Figure S3: Mycobiome divergence among ancient and modern cultures.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available as we are working on another communication. Publicly available datasets analyzed in this study are available under NCBI-SRA BioProjects IDs: PRJEB31971 (Mexican coprolites and The Iceman large intestine), PRJNA268964 (Matses and Tunapuco), and PRJNA48479 (United States).

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Conflicts of Interest: The authors declare no conflict of interest. Tasha M. Santiago-Rodriguez is a current employee of Diversigen Inc., a microbiome services company.

Post-publication discussions:

DNA contamination

It is highly unlikely, or even possible that anything is "introduced" into the fecal material prior to them going through the taphonomic processes to become coprolites. If there is any microbiota that actually contaminate the fecal material, then the possibility of this matter becoming a coprolite is nil. The taphonomic processes include (likely, but not only) rapid dehydration and thus the fecal material is not degraded in any way, and this is demonstrated by the presence of fecal microbiota-derived DNA.

DNA degradation

Regarding the possible detection of microbial DNA after thousands of years, we can only presume that some microorganisms will die in short periods of time due to cell lysis and subsequent degradation of free DNA. However, microorganisms may become dehydrated, and the cells not lysed. This will protect the intracellular DNA making it more "resistant" to DNAses. The presence of coprolites is evidence of rapid dehydration and, thus protection of microbial DNA.

Arbuscular mycorrhizal fungi

Coprolites from the Saladoid culture were dominated by arbuscular mycorrhizal fungi compared to the Huecoid coprolites. One possible explanation is that the Saladoids consumed fine roots or undercooked animals that consumed. Alternatively, the Saladoids ingested soil (geophagy).

Production of mycotoxins

The Huecoid and Saladoid coprolites exhibited a high abundance of the genera *Aspergillus*, *Penicillium* and *Fusarium*, which are mycotoxigenic fungi found in human foodstuffs. Mycotoxins produced by these fungi could be consumed through foods of plant origin, possibly causing a toxigenic response in the Huecoids and Saladoids. However, it is highly likely that many DNA sequences from the coprolites came from coprophilic fungi that colonized the feces after being deposited.

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Supplementary figures

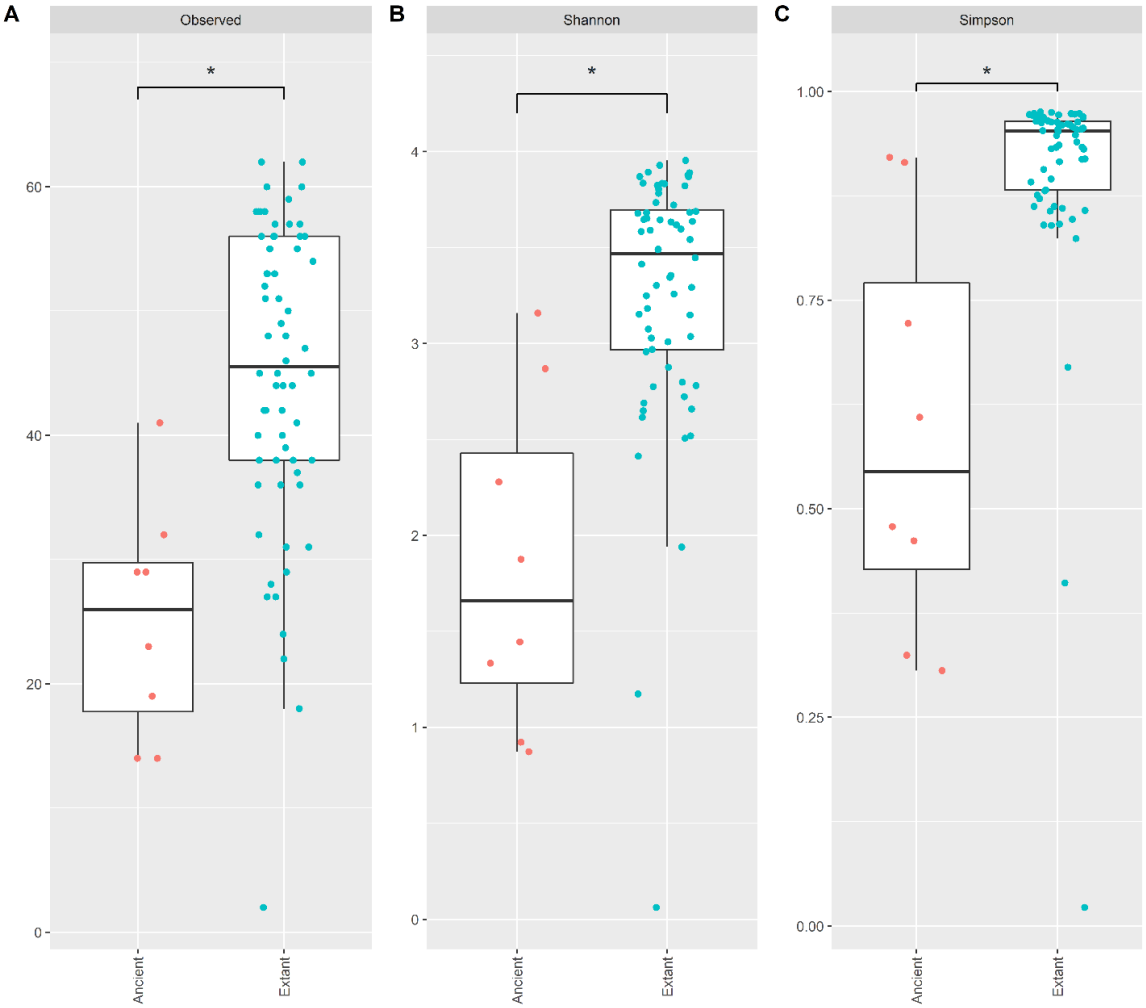

Figure S2.1: Alpha-diversity comparisons of the gut mycobiomes of ancient and extant cultures

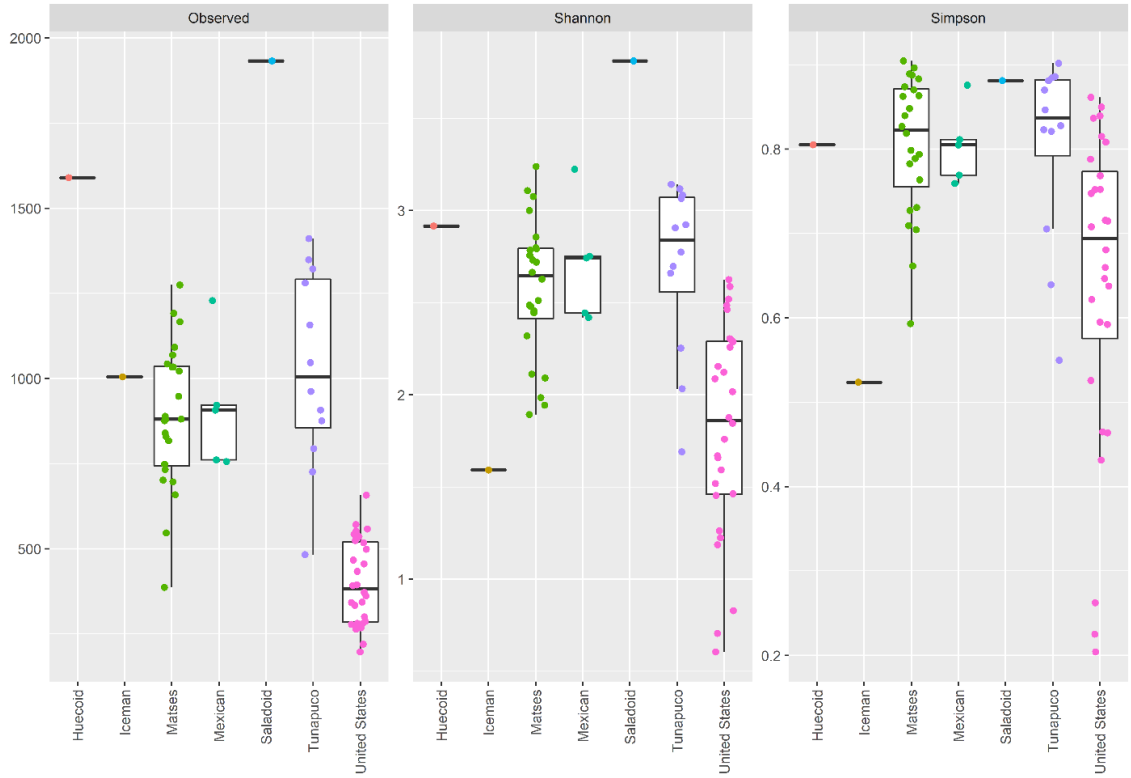

Figure S2.2: Alpha-diversity comparisons of the gut bacterial microbiomes of each ethnic group

