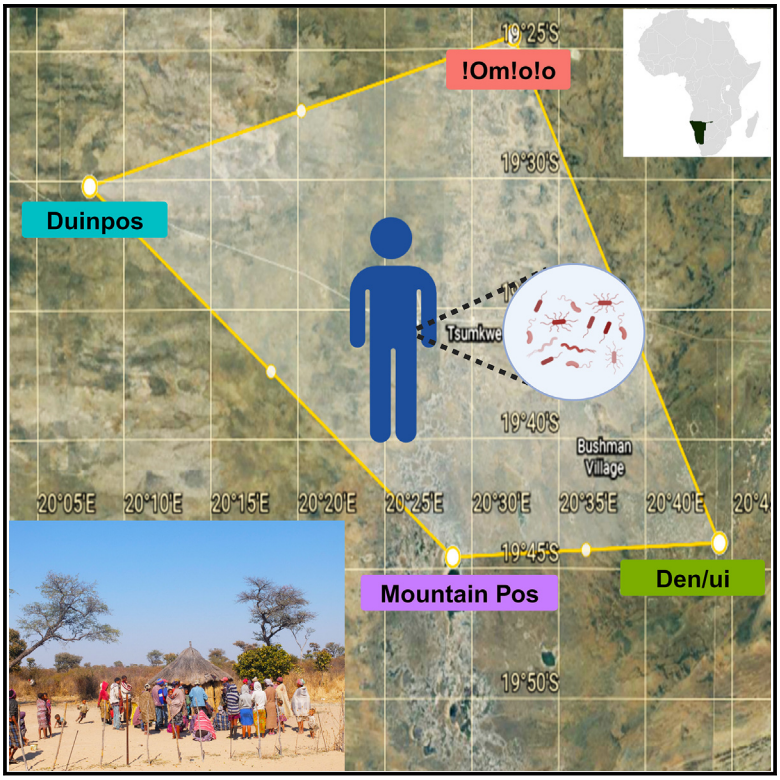
Cell Reports

Article

Documenting the diversity of the Namibian Jul'hoansi intestinal microbiome

Graphical abstract



Highlights

- The Jul'hoansi IM resembles that of non-industrialized societies
- Jul'hoansi IM composition is influenced mostly by lifestyle and culture practices
- The Jul'hoansi core intestinal microbiome is divergent from that of a global cohort

Authors

Mia Truter, Jessica E. Koopman, Karen Jordaan, ..., Simon J. Underdown, Jean-Baptiste Ramond, Riaan F. Rifkin

Correspondence

riaanrifkin@gmail.com

In brief

Truter et al. investigate the prevalence and functionality of bacteria and fungi in the Jul'hoansi intestinal microbiome, a southern African hunter-gatherer community shifting toward more industrialized food sources. The authors find that, while unique, the Jul'hoansi IM resembles that of other hunter-gatherer populations.

Truter et al., 2024, Cell Reports *43*, 113690
February 27, 2024
https://doi.org/10.1016/j.celrep.2024.113690

CellPress

Cell Reports

CellPress
OPEN ACCESS

Article

Documenting the diversity of the Namibian Ju|'hoansi intestinal microbiome

Mia Truter,[1,2,6] Jessica E. Koopman,[1,6] Karen Jordaan,[1,6] Leon Oma Tsamkxao,[3] Don A. Cowan,[1] Simon J. Underdown,[1,4] Jean-Baptiste Ramond,[1,4,5] and Riaan F. Rifkin[1,3,4,7,*]

[1]Center for Microbial Ecology and Genomics, Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Hatfield 0028, South Africa

[2]Scientific Computing Research Unit, Department of Chemistry, University of Cape Town, Rondebosch 7700, South Africa

[3]Jul'hoan Traditional Authority (JUTA), Tsumkwe, Otjozondjupa Region, Namibia

[4]Department of Anthropology and Geography, Human Origins and Palaeoenvironmental Research Group, Oxford Brookes University, Oxford OX3 0BP, UK

[5]Extreme Ecosystem Microbiomics & Ecogenomics (E[2]ME) Lab., Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 8331150, Chile

[6]These authors contributed equally

[7]Lead contact

*Correspondence: riaanrifkin@gmail.com

https://doi.org/10.1016/j.celrep.2024.113690

SUMMARY

We investigate the bacterial and fungal composition and functionality of the Jul'hoansi intestinal microbiome (IM). The Jul'hoansi are a hunter-gatherer community residing in northeastern Namibia. They formerly subsisted by hunting and gathering but have been increasingly exposed to industrial dietary sources, medicines, and lifestyle features. They present an opportunity to study the evolution of the human IM *in situ*, from a predominantly hunter-gatherer to an increasingly Western urban-forager-farmer lifestyle. Their bacterial IM resembles that of typical hunter-gatherers, being enriched for genera such as *Prevotella*, *Blautia*, *Faecalibacterium*, *Succinivibrio*, and *Treponema*. Fungal IM inhabitants include animal pathogens and plant saprotrophs such as *Fusarium*, *Issatchenkia*, and *Panellus*. Our results suggest that diet and culture exert a greater influence on Jul'hoansi IM composition than age, self-identified biological sex, and medical history. The Jul'hoansi exhibit a unique core IM composition that diverges from the core IMs of other populations.

INTRODUCTION

The human gastrointestinal tract (GIT) harbors a dynamic population of bacteria, archaea, fungi, protozoa, and viruses, i.e., the intestinal microbiota. The human intestinal microbiome (IM)[1] performs critical functions in digestion, development, and immunity.[2] Dysbiosis of the IM has been associated with inflammatory and auto-immune diseases, including allergies,[3] obesity,[4] diabetes,[5] and inflammatory bowel disease.[6] In addition, the IM played a key role in facilitating human adaptation to novel environments, in part facilitating the global dispersal of our species.[7] The impacts of changing diets, lifestyles, and environmental exposure to novel pathogens and pollutants on the human IM, therefore, relate directly to long-term human health and well-being.

Prior to the Neolithic Age, humans subsisted solely by hunting and gathering. Although the lifestyle changes associated with the advent of sedentary communities and farming significantly impacted hunter-gatherer IM taxonomic composition and metabolic capacity,[8] the impacts from the 1700s, of the Industrial Revolution, and the resultant process of global "Westernization" on the human IM are particularly marked.[9–14] It is widely held that contemporary hunter-gatherers provide insight into the configuration of the preindustrial human IM, owing largely to their comparatively limited exposure to Western lifestyle factors such as novel food sources, allopathic medication, and pollutants. However, even communities such as the Tanzanian Hadza hunter-gatherers,[15] the Venezuelan Yanomami Amerindians,[16] the BaAka in the Central African Republic,[17] and the Arctic Inuit[18] are and have been subject to the influences of Westernization, including urbanization and industrialization. As such, these communities represent a small window of opportunity to study the evolution of the IM during the transition from a non-industrialized, rural subsistence-based to an urban-industrialized lifestyle.[19]