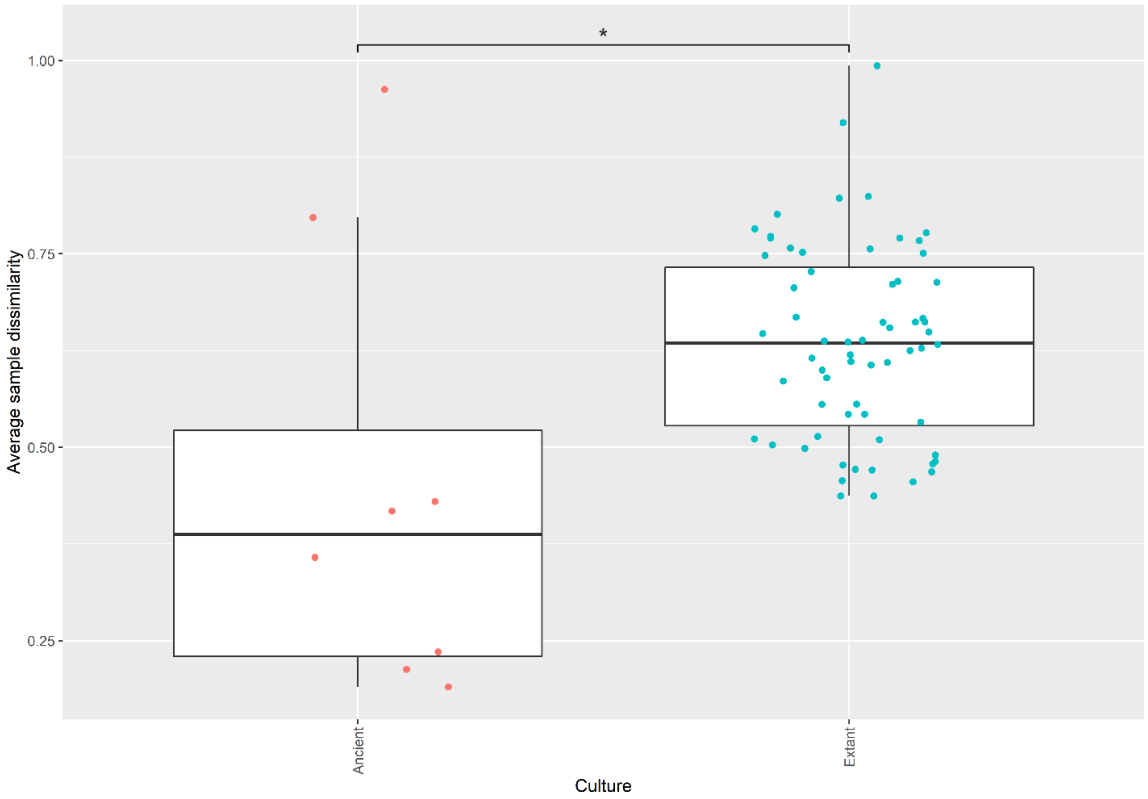

Figure S2.3: Mycobiome divergence among ancient and modern cultures.

Chapter 3: A glance at ancient flora present in pre-Columbian Puerto Rico using plant and fungal phytopathogen sequences found in human coprolites

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Chapter 3: A glance at ancient flora present in pre-Columbian Puerto Rico using plant and fungal phytopathogen sequences found in human coprolites

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Abstract

Coprolites continue to be valuable sources of diet information about ancient cultures. In our study, plant DNA from coprolites from two pre-Columbian cultures (Huecoid and Saladoid) from Vieques, Puerto Rico were analyzed using shotgun metagenomic sequencing to determine diet and lifestyles. DNA sequences of putative phytopathogenic fungi likely ingested during food consumption were also analyzed to confirm dietary habits. We found plant sequences assigned to maize (*Zea mays*), sweet potato (*Ipomoea batatas*), chili pepper (*Capsicum annuum*), peanut (*Arachis* spp.), papaya (*Carica papaya*), and, surprisingly, tobacco (*Nicotiana sylvestris*). Modelling of putative phytopathogenic fungi and plant interactions confirmed the consumption of these plants and even edible fungi. Particularly, sequences of *Ustilago* spp., an edible fungus that causes maize smut, suggest the consumption of maize and huitlacoche in the pre-Columbian Caribbean. The DNA sequences in the coprolites suggested that a variety of dietary, medicinal, and hallucinogenic plants played an important role in ancient human subsistence and societal customs. This is in agreement with previous starch grain analysis on artifacts and dental calculus. As a means of comparison and contrast, sequence data obtained from coprolites found in Mexico and the United States, as well as present-day

feces from Mexico, Peru, and the United States were analyzed. Results suggest that the diet of pre-Columbian cultures differs greatly from that of present-day cultures, likely due to the different cultures, available resources, and likely temporal periods. The present study augments insights into the dietary habits of ancient cultures as well as differences in dietary patterns related to social environments and historical periods. Importantly, data from ancient fecal specimens show the importance of ancient DNA studies to better understand pre-Columbian populations. This study also suggests the flora in the pre-Columbian Caribbean area.

Introduction

Two pre-Columbian indigenous cultures, the Huecoid and the Saladoid, migrated from different regions of the Americas in independent migratory waves many centuries ago to settle in Caribbean islands [1–3]. It has been hypothesized that the Huecoid culture represented a different cultural expression from the Saladoid culture [4]. However, an alternative hypothesis suggested that each culture had different origins based on differences in pottery and lapidary [3], and both co-habited Puerto Rico for more than 1,000 years [5]. While the Saladoid culture migrated from the Orinoco River Valley of Venezuela [6] and inhabited the island of Vieques around the sixth century B.C. [7], the Huecoid culture is believed to have originated on the eastern slopes of the Andean mountains of present-day Bolivia and Peru [8] and to have arrived to Puerto Rico in the third century B.C. Plain pottery and a considerable amount of semiprecious stone ornaments which include the jadeite condor distinguished the Huecoid culture and supports the proposed Andean origin of this culture [9,10]. In contrast, the Saladoid

culture was characterized by polychromic (white and orange over red) painted pottery [6,11].

Highly developed phytocultural practices that connected these pre-Columbian cultures in the Caribbean to South America resulted in complex social systems [12]. Early European chroniclers [13,14] and more recently starch remains stored on plant-processing artifacts (as well as human dental calculus) have shown a complex food system in the Caribbean [15,16]. During the early ceramic age, the ancient South American and Caribbean Amerindians harvested a variety of plants including maize (*Zea mays*), sweet potato (*Ipomoea batatas),* common bean (*Phaseolus vulgaris*), manioc (*Manihot esculenta*), marunguey (*Zamia* spp.), cocoyam (*Xanthosoma* sp.), and peanut (*Arachis hypogaea*) [15–17]. Minor dietary components included achira (Cannaceae) and arrowroot *(Maranta arundinacea)* [18], while chili pepper (*Capsicum* spp.) was used as a condiment. Many of these plants continued being used during the Late Ceramic Age, although indigenous people included a larger repertoire of plants, including fruits [17,19], and the importance of Cannaceae and Marantaceae increased. While paleoethnobotanical data are corroborating the information given by the Spanish chroniclers, significant gaps in knowledge of the pre-Columbian diet, and regional and temporal differences in dietary habits remain. Insights into edible plants contributing to the diet of pre-Columbian and present-day ethnic groups with contrasting geocultural regions and temporal scales are needed for a better understanding of diet as an important part of better understanding present culture and identity.

Coprolites (mummified feces) obtained from archaeological deposits have contributed tremendously valuable information on pre-Columbian diets and the paleoenvironment

where they lived [20]. For instance, the micro remains (e.g., pollen) along with macroscopic remains (bones, seeds, and fibers) recovered from coprolites can provide dietary information [21,22]. In addition, pollen of famine foods in coprolites suggests that the defecating individual lived in arid environments [20,23]. Ancient DNA sequencing of coprolites can provide even more evidence on paleodiets. DNA analysis has revealed the diet of extinct sloths [24,25], dogs [26,27], moas [28,29], and mummies [30,31]. Moreover, well-preserved DNA in coprolites has been used to reconstruct ancient human diets as inferred from the gut microbiota [32,33], virome [34], parasitome [35], and mycobiome [36]. Ancient microbial communities from coprolites can also reflect the evolution of human lifestyles through time [23,36–38]. Plant remains are difficult to identify, thus plant DNA sequences provide a deeper and more defined taxonomic classification and complement archaeological studies.

We present data on plant DNA recovered from coprolites (Vieques, Puerto Rico; ca. 1500 years old) to reconstruct the diet and ambient flora of the pre-Columbian Huecoid and Saladoid cultures and compared the data to those obtained from coprolites found in Loma San Gabriel culture (Rio Zape, Mexico), the Ancestral Puebloans in the Arid West Cave (Arizona, USA) and the Boomerang Shelter (Utah, USA). We also included data obtained from present-day fecal samples from native indigenous people, including the Matses (hunter-gatherers from Peru), Tunapuco traditional (agriculturalists from Peru), and Mazahua (farmers from Mexico) as well as urban-industrial individuals (United States). During food ingestion, phytopathogenic fungi can also be ingested unintentionally, thus fungal DNA could help confirm ancient DNA from plants consumed. Essentially, plant and fungal DNA recovered and analyzed from coprolites might provide important

insights to better understand ancient dietary habits of pre-Columbian cultures of Puerto Rico as well as differences in diet related to geoculture and historical periods.

Materials and Methods

Archaeological samples and site

Coprolites from the Huecoid and Saladoid cultures from La Hueca archeological site on Sorcé, Vieques (18º 05' 56" Latitude North and 65º 29' 34" Longitude West), a semi-arid island located about 13 km southeast of the main island of Puerto Rico (**Figure 3.1**) were used. Archaeologists Luis Chanlatte and Yvonne Narganes conducted the excavations on private land with the approval of the owner and followed all relevant regulations. In total, ten coprolites from the two pre-Columbian cultures were used: six coprolites were from the Huecoids and four from the Saladoids. Detailed information about the samples is presented in Table S3.1. The age of the coprolites was estimated using radiocarbon dating from adjacent archeological material (charcoal and shells) [8]. All samples were carbon-dated at Teledyne Isotopes (Westwood, NJ) and BETA Analytic, Inc. (Miami, FL) using a standard protocol. Radiocarbon dating estimates for the Huecoid coprolites ranged from 245 to 600 A.D., whereas the Saladoid coprolites ranged from 230 to 395 A.D. [34]. Coprolites have yielded well-preserved gut microbiome DNA [34,35] as well as human and plant DNA.

DNA extraction and contamination prevention

DNA was extracted from all coprolites samples previously [34]. Briefly, ten coprolites from the Huecoid (n = 6) and Saladoid (n = 4) cultures were processed in a class II

biological safety cabinet exclusively dedicated to ancient DNA following strict procedures: protective clothes, disinfection of surfaces, sterilization of instruments, and ultraviolet radiation. The class II biosafety cabinet was cleaned with 70% ethanol and exposed to ultraviolet radiation for 30 minutes before and after use. To avoid modern exogenous contamination, DNA extraction was performed using only the inner core of each coprolite after the removal of the exterior portion using sterile and flamed scalpels. Total DNA was extracted using the PowerSoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The inner part of the coprolites was pulverized using a sterile mortar and pestle and hydrated overnight in sterile C1 solution at 4 °C. Because of low concentrations of DNA, samples were then pooled into one composite per culture using standard glycogen precipitation protocols (Thermo Scientific).

Metagenomic library construction and shotgun sequencing

Whole-genome amplification from small quantities of DNA was performed using a REPLI-g Midi kit (Qiagen). Amplified DNA was purified using the PowerClean DNAClean-Up Kit (MO BIO Laboratories) and sample concentrations were calculated using the Qubit® dsDNA HS Assay Kit (Life Technologies). Library preparation was completed using the Nextera DNA Sample Preparation kit (Illumina) according to the manufacturer's recommendations. Libraries concentrations were evaluated using the Qubit® dsDNA HS Assay Kit (Life Technologies) and the average library size was quantified using Experion (Bio-Rad). Libraries were then pooled in equimolar ratios and shotgun sequenced on the Illumina Miseq sequencing platform at MR DNA Research lab (Shallowater, TX) [34]. These libraries are available at MG-RAST

(http://metagenomics.anl.gov) under the project name “Pre-Columbian Coprolite Metagenomes Merged Only” (MG-RAST library numbers mgl386790 and mgl386787).

Comparison with other coprolite and present-day fecal sequences

Publicly available sequence data were obtained from the NCBI’s Sequence Archive (SRA) using the SRA Toolkit (v2.10.4). Archaeological samples constitute sequencing data from 13 coprolites, including coprolites from the Loma San Gabriel culture in La Cueva de Los Muertos Chiquitos (n = 8, Rio Zape, Mexico; under BioProject ID: PRJEB31971, PRJEB33577, and PRJEB35362) [23,39,40]; from the Ancestral Puebloans from the Arid West Cave (n = 3, Arizona, USA; BioProject ID: PRJNA561510) [23]; and from the Ancestral Puebloans from the Boomerang Shelter (n = 2, Utah, USA; BioProject ID: PRJNA561510)[23] (**Table 3.1**). Present-day samples comprised published sequence data from 86 extant stools, including feces from the Matses hunter-gatherers (n = 24, Peru, BioProject ID: PRJNA268964)[41]; the Tunapuco farmers (n = 12, BioProject ID: PRJNA268964); the Mazahua farmers (n =22, Mexico, BioProject ID: PRJNA561510)[23]; and United States individuals from the Human Microbiome Project (n = 28, USA, BioProject ID: PRJNA48479)[42] (**Table 3.2**). All samples were computationally analyzed along with the data from our study [43].

Table 3.1: Description of the archaeological samples analyzed in this study

Pre-Columbian culture	Archaeological site	Geographical regions	Coprolite C-14 data (range)	Reference
Huecoid	La Hueca Sorcé	Vieques, Puerto Rico	1500 BP	This study, [34,35]

Saladoid	La Hueca, Sorcé	Vieques, Puerto Rico	1500 BP	This study, [34,35]
Loma San Gabriel	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	[39,40]
Puebloans	Arid West Cave	Arizona, United States	2500 - 1500 BP	[23]
Puebloans	Boomerang Shelter	Utah, United States	2000 - 1000 BP	[23]

Table 3.2. Description of the published present-day samples analyzed in this study

Present-day culture	**N**	**Subsistence strategy**	**Geographical region**	**Reference**
Matses	24	Hunter-gatherers	Peru	[41]
Tunapuco	12	Agriculturalists	Peru	[41]
Mazahua	22	Agriculturalists	Mexico	[23]
United States	28	Urban-industrial	United States	[42]

Bioinformatics and statistical analysis

Read processing and quality control

Raw paired-end reads were trimmed and filtered from adapters and low-quality reads (Phred score < 20) through trim-galore using default parameters as implemented in the metaWRAP Read_qc module (v1.2.4) [44]. Contaminating human DNA sequences were then removed from the metagenomic datasets through alignment of reads to the *Homo sapiens* reference genome (build Hg38) using the BMTagger approach implemented in

the metaWRAP Read_qc module [44]. Quality control improvement on sequencing reads was assessed using FastQC [45]. Pre-processed reads were considered for all downstream analyses.

Metagenomic profiling

Taxonomic assignment of high-quality sequencing reads was performed through Kaiju as implemented in command-line (v1.5.0) [46] using the following parameters: −a greedy −E 0.05 for e-value filtering. Kaiju classified reads using a subset of the NCBI BLAST non-redundant (nr) reference database (argument -nr_euk) comprising annotated protein-coding genes from bacteria, archaea, viruses, and fungi (accessed on 25 May 2020). Taxon IDs from plant sequences from the NCBI nr database were also included. It has been shown that a database comprising all domains of life is better suited for taxonomic profiling of microbial eukaryotes [47].

Functional ecological guilds

Ecological functions (trophic and guilds) of fungal genera were parsed using FUNGuild (v.1.2) (https://github.com/UMNFuN/FUNGuild) [48]. Fungal genera that classified within the plant pathogen functional guild were considered for further analysis.

Source tracking of microbial communities

The proportion of DNA reads from each potential source contributing to the Huecoid and Saladoid sink coprolite samples was estimated using Meta-SourceTracker (mSourceTracker) [49]. Publicly available shotgun libraries from human feces, coprolites, and human skin were downloaded from the Sequence Read Archive (SRA)

using SRA Toolkit (v2.10.4) and the soil metagenomes were downloaded from MG-RAST using grabseqs [50]. These reference metagenomes were selected as potential sources and contaminants (i.e., soil and skin) for coprolite samples. The environmental source samples included: 58 non-industrial human feces, 28 industrial feces, 13 coprolites, 16 human skin, and 16 soil samples. All samples were processed using the metagenome classifier Kaiju and then combined using the mSourceTracker script kaiju_table_to_OTU_table.py. The resulting table for the Eukaryotic domain was converted to HDF5 biom format using the biom-format python package (v.2.1.10) and then used as an input for mSourceTracker.

Plant-pathogen interaction network

We used the rglobi (global biotic interactions) R package to extract all the interactions between the plants and phytopathogenic fungi (queried as "Fungi") in the dataset using the get_interactions_by_taxa function. In addition, we retrieved plant disease data from the American Phytopathological Society website (https://www.apsnet.org/edcenter/resources/commonnames/Pages/default.aspx). We only retained potential phytopathogenic fungi that were identified through Kaiju in our dataset. Pathogen-host interaction network was constructed from a taxonomic table by generating a directional data frame of pathogen-host interactions as identified using rglobi and plant disease data. We then imported the dataset table into Cytoscape to build a directed network. For the resulting network, we calculated the degree of connectivity, and the eigenvector centrality using CytoNCA, to understand the importance of each node.