Despite these transformations, differences in IM adaptations to diverse lifestyles remain prevalent between urban-industrialized societies and non-industrialized rural populations. Non-industrialized rural populations, whose subsistence is based primarily on foraging and small-scale subsistence-based agriculture and pastoralism, typically adhere to a high-fiber, low-fat, and low-sugar diet and generally have limited access to allopathic medication.[20] Moreover, in these contexts, people typically tend to associate more closely with one another, their pets, livestock and wildlife, and environmental microbes.[21,22]

In contrast, Western diets, as is most common among urban-industrialized societies, tend to comprise processed, high-fat, low-fiber foods combined with increased sedentarism and easier access to allopathic medication.[23] Western communities typically tend to experience less exposure to the natural environment and associated microbes. These socioeconomic and subsistence-related factors are understood to account for observed compositional differences between the IMs of rural non-industrial and urban industrial populations.[24]

Non-industrialized populations tend to harbor taxonomically more diverse IMs, containing higher abundances of short-chain fatty acid (SCFA)-producing bacteria, such as *Prevotella*, *Succinivibrio*, and *Treponema*.[24–26] The changes in taxonomic composition and metabolic capacity, including the loss of various "cornerstone" IM members resulting from urbanization and industrialization, are suspected to contribute to the increasing prevalence of inflammatory diseases typically seen in Westernized populations.[7,27,28]

Studies concerning the Hadza,[15] Amerindians,[16] the BaAka,[17] and the Inuit[18] have provided insight into the IM composition of non-industrialized forager-farmer societies. To date, comparable research has not been conducted in Namibia. Moreover, few studies explicitly investigate the influence of lifestyle factors, such as medical history and residential mobility, on observed IM taxonomic and metabolic variability.

The Jul'hoansi of the Nyae Nyae

To elucidate the taxonomic composition and metabolic capacity of the bacterial and fungal IMs of a former southern African hunter-gatherer community, we sequenced and analyzed the 16S rRNA gene (V3-V4) and the ITS1-ITS2 (internal transcribed spacer) region of fecal samples derived from 40 Jul'hoansi (pronounced "Zhu-*t*-wasi") hunter-gatherers. The Ju|'hoansi, formerly known as the "!Kung Bushmen," inhabit the Nyae Nyae Conservancy (NNC) in northeastern Namibia. Established in 1998, the NNC covers ~9,000 km^2 and is home to 2,300 Jul'hoansi and Bantu-speaking Herero agro-pastoralists.

Even though the Jul'hoansi have long been portrayed as "pristine" hunter-gatherers representative of prehistoric southern African foragers, it is emphasized that Neolithic Iron Age farmers have sporadically entered the Kalahari since at least 500 AD.[29] Contact with agrarian societies, including the associated dietary aspects, has long been part of Kalahari hunter-gatherer history. Despite long-standing interactions with Bantu-speaking farmers and, since the 19th century, European hunters and traders, many Jul'hoansi do maintain hunting and gathering practices, particularly as Nyae Nyae is one of the few remaining regions where an indigenous African community retains rights to their lands and where they may still forage throughout the year.

The Ju|'hoansi have experienced a dietary transition, increasing their consumption of domestic plants, milk, and meat from livestock while reducing their dependence on wild plants and animals.[30] Prior to the 1960s, the Ju|'hoansi subsisted by hunting and gathering no less than 85 species of wild plant foods.[31] Up to 80% of all identified food species consumed comprised plants, with the remainder of their diet comprising meat obtained via the hunting and trapping of approximately 50 different animal species.[31]

From the 1960s onwards, several stores selling food, liquor, and other facilities, such as a medical clinic, were introduced to the NNC. This exposed the Ju|'hoansi to Western commodities, including sugar, canned foods, coffee, tea, and maize porridge. Since the 1980s, several foundations have assisted the Ju|'hoansi to plant vegetable gardens and raise livestock.[32] Such initiatives include the planting of papaya (*Carica papaya*), beetroot (*Beta vulgaris*), carrots (*Daucus carota*), onions (*Allium cepa*), and tomatoes (*Solanum lycopersicum*) and the ownership of cattle and goats.

Currently, following the onset of the summer rains in December, the Jul'hoansi diet largely comprises "bush food," various species of geophytes termed "wild onions" (such as *Babiana*, *Dipcadi*, *Eulophia*, etc.), wild melons (e.g., *Citrullus lanatus* and *Cucumis hookeri*), mongongo nuts (*Schinziophyton rautanenii*), Grewia berries and baobab (*Adansonia digitata*), marula (*Sclerocarya caffra*) fruits, tree gums (e.g., *Acacia*, *Combretum*, and *Terminalia*), mushrooms (e.g., *Terfezia*), and honey.

By July, foraging becomes less important, as natural resources become less abundant, although rhizomes and Acacia tree resins are still collected.[33] The Ju|'hoansi are very fond of meat and will consume it at every opportunity. Hunting and trapping provide the Jul'hoansi with kori bustard (*Ardeotis kori*), helmeted guineafowl (*Numida meleagris*), steenbok (*Raphicerus campestris*), springhare (*Pedetes capensis*), porcupine (*Hystrix africaeaustralis*), and other taxa. During the dry season, when foraging opportunities become scarcer, the Ju|'hoansi will more frequently hunt and trap animals. Sometimes, if their hunting is successful, they will eat meat up to three times a day.

Given these seasonally dependent dietary variations, it is probable that the taxonomic composition of the Ju|'hoansi IM is influenced by seasonality,[34] although this does not form part of the scope of this analysis. It must be noted that sugar, tea, coffee, and, rarely, chocolate form part of their diet throughout the year, and increasing amounts of chili, pepper, and salt are consumed. Food sources available from the stores in Tsumkwe, the central village, substitute a significant proportion of foraging as the primary means of subsistence, and, consequently, the Ju|'hoansi have become reliant on a combination of both hunter-gatherer and contemporary-market-based subsistence strategies.[35]

We aimed to determine how observed taxonomic variations in the Jul'hoansi IM might relate to eight variables, namely (1) the ages of research participants, (2) their former use of antibiotic treatment for tuberculosis, (3) their self-identified biological sex (i.e., male or female), (4) whether diarrhea is or had been experienced following the consumption of certain foods, (5) whether participants have ever experienced an intestinal infection, (6) their former or current use of malaria medication, (7) their exposure to local, regional, and international travel, and (8) the village of primary residence of each research participant (i.e., Duinpos, Mountain Pos, Den/ui, and !Om!o!o). We also aimed to ascertain whether a core bacterial and fungal Jul'hoansi IM could be identified and the degree to which this might approximate the core IM identified on a global scale.

RESULTS

Characterizing the Jul'hoansi IM by 16S rRNA and ITS sequencing

In addition to the 40 fecal samples, two control samples, namely KIT-CTRL (kit control; i.e., the buffers of the used extraction kit) and CON-CTRL (a sampling container control), were analyzed. A total of 4,679,902 16S forward and reverse reads were imported into QIIME2-2021.2[36] and merged, resulting in a mean read count of 38,031 reads per sample. A total of 5,938,170 ITS forward reads were imported, resulting in a mean read count of 88,116 reads per sample. Six individuals were removed from the study on account of being outliers following quality control, resulting in 34 individuals. After filtering and quality control, 4,184 bacterial ASVs (amplicon sequence variants) remained. The fungal reads were clustered due to insufficient resolution for an ASV-level analysis, resulting in 167 OTUs (operational taxonomic units).

ASV/OTU and taxonomy tables were imported into R.[37] Contaminant ASVs/OTUs were identified using decontam[38] based on their prevalence in the control samples. Twelve ASVs from three bacterial species were identified as contaminants: *Streptococcus salivarius*, *Parabacteroides merdae*, and members of the *Eubacterium coprostanoligenes* group. Fungal contaminant identification yielded four OTUs, namely *Malassezia globosa*, *Pleosporales* sp., *Saccharomycetales* sp., and *Candida albicans.*

Following the removal of these contaminants, 17 bacterial phyla were identified, with *Firmicutes* (66%) and *Bacteroidota* (25%) being the two most abundant, resulting in a *Firmicutes*/*Bacteroidota* (F/B) ratio of ~2.64. Other phyla included *Proteobacteria* (7.4%), *Spirochaetota* (0.84%), and *Actinobacteria* (0.29%) (Figure 1). In total, 125 bacterial genera and 120 bacterial species were identified in the Jul'hoansi IM, with the top five genera comprising *Prevotella* (23%), *Blautia* (9.53%), *Faecalibacterium* (7%), *Succinivibrio* (6%), and *Ruminococcus* (5%). *Treponema* occurred at an abundance of 0.42%.

The two most abundant fungal phyla were *Ascomycota* (54%) and *Basidiomycota* (46%), with *Chytridiomycota* (0.01%) comprising the remainder (Figure 2). In total, 82 fungal genera representing 95 species were identified, with the top three genera comprising *Malassezia* (21%), *Candida* (20%), and *Naganishia* (14%).

Community composition and differentially abundant taxa of the Jul'hoansi IM

No statistically significant differences in α-diversity were detected between groups for the variable factors tested, i.e., age, biological sex, mobility, medication use, medical history, and the village of primary residence.

Bacterial and fungal combined β-diversity was measured using the Bray-Curtis metric, and a significant difference was evident for communities between !Om!o!o and Mountain Pos ($p = 0.004$), Den/ui and Duinpos ($p = 0.002$), and Den/ui and Mountain Pos ($p = 0.001$) using ANOSIM. (Figure 3A). β-Diversity was also significantly different when considering bacterial and fungal communities individually (Figure S1).

Differentially abundant genera were identified using ALDEx2 Welch's t test. *Rikenellaceae RC9 gut group* was more abundant in Mountain Pos than !Om!o!o ($p = 0.02$). *Cladosporium* was more abundant in !Om!o!o than Duinpos ($p = 0.09$) and more abundant in Den/ui than Duinpos ($p = 0.04$). *Candida* was more abundant in Duinpos than Den/ui ($p = 0.08$) (Figure 3B). Differential abundance for travel, the use of malaria medication, and whether participants experienced frequent diarrhea could not be tested due to class imbalance. There were no differentially abundant genera between participants of different ages or biological sexes or whether participants experienced intestinal infections. We observed an interaction effect between the village of primary residence and antibiotic usage: most Duinpos (88%) and Den/ui (100%) villagers had used antibiotics, while far fewer villagers from !Om!o!o (13%) and Mountain Pos (50%) made use of antibiotics (chi-squared $p = 0.0004$).

Core bacterial and fungal genera of the Jul'hoansi IM

The ongoing search for a core IM—a set of taxa shared across human populations[39]—will further our understanding of the evolutionary pressures that govern host-microbe interactions, as well as the organization and functional importance of these interactions.[40]

The Ju|'hoansi bacterial and fungal core IM was elucidated at 90% (hard core), 70% (medium core), and 50% (soft core) prevalences, and a detection threshold of 0.1% was used to exclude very rare taxa from the core IM. The Ju|'hoansi bacterial and fungal intestinal medium-core IMs comprised five and six taxa (Figure S2), respectively, whereas the soft core consisted of 11 bacterial and seven fungal taxa (Figure 4). Noticeable were the high relative abundances of *Blautia* and *Malassezia* in the medium core and of *Prevotella*, *Faecalibacterium*, *Malassezia*, and *Naganishia* in the soft core. No intestinal hard core was detected at a 90% prevalence cutoff.

A few bacterial genera uniquely comprised the soft-core microbiome of one or two villages only, such as the *Ruminococcus gnavus* group, a core member of Mountain Pos alone. This was also the case for several fungal soft-core genera, such as *Candida* being unique to Duinpos and !Om!o!o and *Vishniacozyma* being unique to Den/ui (Tables S1 and S2).

Metabolic enrichment of the Jul'hoansi IM

To explore the bacterial functional enrichment of the Ju|'hoansi IM, we established putative metabolically functional profiles for both fungal and bacterial datasets and determined which pathways are differentially expressed. Only two villages, Mountain Pos and !Om!o!o, showed differences in abundances for pathways involved in (1) amino acid biosynthesis, (2) biotin biosynthesis, (3) co-factor, carrier, and vitamin biosynthesis, (4) fatty acid biosynthesis, (5) proteinogenic amino acids biosynthesis, (6) sugar biosynthesis, and (7) other biosyntheses. Interestingly, we also detected pathways for pathogenicity, particularly for polymyxin resistance in *E. coli* and peptidoglycan biosynthesis (β-lactam resistance) in *Enterococcus* and *Staphylococcus*. It should also be mentioned that fewer participants living in Mountain Pos (50%) and !Om!o!o (11%) indicated they had or were using antibiotics, whereas the opposite was noted for Duinpos (88%) and Den/ui (100%). Additionally, all villages except Mountain Pos reported intestinal infections, although they were more prevalent in Duinpos. Our results suggest that the IMs of