Statistical analysis and visualization

Sequencing data were primarily analyzed and visualized using the R statistical environment, version v4.1.3 (R Foundation for Statistical Computing). For beta diversity, dimensional reduction of Aitchison distances was visualized in a principal coordinates analysis (PCoA) using the phyloseq R package (v.1.38.0) [51]. Statistical differences in beta diversity were tested through Permutational Multivariate Analysis of Variance (PERMANOVA) using the adonis function in the phyloseq R package. A hierarchical clustering dendrogram based on Bray-Curtis dissimilarity distances was constructed on fungal abundance per sample using the Ward's clustering algorithm in the vegan R package (v. 2.5.7) [52]. Maps and piedonut plots were generated using the R packages sf (v.1.0.7), webr (v.0.1.6), and ggplot2 (v. 3.3.5).

Results

General patterns of plant DNA in coprolites from the Huecoid and Saladoid

We studied ten coprolites recovered from an archaeological midden in Vieques, Puerto Rico to reconstruct the dietary habits of the pre-Columbian Huecoid and Saladoid cultures (**Figure 3.1**). We analyzed DNA sequences from plants and their potential phytopathogenic fungi using shotgun metagenomic sequencing, which may contain fewer biases in ancient microbiome reconstruction compared to amplicon-based sequencing [53]. Following bioinformatic processing, plant sequence reads were classified into one phylum, one class, four orders, four families, six genera, and seven species (**Table 3.3**).

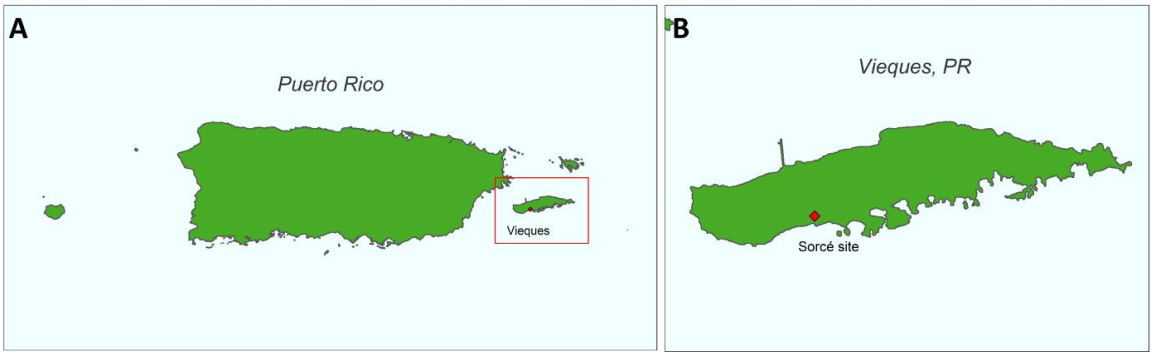

Figure 3.1. Physical map of Vieques, Puerto Rico showing the Huecoid and Saladoid archaeological settlements. Panel **(A)** Island of Vieques, an island municipality of Puerto Rico, is located about 13 km off the east coast and is highlighted with a red square. Panel **(B**) Magnified Island of Vieques showing La Hueca archeological site on Sorcé Estate, where coprolite samples used in the study were retrieved. Seven Huecoid archaeological deposits and fourteen Saladoid archaeological deposits were identified. Image created using the sf and ggplot R packages.

A high proportion of eukaryote reads from the Huecoid and Saladoid coprolite samples came from coprolite and unknown sources

We performed a source tracking analysis to estimate the environmental source (coprolites, rural and industrial feces, soil, and skin) of eukaryotes from the Huecoid and Saladoid coprolite sink samples. Overall, mSourceTracker showed that unknown sources contributed the highest proportions of Eukaryote reads in the Huecoid (0.41%) and Saladoid (0.68%) coprolites. Besides unknown sources, mSourceTracker estimated that a high proportion of the eukaryote reads of the Huecoid coprolite sink sample came from well-preserved coprolite source samples (0.33%). Conversely, a high proportion of

eukaryote had soil (0.24%) and coprolite (0.07%) origin in the Saladoid coprolite sink sample (**Figure 3.2**).

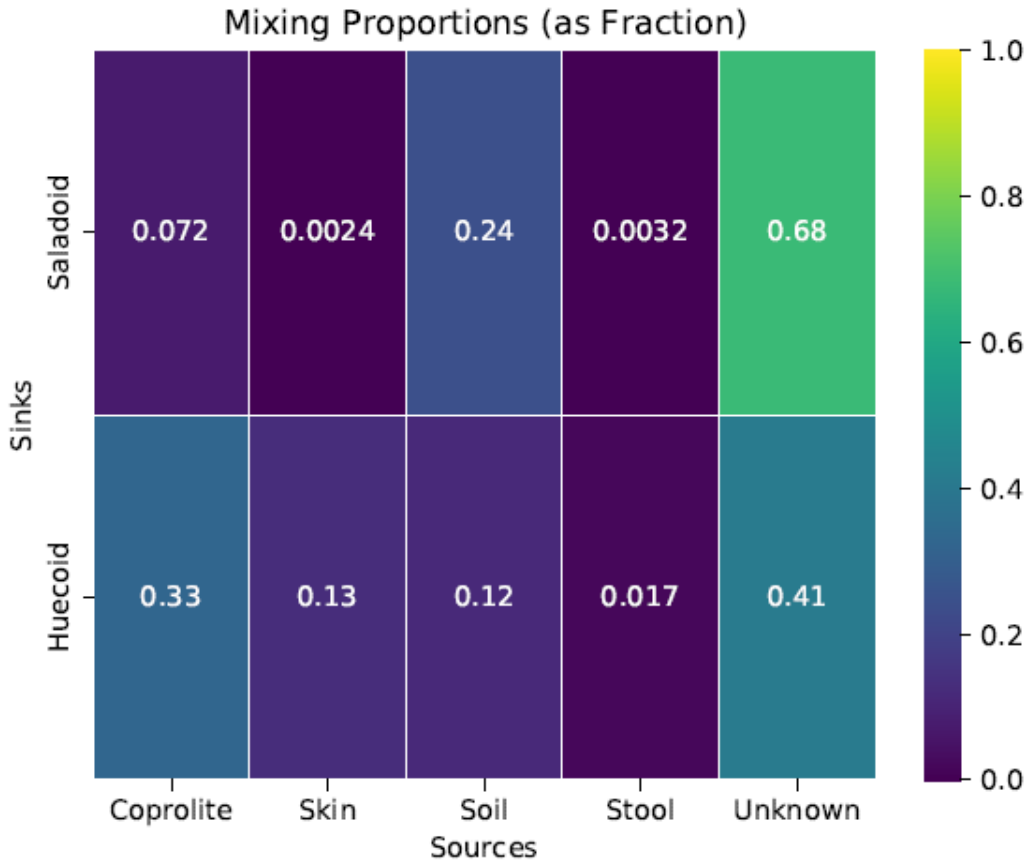

Figure 3.2: Source proportion estimates for the Huecoid and Saladoid coprolite samples (sink) using reference datasets of environmental samples (source). Meta-SourceTracker showed the proportion of Eukaryote domain sequencing data that each environmental source sample contributed to the Huecoid and Saladoid coprolite sink samples.

Starchy tubers, legumes, pseudograins, fruits, and a hallucinogenic plant were part of the Huecoid and Saladoid vegetal diet and culture

Plant sequencing reads analyzed in this study revealed a variety of food plants in the Huecoid and Saladoid coprolites. We found in the Huecoid coprolite sample a high

abundance of maize (*Zea mays*; relative abundance = 51.7%) followed by chili pepper (*Capsicum annuum*; 31.0%), sweet potato (*Ipomoea batatas*; 6.9%), wild peanut (*Arachis duranensis*; 6%), and domesticated peanut (*Arachis hypogaea*; 3%). For the Saladoid coprolite sample, the diversity of plants in the meal was different than the Huecoid coprolite sample. Plant sequencing reads identified in the Saladoid coprolite sample included chili pepper (*Capsicum annuum*; 63.2%), tobacco (*Nicotiana sylvestris*; 21.1%), and papaya (*Carica papaya*; 15.8%). Sequencing reads of chili peppers were shared between the Huecoid and Saladoid coprolite samples, while six plant taxa were only identified in one sample (**Figure 3.3A**).

Table 3.3 Description of the identified taxa from DNA sequencing of the Huecoid and Saladoid coprolites

Order	**Family**	**Genus**	**Species**	**Common name**	**Possible Origin[1]**	**Uses**
Poales	Poaceae	*Zea*	*Zea mays*	Maize	Mesoamerica	Foodstuff
Brassicales	Caricaceae	*Carica**	*Carica papaya*	Papaya	Tropical America	Foodstuff
Fabales	Fabaceae	*Arachis**	*Arachis hypogaea*	Domesticated peanut	Brazilian–Paraguayan Center	Foodstuff
Fabales	Fabaceae	*Arachis**	*Arachis duranensis*	Wild peanut	Brazilian–Paraguayan Center	Foodstuff
Solanales	Solanaceae	*Ipomoea*	*Ipomoea batatas*	Sweet potato	Central America	Foodstuff
Solanales	Solanaceae	*Capsicum*	*Capsicum annuum*	Chili pepper	South America, northern Peru	Condiment and medicinal

Solanales	Solanaceae	*Nicotiana**	*Nicotiana sylvestris*	Tobacco	Probably Mexico, Central America	Narcotic and hallucinogenic

*Tentative taxonomic assignment, taxa could be another genus in the same family.

[1] Data obtained from [54]

Network analysis of fungal pathogens and plant hosts for the Huecoid and Saladoid coprolites was implemented considering previous information from the archaeological record. Because of the low taxa in the Saladoid coprolite sample, we merged the ethnic groups and separated them into clusters in the network modeling, resulting in the highest degree of connectivity (degree = 25) and eigenvector centrality (eigenvalue = 0.40) in the maize node. This result shows the importance of this node in connecting others and that maize could be a possible host for many fungi. Tobacco node showed the second highest degree of connectivity (degree = 13), followed by sweet potato (degree = 20), and peanuts (degree = 18). Nonetheless, sweet potato showed a higher eigenvector centrality (eigenvalue = 0.35) than tobacco (eigenvalue = 0.34), suggesting that the node of sweet potato is more influential within the network. Plants with the lowest degree of connectivity and eigenvector centrality included Papaya (degree = 3, eigenvalue = 0.12), and chili pepper (*Capsicum* spp.; degree = 2, eigenvalue = 0.07) and tobacco (*Nicotiana sylvestris*; degree = 6, eigenvalue = 0.05) (**Figure 3.3B**).

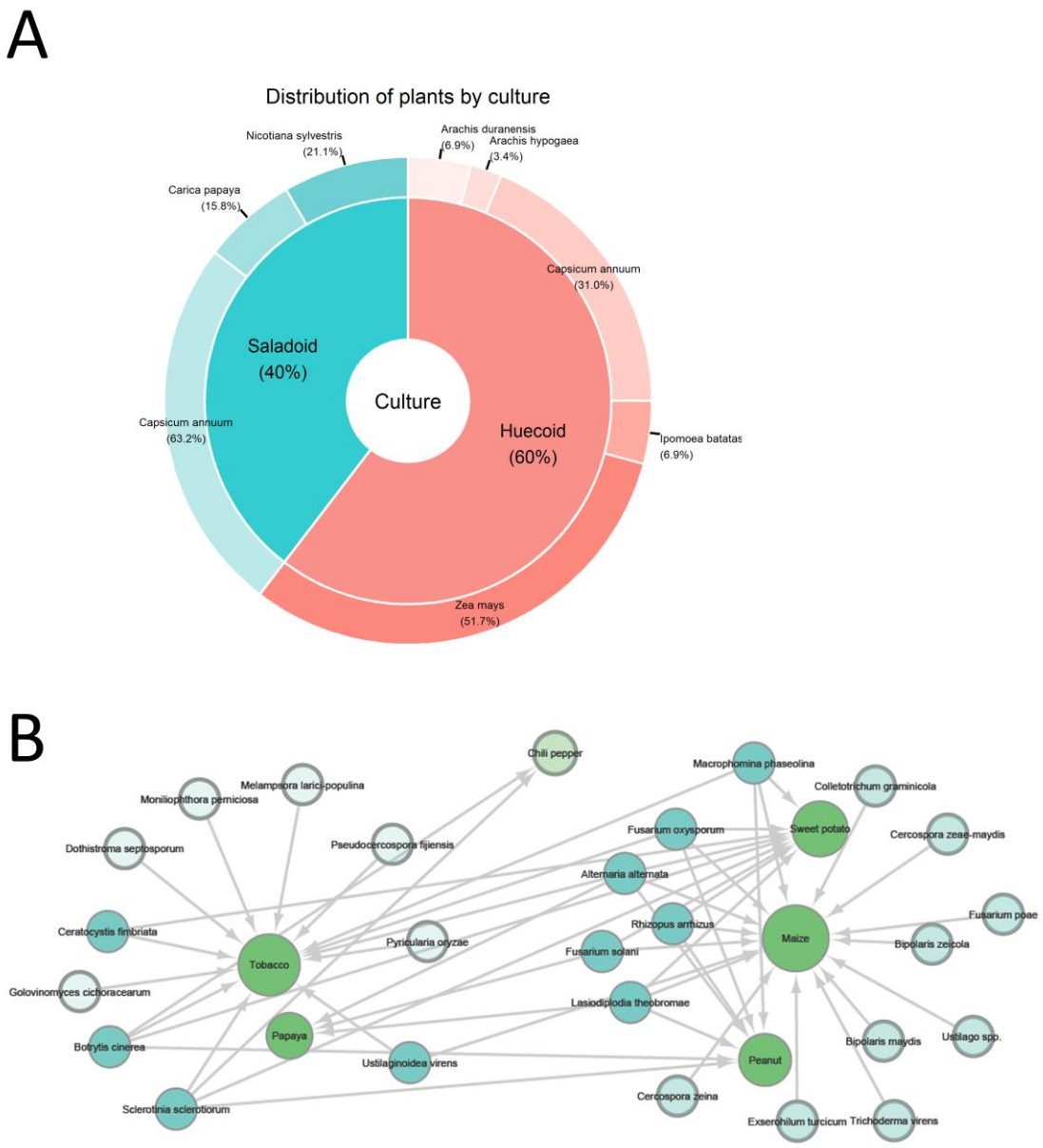

Figure 3.3: Piedonut diagram for plant distribution and directed network analysis of pathogen-host interactions of the pre-Columbian Huecoid and Saladoid cultures. Panel **(A)** Inner pie chart represents the percentage of plants identified by culture, whereas the outer donut shows the distribution of the plants. Panel **(B)** Pathogen-host interaction network constructed using the rglobi (global biotic interactions) database. Relationships between fungal pathogens and plant hosts are represented as directed edges from source

(fungi) to target (plants). Each node represents either plant (green) or fungal (blue) taxa. Plant node size represents indegree and plant node transparency depicts the Eigenvector centrality.

Phytocultural practices of ancient cultures are different from those of present-day cultures

For comparative purposes, we analyzed publicly available coprolite sequence data from Rio Zape Cave (Mexico), Boomerang Shelter (United States), and Arid West Cave (United States) as well as present-day feces from the Matses hunter-gatherers (Peru), Tunapuco (Peru) and Mazahuas (Mexico) agricultural communities, and industrial populations (from the United States).

We quantified Bray-Curtis dissimilarity and Aitchison distances to investigate differences in the plant species beta diversity across the samples. PCoA based on Aitchison distances and relative abundance of plant species showed significant segregation (PERMANOVA, $R2 = 0.23$ and p-value = 0.001) in the plant species across samples from the Amerindian groups (**Figure 3.4A**), suggesting differences in community structure. However, coprolites from Arid West, Boomerang Shelter, Loma San Gabriel, Huecoid, and Saladoid coprolites have more similar plants to each other than to present-day feces from Matses, Mazahua, Tunapuco, and the United States (**Figure 3.4A**). Hierarchical cluster analysis based on Bray-Custis, and the fungal relative abundance of each sample reflected two main clusters; combining coprolites from Ancestral Puebloans, Loma San Gabriel, Huecoid, and Saladoid (Cluster 1); and present-day feces from Matses, Tunapuco, Mazahua and United States (Cluster 2) (**Figure 3.4B**).