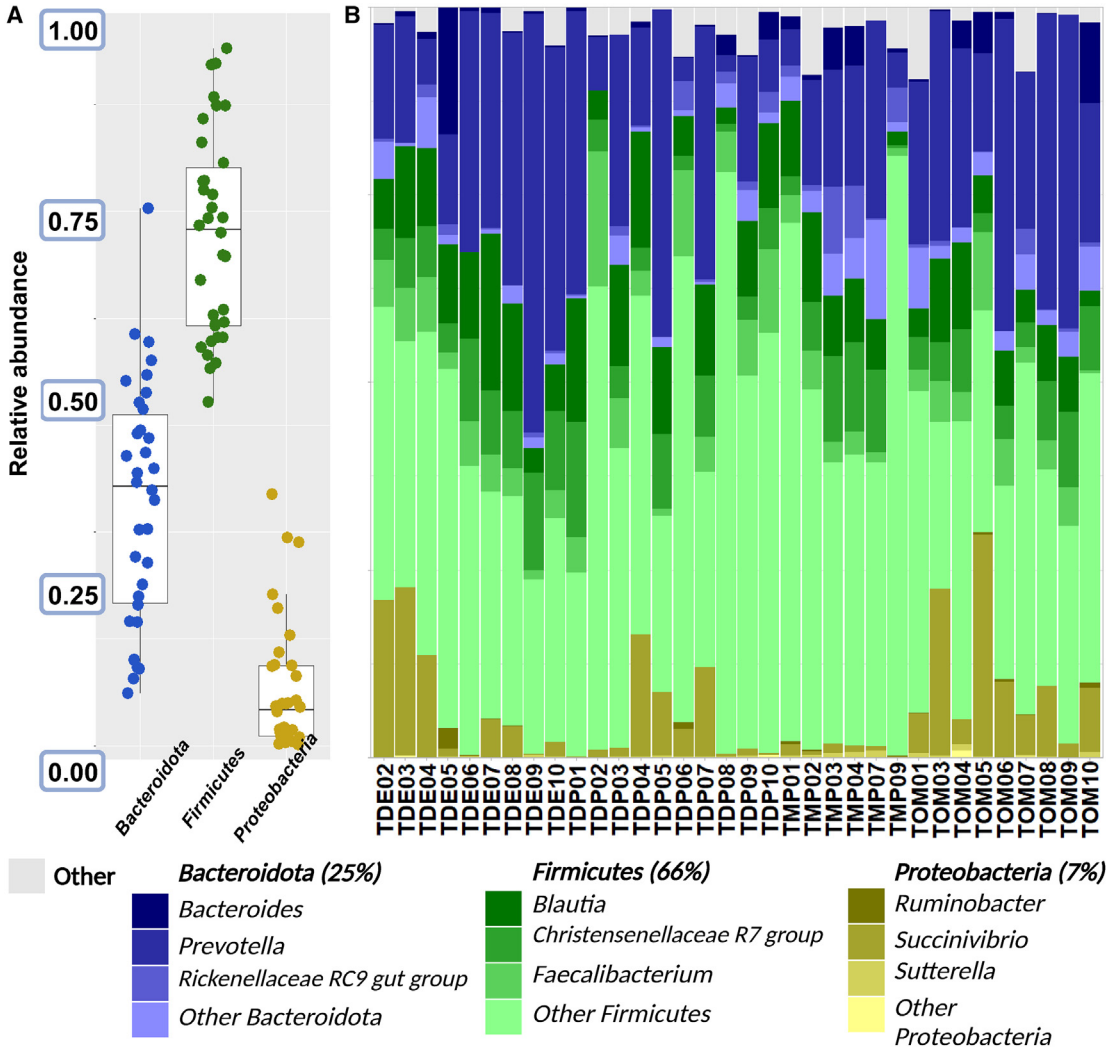


Figure 1. The bacterial taxonomic IM profiles of the 34 Ju|'hoansi research participants

(A) Phylum level and (B) Genus level. TDE, Den/ui; TDP, Duinpos; TMP, Mountain Pos; TOM, !Om!o!o.

Mountain Pos and !Om!o!o participants potentially have more pronounced amino acid, fatty acid and lipid, enzyme co-factor, and carbohydrate metabolisms than the other villages (Figure S3).

We detected the functional profiles of fungal IM inhabitants using FUNGuild.[41] Most of the fungal IM inhabitants were animal pathogens and wood or leaf saprotrophs. This included *Fusarium*, which is an opportunistic human pathogen, and genera such as *Amyloporia*, *Botryobasidium*, and *Wojnowiciella*,[42] which are wood saprotrophs commonly found on dead plant material. Other fungi, such as *Podospora*,[43] can also be found on the dung of wild animals (Table S3).

Interaction between the Jul'hoansi bacterial and fungal IMs

Since fungi and bacteria commensally co-inhabit the human IM, the interactions between these taxa are of interest. It has been shown that the diet-microbe association in the human IM is not exclusively limited to a particular microbial kingdom and that interactions with other microbes, such as fungi, also play a role in human health and disease.[44] To explore the co-occurrence of fungal and bacterial taxa in the Jul'hoansi IM, we performed Spearman correlation analysis to explore the incidence of fungal-bacterial co-occurrence networks in the Jul'hoansi IM. Only statistically significant correlations ($p < 0.01$) with a high correlation coefficient (ρ) $\geq \pm 0.7$ were selected and translated into a network (Figure 5). The network was further analyzed to identify the network statistics and modular structures of highly interconnected nodes. Due to the effects of antibiotics on the gut microbiome, the network was colored according to antibiotic use, and the nodes were shaped by village (see Figure S4 to visualize the network with its nodes identified by their microbial phyla of origin).

The network was highly modular and consisted of 754 nodes (bacteria: 682 [90.45%] and fungi: 72 [9.55%]) and 5,887 edges (99.9% positive [5,886/5,887] and 0.01% negative [1/5,887]) (Figure 5; Table S4). Interestingly, the network comprised several

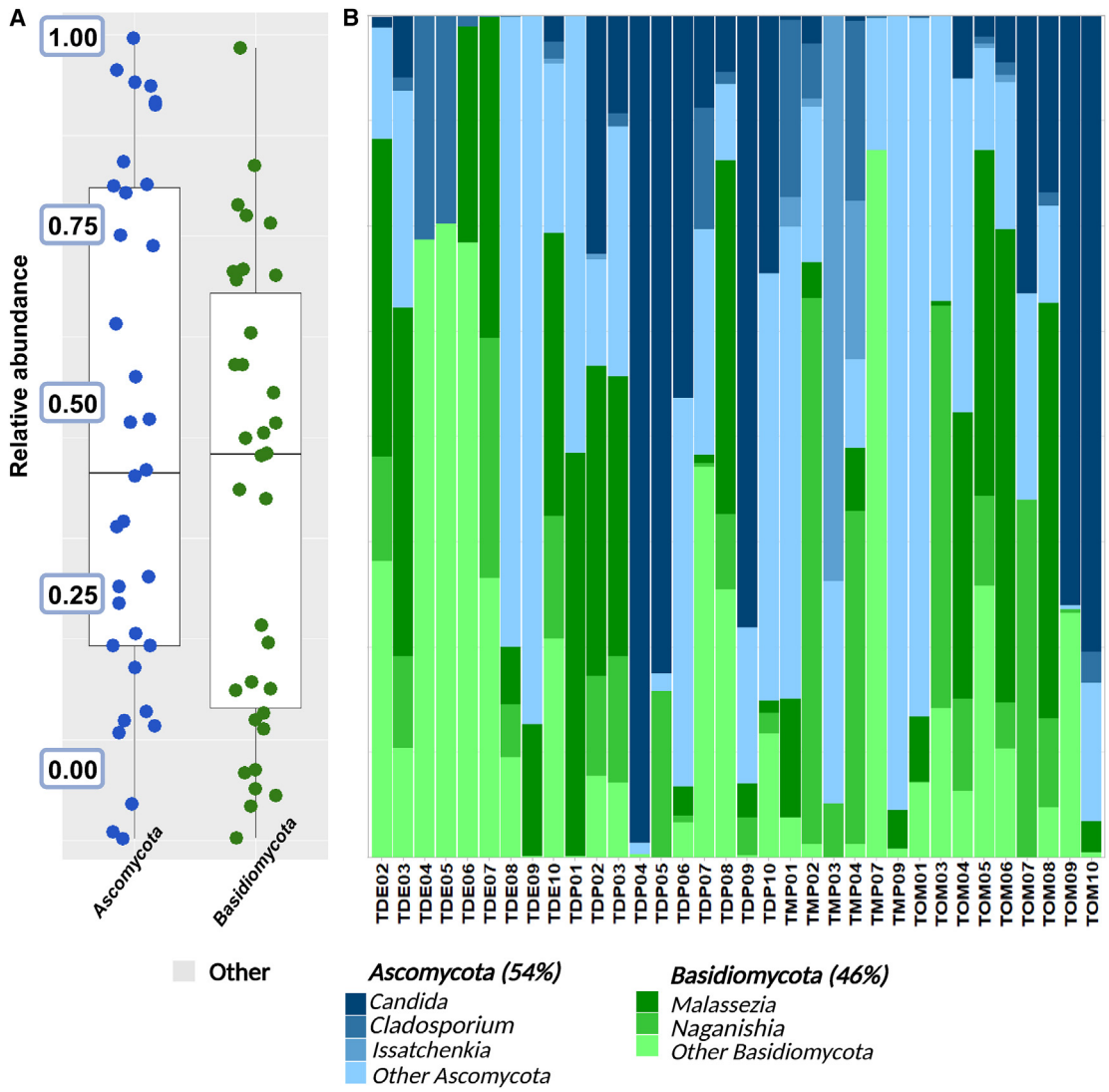


Figure 2. The fungal taxonomic IM profiles of the 34 Ju|'hoansi research participants

A) Phylum level and (B) Genus level. TDE, Den/ui; TDP, Duinpos; TMP, Mountain Pos; TOM, !Om!o!o.

smaller groups of nodes (i.e., ASVs/OTUs) assigned to a specific village with or without antibiotic use. These smaller groups were linked by "universal" nodes, classified as those observed at multiple villages, irrespective of antibiotic use (i.e., some participants have used antibiotics and others have not). Expectedly, the groups were clustered into several modules by MCODE,[45] with village as the primary grouping factor; the largest module consisted of nodes only found in !Om!o!o with a few universal nodes (module I) (Figure 5). Only the most important modules are illustrated in Figure 5.

The dominant phyla in the network included *Firmicutes* (56.36%), *Bacteroidota* (28.91%), and *Ascomycota* (7.82%), and the majority of interactions were also within and between these three groups (Figure 5). For example, positive interactions between phylotypes within (1) *Firmicutes* (e.g., *Faecalibacterium*, *Eubacterium* sp., and *Clostridia* sp.), (2) *Bacteroidota* (e.g., *Prevotella*, *Alloprevotella*, *and Bacteroides*), and (3) *Ascomycota* (*Aspergillus* sp., *Candida* sp., *Didymella* sp., *Epicoccum* sp., and *Fusarium* sp.) and (4) between *Firmicutes* and *Bacteroidota* comprised 63.28% of the total interactions. Their prevalence in the network was not surprising since these are common taxa of the IM[39,46] and play important roles in carbohydrate and amino acid metabolism and energy production.[47,48] Although these common taxa and their associations were prevalent throughout the network, we noticed interesting co-occurrences only for modules where participants have used antibiotics and/or had inflammation, specifically the genera *Sutterella*, *Dialister*, *Alistipes*, *Epicoccum*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Knufia*, *Paraprevotella*, and *Streptococcus*.

We also identified keystone species since alteration in their abundance is expected to induce changes in the abundance of

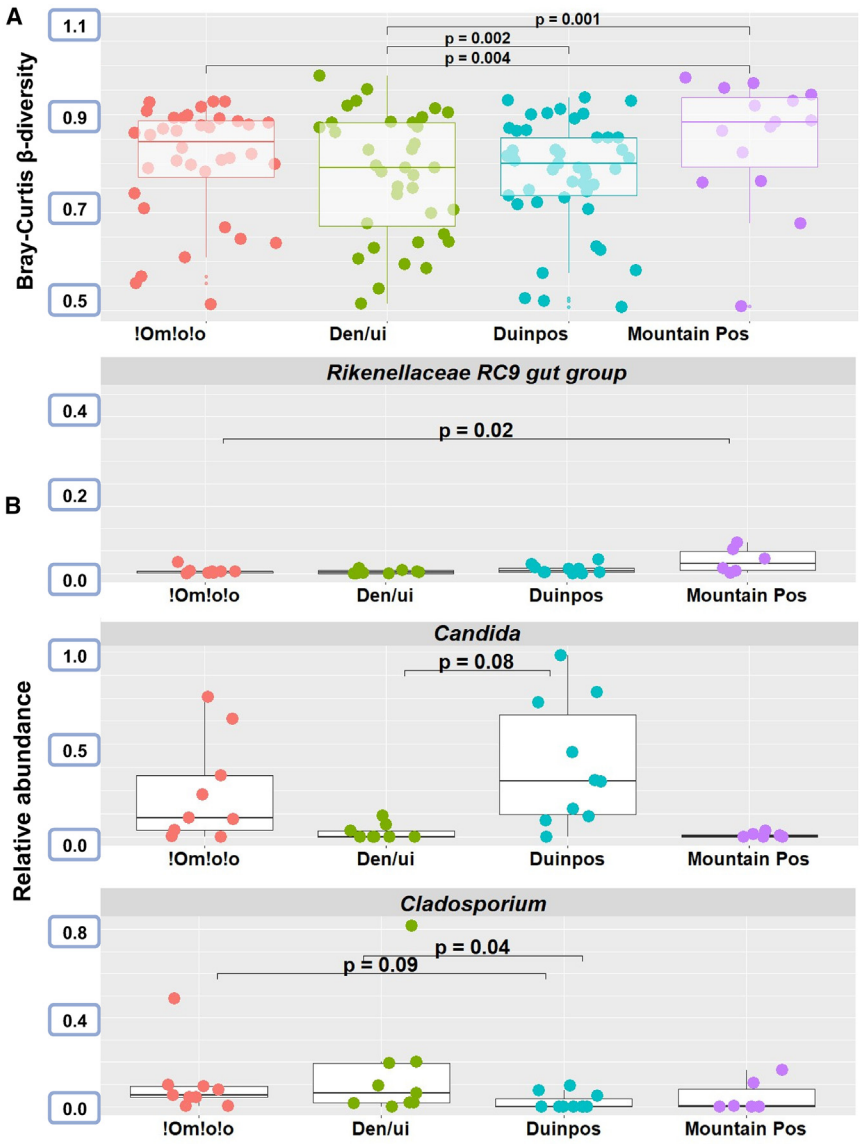


Figure 3. Community composition of the Ju|'hoansi IM

(A) Bray-Curtis β-diversity between villages, the only group for which a significant difference in community composition was detected.