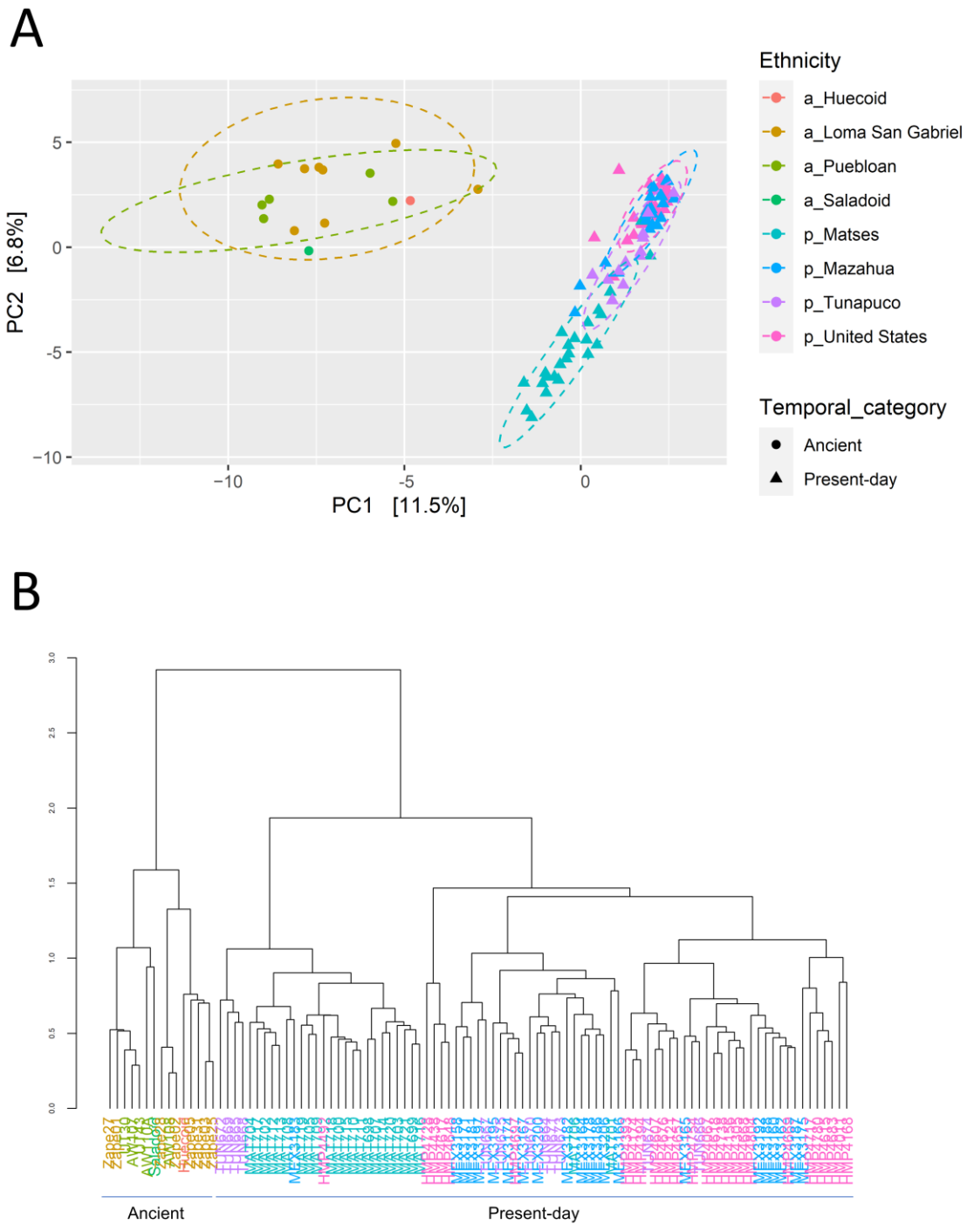

Figure 3.4. Plant species composition and structure differentiate the ethnic groups according to temporal category. Panel **(A)** Principal component analysis of Aitchison distances showing that plant species beta-diversity segregated pre-Columbian ethnic

groups from present-day ethnic groups. Each color code represents the ethnicity of each group, whereas the circle and triangles symbols represent plant species communities of each sample in ancient and present-day ethnic groups, respectively. Adonis test was performed on Aitchison distances. Panel **(B)** Bray-Curtis distance dendrogram constructed on fungal genera abundance showing hierarchical clustering/relationships between similar samples.

Discussion

Sophisticated agricultural ecosystems were developed by pre-Columbian indigenous people, which allow them to evolve into complex social cultures [12]. In the Caribbean, the Ceramic Age began with the dispersals of the pre-Columbian Huecoid and Saladoid cultures [1–3]. In addition to the traffic of ideas and a complex toolkit (including the *burén*, stones, and shells), an advanced horticulture distinguished these indigenous cultures that migrated from South America. Unfortunately, vegetal components of the Huecoid and Saladoid cultures' diets remain poorly studied. Furthermore, regional, and temporal variations in dietary patterns of ancient culture compared to present-day cultures have not been studied. The presence of plant DNA sequences preserved in coprolites presents a unique opportunity and a window through which information not available in any other way can be obtained. Employing a combination of molecular data, pathogen-host interaction modeling, and published literature, we report the first diet reconstruction of pre-Columbian cultures of Puerto Rico as inferred from preserved ancient DNA sequences and compared the phyto-cultural diversity between ancient and present-day populations with varying social environments. We analyzed plant sequence reads recovered from coprolites of the Huecoid and Saladoid and confirmed the plant

DNA identity using phytopathogenic fungi DNA that possibly impacted their horticultural ecosystem. We then compared results with previously published coprolite sequence data from Mexico and the United States, as well as present-day feces from Mexico, Peru, and the United States. Our study provides insights into the Huecoid and Saladoid cultures' lifestyles and diets that structured the present-day Caribbeans' eating habits and cultural identity, by identifying plants that were used for consumption by the mentioned pre-Columbian groups.

Overcoming challenges with contamination

Sample contamination with modern exogenous DNA is a major challenge in the analysis of ancient DNA from coprolites [55,56]. To test contamination and verify the authenticity of DNA, we used mSourceTracker and found that unknown sources contributed the highest number of eukaryotes, suggesting that no environmental contamination affected the results. Moreover, the high proportion of unknown sources contributing taxa is consistent with previous studies on coprolites and mummies [30,38]. We also found that published metagenomes of coprolites were the main known sources contributing to the Huecoid coprolite, indicating that the Huecoid eukaryote sequencing reads are endogenous to the coprolite sample. In contrast, soil was the primarily source contributing to the Saladoid coprolite followed by coprolites, suggesting either possible soil contamination or soil as an important microbial seeding source (possible geophagy) [57]. Geophagy (ingestion of soil intentionally or unintentionally), has been used by humans to protect them from dietary chemicals and pathogens [58]. It has been observed in many different cultures around the world [58] and archaeological evidence suggests that geophagy dates back to *Homo habilis* [59,60].

Fragmentation of ancient DNA sequences may also favor the amplification of modern DNA (contamination). Hence, shotgun metagenomics sequencing was used in this study.

Evidence of various food items

Previous studies of preserved starch grains in lithic artifacts have shown a broad spectrum of plants processed with these tools, suggesting a complex food system [19] contrasting with historical narratives documenting the indigenous cultures reliance on manioc [61–64]. Consistent with this early archaeobotanical study, we identified a diversity of plants, including a starchy tuber (sweet potato), legume (peanut), solanaceous fruit (chili peppers), caricaceous fruit (papaya), pseudograin (maize), and an industrial crop (tobacco). Such plants identified in the Huecoid and Saladoid coprolites analyzed suggest a variety of dietary, medicinal, and hallucinogenic plants as part of these pre-Columbian cultures diet and culture. Although very useful and insightful, the results and conclusions in the present study are limited by the fact that there are relatively few available sequences in the current databases. As the available databases increase, the sequences obtained from coprolites will be more defined.

Tobacco

At first glance, it is difficult to imagine how tobacco sequences could be present in the coprolites, since only smoking has been described in the chronicles. Smoking will not result in any DNA being ingested, or, at best, it would be very unlikely. However, tobacco is also chewed, and although we could not find any references to this practice in the pre-Columbian chronicles, it is highly likely that tobacco could have been consumed in this manner. A second manner in which the tobacco could have been ingested is in the

still-used form of wood or ceramic inhalers where the tobacco (and other herbs, or mixtures) can be placed and the inhaler inserted in the nostrils of the recipient and then a second person would blow into the inhaler to force the powder deep into the nostrils. These two are the most likely explanations for the presence of tobacco DNA in the coprolites.

Sweet potato and legumes

Sweet potato and legumes (including common beans) fulfilled an important function in the agricultural economies of ancient Puerto Rico. We identified sequencing reads of sweet potato in the Huecoid coprolite sample. In addition, we observed sequencing reads of Fabaceae (tentatively assigned as peanut), suggesting that it was a component of the Huecoid diet. Sweet potato and legumes were also found on lithic and shell artifacts, and dental calculus from Puerto Rico and the Caribbean [18,19,65,66], indicating the consumption of these food plants. Legumes persisted in the ancient Caribbean diet of pre-Columbian cultures from the early ceramic age to the early colonial period, whereas the sweet potato was a key starchy crop during the pre-Columbian era, although its presence was higher in the early ceramic age [18]. Since the components of one meal can coincide with those of other meal [21], the low abundance of sweet potato sequences in the Huecoid coprolite sample may be because it is a food trace of a previous meal that was obscured by the abundant foods of the last meal.