(B) Differentially abundant genera between villages as identified by ALDEx2. Genera were considered significant if Benjamini-Hochberg-corrected p values for the Welch's t test were <0.1.

other species and possibly impact host metabolism and health. Consistent with the topology of the network, most keystone species were positioned in the main module (module I) and included the three dominant taxa with genera *Prevotella*, *Parabacteroides*, *Alloprevotella* (*Bacteroidota*), *Faecalibacterium*, *Phascolarctobacterium*, *Anaerovibrio*, *Blautia*, *UCG-002*, *CAG-352*, *Holdemanella*, *Eubacterium ventriosum group*, *Ruminococcus torques group*, *Lachnoclostridium*, *Clostridia UCG-014* (*Firmicutes*), and *Aspergillus* (*Ascomycota*), as well as three minor taxa (*Elusimicrobiota*, *Elusimicrobium*; *Proteobacteria*, *Succinivibrio*; and *Basidiomycota*, *Tremellales*). The remainder of the keystones were primarily universal nodes present in multiple villages. Taxa for the latter differed slightly from the keystone species in module I and included *CAG-873*, *Prevotellaceae NK3B31 group*, *Rikenellaceae RC9 gut group* (*Bacteroidota*), *UCG-005*, *Ruminococcus torques group*, *Roseburia*, *Subdoligranulum*, *Christensenellaceae R-7 group* (*Firmicutes*), *Succinivibrio* (*Proteobacteria*), *Aspergillus*, *Didymella*, *Phaeosphaeria*, *Exserohilum*, *Wojnowiciella*, and *Sclerostagonospora* (*Ascomycota*). Our results indicate that some keystone species of the Ju|'hoansi IM might be village dependent, while others are common between villages; nevertheless, *Firmicutes*, *Bacteroidota*, and *Ascomycota* appear to influence the IM of the Ju|'hoansi most strongly.

Global comparative analysis of the Ju|'hoansi IM

To determine how the Ju|'hoansi IM compares to the IM of a global cohort, we incorporated bacterial IM data from the BaAka hunter-gatherers, Bantu and Papua New Guinean agriculturalists, and United States industrialists,[17,49] as well as fungal IM data from rural and urbanized South Africans.[50] We identified the soft-core microbiome at a prevalence of 50% and a detection threshold of 0.1% (Table S5).

We first considered the global cohort as a whole to identify features unique to the Ju|'hoansi core IM. The Ju|'hoansi IM harbors several unique core IM residents, including 20 bacterial genera such as *Bacteroides*, *Colidextribacter*, *Oribacterium*, *Desulfovibrio*, and *Sarcina*, as well as four unique fungal genera: *Malassezia*, *Fusarium*, *Naganishia*, and *Panellus*. The only two fungal genera that were part of the core South African microbiome were *Candida* and *Cladosporium*, which also formed part of the Ju|'hoansi core fungal IM (Figures 6A and 6B).

To determine which bacterial features were shared between individual populations and the Ju|'hoansi, the core IMs of the populations were analyzed individually at the same detection and prevalence thresholds mentioned above. The Ju|'hoansi share only two core genera with the BaAka hunter-gatherers: *Butyrivibrio* and *Anaerovibrio*. *Marvinbryantia* is common between the Ju|'hoansi and the agriculturalists from Papua New Guinea (Figure 6C). These are the only core bacterial genera common between the Ju|'hoansi and other populations, suggesting that the Ju|'hoansi core IM is somewhat unique compared to the global cohort.

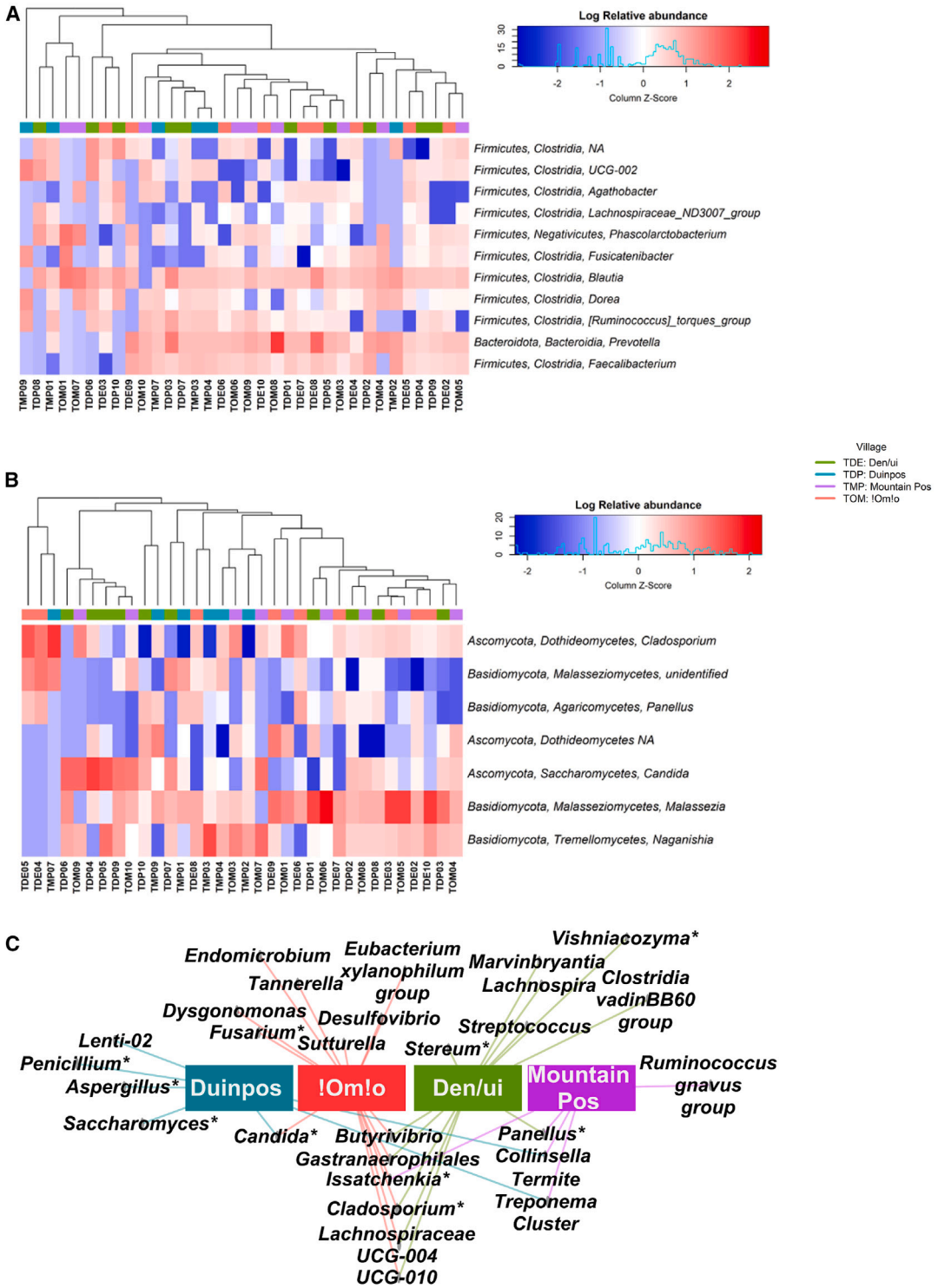


Figure 4. The Ju|'hoansi soft-core IM

(A and B) Abundance of the Ju|'hoansi (A) bacterial and (B) fungal IM core genera indicated per village and research participant, detected at a prevalence and detection threshold of 50% and 0.1%, respectively.

(C) Network of the shared Ju|'hoansi bacterial and fungal core genera between the four villages. Fungal genera are indicated with an asterisk. Genera shared between more than two villages were excluded from the figure (Tables S1 and S2).

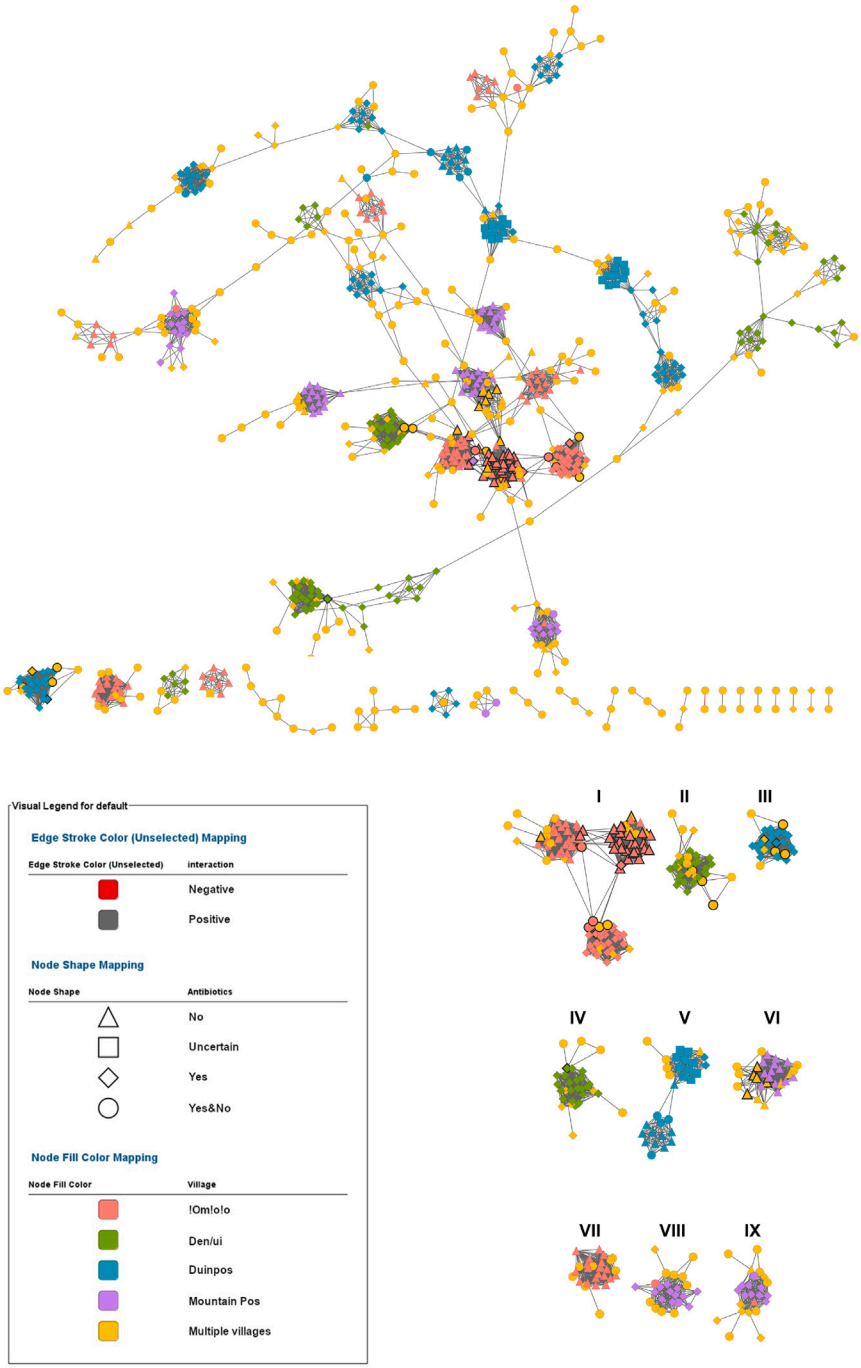


Figure 5. Multidomain co-occurrence networks of the Ju|'hoansi IM

Relationships were considered statistically significant if Spearman $\rho \geq \pm 0.7$ and $p < 0.01$. The nodes are colored according to village and shaped by antibiotic use. The keystone species are nodes indicated with thick black borders. The most densely connected regions of the network, the modules, are shown to the right of the complete network.

DISCUSSION

Based on our results, we conclude that (1) the Ju|'hoansi IM is enriched for bacterial taxa commonly associated with other hunter-gatherer populations, (2) overall bacterial and fungal IM composition was significantly different between residents of different villages, (3) *Rikenenellaceae RC9 gut group*, *Candida*, and *Cladosporium* were differentially abundant between participants from different villages of residence, and (4) unique Ju|'hoansi dietary and lifestyle characteristics are associated with a similarly unique core IM compared to those of other populations.

The Jul'hoansi bacterial IM broadly resembles that of other non-industrialized societies

Firmicutes and *Bacteroidota* are the dominant phyla in the Ju|'hoansi IM, resulting in an F/B ratio of 2.64. While the significance of the F/B ratio is controversial, it has been associated with the onset of inflammation, obesity, and various metabolic diseases.[51] The Ju|'hoansi F/B ratio broadly resembles those reported for Bantu-speaking Africans in Burkina Faso[26] (2.8) and the East African Hadza[15] (2.6). The Ju|'hoansi F/B ratio does not resemble that reported for a pre-industrial (i.e., archaeological) Neolithic agro-pastoralist South African IM, which has an F/B ratio of 0.4.[14] The increased presence of *Firmicutes* in the Ju|'hoansi IM can be attributed to the fact that diets rich in starches have been shown to increase the F/B ratio, corresponding to increases in enzymatic pathways and metabolites involved in lipid metabolism.[52] Additionally, the presence of *Treponema* in the Ju|'hoansi IM is expected, as this taxon normally occurs in the IMs of rural forager-farmer societies, while it is rare in the IMs of urban-industrialized populations.[25,53]