Maize

Maize (*Zea Mays*), a plant domesticated in Mesoamerica [67,68], was introduced from the circum-Caribbean region (Central America and the northern countries of South

America) to Puerto Rico probably during the archaic age approximately 5,000 B.P [69]. Early European chroniclers indicate that indigenous cultures cultivated maize twice a year and consumed it tender for fruit, raw, and roasted when it is in milk. They also made certain stews, ground and with water [13]. Maize was previously considered a restricted crop [17,70]. However, evidence of human isotope and pre-Columbian dental calculus from Puerto Rico and the Caribbean suggest that maize was frequently consumed [15,71]. The authors also suggest that maize could be grounded or pounded and further baked, grilled, or toasted by these pre-Columbian cultures to possibly prepare bread [15,72]. Such findings were further extended by Pagan and Mickleburgh (2022), who suggest that maize was the most ubiquitous edible crop in all the time periods of the insular Caribbean [18]. Overall, these results suggest that maize had an important role in pre-Columbian dietary habits. We detected a high abundance of maize in the Huecoid coprolite sample, suggesting that maize was an important crop in the Huecoid culture, being consumed possibly daily, which is consistent with previous paleomicrobiological findings [32]. In addition, the analysis of starch residues in lithic tools from two Huecoid settlements in Puerto Rico demonstrated that the Huecoid culture maintained and used this plant [19]. Some plants were detected in a single culture, not because they are not in the diet of the other culture but because of possible food preferences and possibly even sporadical consumption.

Ustilago spp. sequence reads in the coprolites not only provided further evidence of maize consumption but possibly point to the consumption of huitlacoche, a common fungal phytopathogen that is priced as a delicacy even by today's cultures. Several plants were only detected in one culture, probably because of the different cultural background

that favors preferences for different plants and not because the plants are part of the other cultures diet.

Chili peppers

Chili peppers have been used for medicinal and religious purposes throughout the Americas [54]. Paleo-biolinguistics along with genetic and archaeobotanical evidence have shown that domesticated chili pepper originated in central-east Mexico approximately 6,500 years ago [73]. Chili peppers are not frequently found in the archaeological record likely due to poor starch or capsain resiliency over time [74]. However, starches of chili pepper have been detected in food-processing tools of the early southern Caribbean and the late pre-Columbian period of the northern Caribbean [18,65]. We identified sequencing reads of chili peppers in the Huecoid and Saladoid coprolites. Chili peppers were consumed as a condiment, stimulant and medicine in the pre-Columbian era [13,14]. It has been shown that chili peppers and maize occurred together in food-processing tools, suggesting that represented a food-complex [74]. Consistent with this data, we found a high abundance of maize and chili pepper DNA sequences in the Huecoid coprolite sample, pointing, once again to a likely Andean cultural origin of the Huecoid.

Lack of DNA sequences of cassava

Many archaeological narratives of the Caribbean suggest that the subsistence strategies of the pre-Columbian Huecoid and Saladoid ethnic groups were primarily based on cassava/yucca/manioc (*Manihot esculenta*) [61–63]. Plant sequencing reads of sweet potato were detected but not cassava; possibly because of the pretreatment these go

through to get rid of toxins present, something that clearly leads to the conclusion that detection of sequences depends on food preparation and mode of consumption. Methods of preparation can degrade dietary DNA [34,75], which could be further degraded by enzymes and microbes during digestion [76] as well as taphonomic effects. Alternatively, (although highly speculative) the meals represented in the coprolites possibly did not include cassava, as coprolites only show what was consumed in a few previous meals. The absence of some plants could also be due to seasonal variation in food resources [26].

The importance of cassava has been debated; the study of fifty-eight Huecoid lithic tools from La Hueca in Vieques showed that ancient cassava starches were recovered from a single tool [19]. In contrast, sweet potato, and other plants (including maize), were identified in several lithic tools, suggesting that cassava was only part of a diverse spectrum of plants contributing to the diet [19]. Interestingly, cassava starch grains were undetected in twenty-four tools from the Saladoid culture, although archaeological narratives suggest that cassava was introduced to Puerto Rico by the Saladoid culture [77,78]. Similarly, cassava starches were unidentified in “burenes” (artefacts often associated with the cooking of cassava) from the Saladoid culture where sweet potato and other plants (maize and beans and others) are frequently found, suggesting the processing of diverse single or mixed flours. Based on the data, the absence of sequencing reads from cassava in the coprolites could be explained by the suggested important role of other plants [18]. Recently, archaeobotanical data from dental calculus showed that cassava was detected throughout the insular Caribbean from the Archaic/Early Ceramic Age to the Early Colonial Period. However, the authors argue that pre-Columbian cultures from

the Caribbean did not rely exclusively on cassava, but on several important food plants that formed part of dynamic phyto-cultural practices [16,66,77,79,80]. During the transition from the Late Ceramic Age to the Early Colonial Period, however, the Spanish Conquest changed the indigenous people food systems [18,80] and cassava possibly acquired higher importance for subsistence [13,18], largely because the ability of this plant to grow in poor soils.

Spatiotemporal dietary variations

Coprolites and present-day feces showed contrasting diets. Plant communities among the ethnic groups were significantly segregated depending on ethnicity and temporal category, as shown in the PCoA and hierarchical clustering dendrogram. Clustering of plant communities in the samples into pre-Columbian and present-day temporal categories suggests differences in dietary habits. Diet of past populations differs greatly from that of extant populations depending on the environment and available resources [81]. During the Neolithic Era, human dietary lifestyles transitioned from game meat and gathering of unprocessed fruits from the environment (hunter-gatherers) [82], into one based on agriculture and animal domestication (farmers). However, with the industrial revolution, people adopted a western diet, which is high in fats and simple carbohydrates [83,84].

Ancestral Puebloans (the Anasazi) were a prehistoric culture from the Colorado Plateau, which include the States of Colorado, New Mexico, Arizona, and Utah. Macro-remains analysis of coprolites from the Arid West (Arizona) and the Boomerang Shelter (Utah) have shown that maize-derived foods (including huitlacoche) [23,85] and prickly pear

fruit (*Opuntia*) are abundant components of the Ancestral Pueblo diet [23]. On the other hand, coprolites from the Loma San Gabriel culture, a prehistoric population from Rio Zape Valley in Durango, Mexico, showed that they subsisted mainly on *Agave* and maize [21,86]. Other plants that supplemented the diet included squash (*Cucurbita* spp.) and beans (*Phaseolus* spp.). In agreement with previous studies, the Huecoid and Saladoid vegetal diet consisted of starchy tubers, maize, and legumes supplemented with fruits. It is well known that food sources can vary due to differing geoculture and environmental factors. However, the Huecoid and Saladoid shared food sources with the Ancestral Pueblo culture and the Loma San Gabriel culture, which may explain the clustering of different pre-Columbian cultures. We should also mention that the presence of the plant sequences also correspond to what was consumed a short time prior to fecal deposition, and therefore what is found in the coprolites only paint part of the types of plants consumed.

In contrast, the present-day Matses (hunter-gatherers from the Peruvian Amazon), have a diet mainly composed of gathered tubers (*Manihot* spp.) and plantains (*Musa* spp.). Potatoes (*Solanum tuberosum* spp.), oca (*Oxalis tuberosa*), and mashua (*Tropaeolum tuberosum*) are part of every Tunapucos meal, who are extant agriculturalists from the Peruvian highlands [41]. On the other hand, the present-day Mazahua farmers from Mexico base their diet on maize and secondarily wheat as well as edible mushrooms [23,87]. Individuals from the United States exhibit the typical western diet composed of processed foods and dairy products [42]. Although rare or limited, the Matses hunter-gatherers and the Tunapuco agriculturalists consume dairy and processed foods [41]. In addition, rice and bread are the main food supplementing the Tunapuco diet, while wheat

contributes the majority of the Mazahua calories after maize. During colonization times there was an extraordinary exchange of plants between continents. Similar plant communities among the Matses, Tunapuco, and United States feces clustered these present-day populations. These results are supported by a recent study showing the segregation of ancient (Huecoid, Saladoid, and Loma San Gabriel) and present-day (Matses, Tunapuco, and the United States) populations based on their gut mycobiome. Studies have shown that dietary lifestyles strongly shape the gut bacteriome composition [88,89] and emerging data shows that diet has even a greater impact on the gut mycobiome [90].

Edible plants identified in a coprolite are detected because the animal had ingested the plants. However, plant DNA from coprolites is not representative of all the plants consumed by the Huecoid and Saladoid cultures. Despite that the coprolites provide relevant information about diet, food plant DNA in each coprolite likely reflect a few meals prior to deposition. Additionally, cooking affects whether plant ancient DNA is preserved in the coprolites [34,91].

Because of the possibility of DNA degradation as a result of cooking or food preparation, results may be biased towards the detection of foods consumed raw or lightly cooked. Furthermore, plant materials metabolized during digestion are hardly identified [92,93]. Maize was commonly identified in the Huecoid coprolite likely due to non-digestible fibers resistant to digestion [94]. The difficulty in the identification of sequences may be due to the current DNA databases limited to commercially important plants and completely sequenced plant genomes. Damage of ancient DNA by taphonomic processess further aggravates and limits matching ancient DNA sequences to those

available in databases. A match or close hit between sequences does not necessarily imply similarities across the species being compared, rather that the ancient DNA sequence could represent a taxon that is unrepresented in the database [26].

These results also indicate that in order to move forward, it would be important to incorporate plant sequences native to specific regions into DNA databases, which in turn could enable the validation and classification of sequences into new species [26]. In addition, further analysis of the dietary materials may provide new insights into ancient human diet and health. Similarly, a wider inclusion of coprolites from the Huecoid and Saladoid along with a combination of methods could provide complementary insights into the diet of these ancient ethnic groups.

Conclusions

Plant sequencing read analyses from coprolites showed the presence of a variety of plants in two pre-Columbian cultures of the Caribbean, as well as ancient and present-day America. The usual suspects (such as cassava and maize) were, of course observed in these ethnic groups: the Huecoid and the Saladoid, as well as others. Even though coprolites represent a limited range of plants ingested, we demonstrated that the Huecoid and Saladoid ethnic groups were consuming maize (*Zea mays*), sweet potato (*Ipomoea batatas*), chili pepper (*Capsicum annuum*), papaya (*Carica papaya*), and peanut (*Arachis* spp.). Some plant sequences were detected in the coprolite sample of a single culture, not because they are not in the diet of the other cultures but rather because there could have been preferences for other plants. In addition, DNA sequences present in the feces (be it coprolites or fresh feces) likely indicate what was consumed recently. It can also be

argued that the diet could have been even more varied, depending on what was consumed at a certain moment. Currently, all our analyses and conclusions are limited by sequence databases, that although growing exponentially, still have focused mostly on commercially important crops, especially those important to the industrialized society. It can be argued that many of the sequences found in coprolites and brushed aside as "false-positives" because those plant families or phyla could not have been present in pre-Columbian America based on historical and archeological records. However, it can also be argued that many families or phyla that served as food for these ethnic groups could be extinct as a result of the colonization of the Americas or could have evolved as a result of domestication. We hope for a larger database in the near future that includes plants that are not necessarily important in modern times; this might allow us to better understand the true diet of these ancient groups. Our data have demonstrated clearly the presence of certain plants and fungi that were part of the ancient ethnic groups diets; hopefully this will serve as a starting point for more research that will give us more information on the diets and lifestyles of pre-Columbian groups in America.

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Supplementary material

Table S3.1: Detailed description of the archaeological samples analyzed in this study

Sample ID	Archaeological site	Geographical region	Radiocarbon date	Reference
Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	470 A.D.	This study, (29,30)
Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	Circa 385 A.D.	This study, (29,30)
Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	Circa 450 A.D.	This study, (29,30)
Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	Circa 245 A.D.	This study, (29,30)
Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	215-220 A.D.	This study, (29,30)

Huecoid	La Hueca, Sorcé	Vieques, Puerto Rico	470-600 A.D.	This study, (29,30)
Saladoid	La Hueca, Sorcé	Vieques, Puerto Rico	270-385 A.D.	This study, (29,30)
Saladoid	La Hueca, Sorcé	Vieques, Puerto Rico	230-385 A.D.	This study, (29,30)
Saladoid	La Hueca, Sorcé	Vieques, Puerto Rico	230-385 A.D.	This study, (29,30)
Saladoid	La Hueca, Sorcé	Vieques, Puerto Rico	335-395 A.D.	This study, (29,30)
UT30.3	Boomerang Shelter	Utah, USA	60 A.D.	(40)
UT43.2	Boomerang Shelter	Utah, USA	10 A.D.	(40)
AW107	Arid West Cave	Arizona, USA	595 A.D.	(40)
AW108	Arizona Cave	Arizona, USA	635 A.D.	(40)
AW110A	Arid West Cave	Arizona, USA	620 A.D.	(40)
Zape1	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	920 A.D.	(40)

Zape2	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	850 A.D.	(40)
Zape3	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	725 AD	(40)
Zape 5	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	(41)
Zape 25	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	(42)
Zape 27	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	(42)
Zape 28	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	(41)
Zape 31	La Cueva de los Muertos Chiquitos, Rio Zape	Durango, Mexico	1300 BP	(42)

Chapter 4: General conclusions

Overall conclusions:

Ancient microbial DNA from coprolites can provide insights into the past of humankind and its microbiome, as well as dietary habits. However, there was an information gap on the impact(s) of modern lifestyle on the gut mycobiome compared with the gut bacteriome. Therefore, shotgun sequencing data from coprolites of ancient indigenous cultures give us a valuable opportunity to study how humans coevolved with the gut mycobiome. We aimed to determine the gut mycobiome from coprolites of the pre-Columbian Huecoid and Saladoid cultures from Puerto Rico and compared them, for the first time, with coprolites from Mexico, intestinal contents from Ötzi, stool samples from extant native populations from Peru, and urbanized populations from the United States. Our results demonstrated that:

- the fecal mycobiome in coprolites from the Huecoid and Saladoid cultures shared fungal genera, but differ in relative abundance, suggesting differences in culture and dietary lifestyles
- the gut mycobiome of ancient populations is significantly less diverse than that of extant populations, which suggests that ethnicity and modern lifestyle (i.e., dietary habits) may affect the gut mycobiome diversity
- the hierarchical dendrogram clustered most coprolites together, separated from the extant feces. Thus, the gut mycobiomes from ancient populations are more similar to each other than to the gut mycobiomes from extant populations

- principal component analysis showed a significant separation between the ethnic groups, suggesting differences in the composition and structure of the gut mycobiome of these populations

- inter-individual variability in the gut mycobiome was lower in coprolites and extant feces from native communities, whereas extant feces from urban individuals showed the highest heterogeneity, suggesting that differences in food processing and hygiene standards associated with modern lifestyle could limit microbial transmission increasing mycobiome dissimilarity among individuals

- the coprolites had a high abundance of Ascomycota, while the abundance of Basidiomycota was higher in extant feces, and the intestinal content from Ötzi, suggesting an adaptation of the gut mycobiome in response to temporal changes in diet and likely the environment

- the overall composition of fungal genera was significantly different between the group of samples Furthermore, fungal genera of the Ascomycota phylum were differentially abundant between ancient populations and extant populations

- a core mycobiome was identified in most of the samples, suggesting that a group of fungal genera could be consistently found in ancient and extant populations despite differences in ethnicity and lifestyle. However, many of these fungi are food-derived and thus, are likely transient colonizers of the gut mycobiome

Shotgun metagenomic sequencing targets all DNA isolated from a sample and thus, enable the analysis of plant DNA to gain further insights into the dietary habits of ancient Caribbean people before the arrival of Europeans. The Huecoid and Saladoid cultures of

Puerto Rico migrated from South America during the pre-Columbian era bringing with them intensive horticultural practices. Yet, little was known about the Huecoid and Saladoid dietary habits. We studied plant DNA along with phytopathogenic fungal DNA from coprolites to reconstruct the meals of the Huecoid and Saladoid using a pathogen-host interaction network modeling and expanded our analysis by including published data from coprolites and present-day feces. The main findings were that:

- the environmental sources contributing the highest eukaryote reads to the Huecoid and Saladoid coprolite sink samples were unknown, followed by coprolites and soil in the Huecoid and Saladoid, respectively. Soil may have had a role in part in the mycobiome and microbiome in terms of geophagy
- the Huecoid and the Saladoid consumed a variety of plants, including sweet potato, chili peppers, maize, peanut, papaya, and tobacco, suggesting that dietary, medicinal, and hallucinogenic plants were part of these ancient cultures' diet and cultural traditions. Furthermore, fungal DNA from coprolites suggests that phytopathogenic fungi threaten the foodstuffs used by these cultures
- Maize was an important part of the diet of the Huecoid and Saladoid cultures in the pre-Columbian Caribbean and the edible maize smut, *Ustilago* spp., was likely consumed as well
- DNA sequences of cassava were not identified in the Huecoid and Saladoid coprolites, probably because of the processing of cassava to reduce cyanide, which suggests that food preparation may affect the recovery of ancient DNA from

coprolites. It may also be that the meals represented in the coprolites did not include cassava

- the hierarchical clustering dendrogram and the principal component analysis showed significant segregation of coprolites and extant feces, suggesting differences in dietary habits according to the historical period

In general, this study provides an outstanding opportunity for understanding pre-Columbian Puerto Rico, where written records are lacking. The pre-Columbian Caribbean was populated by several indigenous cultures that immigrated from South America, including the Huecoid culture and the Saladoid culture. However, little information is available about these pre-Columbian cultures possibly contributing to the dietary habits and cultural identity of the present-day Caribbean. We showed that molecular data from coprolites provides information about the ancient mycobiome, and in turn, how modern lifestyles and dietary habits may impact the composition of the gut mycobiome. In addition, this work provides new insights into the diet of the pre-Columbian Huecoid and Saladoid cultures complementing paleoethnobotanical data of the Caribbean.