The Ju|'hoansi IM harbors an abundance of bacterial taxa that ferment fiber and plant polysaccharides, including *Prevotella*, *Blautia*, *Faecalibacterium*, *Succinivibrio*, and *Treponema*. These convert fiber into metabolically advantageous SCFAs, namely propionate, acetate, and butyrate, which have anti-carcinogenic and anti-inflammatory properties.[54,55] The abundance of fiber-fermenting bacteria in the Ju|'hoansi IM reflects their fiber-rich diet that is relatively low in animal protein and fat. It includes staple food items such as mongongo nuts, which have 3.5 and 2.7 g fiber per 100 g in the flesh and kernel, respectively.[56] The

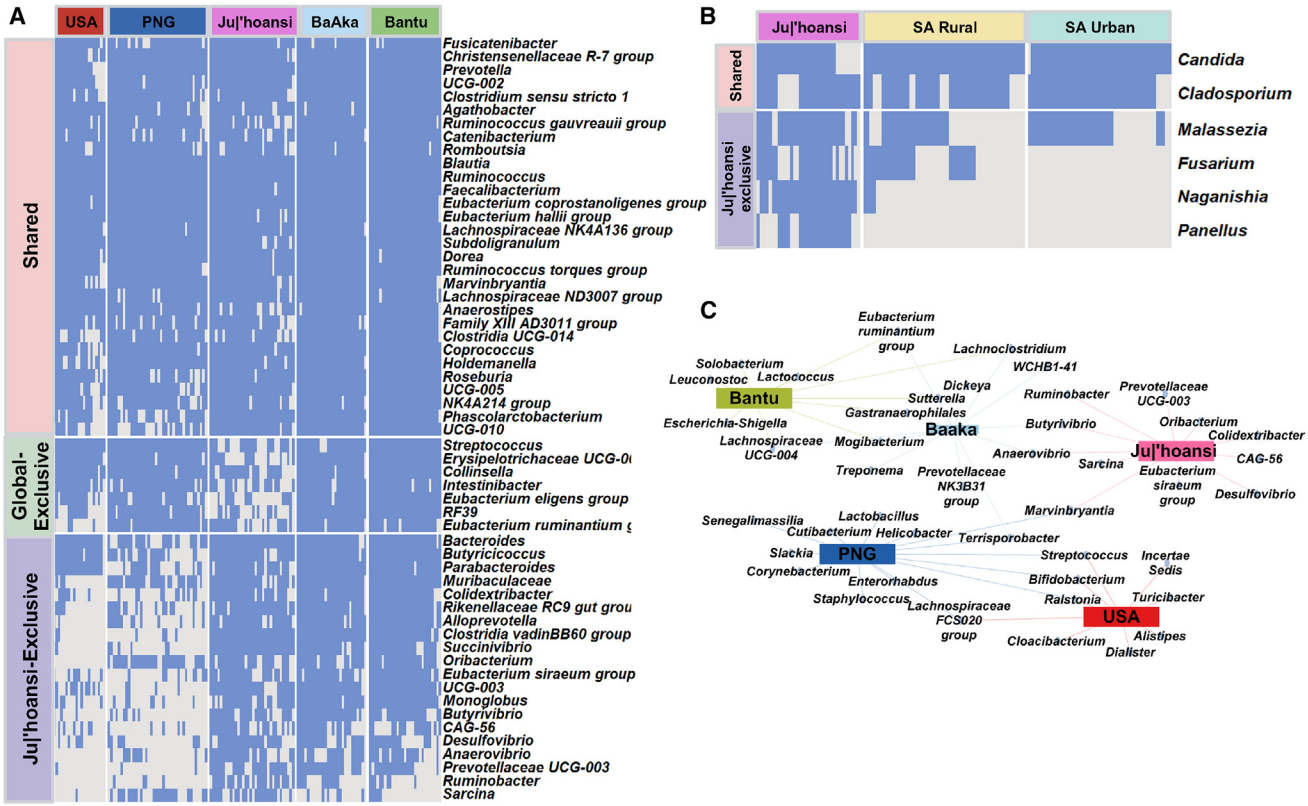


Figure 6. Comparison of the Ju|'hoansi core IM with other populations

(A) The bacterial core IM of the Ju|'hoansi was compared to the core IMs of the United States, Papua New Guinea, the BaAka, and the Bantu.

(B) The fungal core IM of the Ju|'hoansi compared to rural and urban South African core IMs.

(C) Network of the shared bacterial core IM between the various populations. Bacteria shared between three or more populations were excluded (Table S5). Blue: present; gray: absent.

abundance of fiber-fermenting taxa in the Ju|'hoansi IM is comparable to the IMs of other rural forager-farmer societies that adhere to a similar lifestyle. Children from Burkina Faso[26] harbor high abundances of the same taxa as individuals from rural Nigeria[57] and the Tanzanian Hadza.[15] The Hadza are documented to consume tubers, berries, honey, baobab fruit, and wild animals,[58] which is similar to the primary dietary constituents of the Ju|'hoansi.[59] The parallels in diet across these populations may clarify the observed similarities in IM composition.

The Ju|'hoansi IM harbors functional potential relating to several metabolic pathways, of which amino acid and lipid metabolism are the greatest, followed by enzyme co-factor and carbohydrate metabolism (e.g., glucose, galactose, sucrose, starch, hemicellulose). Similar to the BaAka,[17] our results suggest that the Ju|'hoansi incorporate a considerable amount of meat into their diet during the dry season, when foraging becomes less prevalent.

Inflammatory bowel disease or colonic inflammation is often associated with increased amino acid turnover and secondary bile acids due to the high consumption of red meat.[60,61] Interestingly, a moderate percentage of participants (35%) indicated they were or had been experiencing intestinal infections, and several pathways associated with bacterial pathogenicity, such as polymyxin resistance in *E. coli* and peptidoglycan biosynthesis (β-lactam resistance) in *Enterococcus* and *Staphylococcus*, were identified, consistent with specific taxa (e.g., *Sutterella*, *Dialister*, *Alistipes*, *Epicoccum*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Knufia*, *Paraprevotella*, and *Streptococcus*) related to villages with higher antibiotic use and/or inflammation. This is noteworthy since similar results have been observed in the BaAka[17] and, interestingly, parasitism was found here (e.g., *Trichuris trichuira*) and in the gut microbiome of other rural African populations.[17,62] However, it is not clear if antibiotic use and/or evolutionary adaptations in genes in the Ju|'hoansi increase susceptibility to colon infection, as knowledge on this is still lacking. Future research should determine the impact of Ju|'hoansi host genetics in selecting the IM and potential pathogens, host-microbe interactions, and if virulence-associated genes and those associated with host immune response are comparable with other African hunter-gatherers.

Bacterial-fungal interactions are known to occur in the human IM, and associations can influence bacterial/fungal growth and physiology and, ultimately, behavior and survival.[63,64] Since the majority of interactions (99%) between bacteria and fungi in this study were positive, we can infer mutualistic relationships where one species promoted the growth of the other, such as commensal bacteria/fungi influencing the availability of specific biologically important metabolites[65] or fungi (e.g., *Candida*)

enhancing the environment for strict anaerobes like *B. fragilis* and *B. vulgatus.*[66] For example, a recent study investigating the difference in gut IMs between Japanese and Indian participants showed higher abundances of *Candida* and *Prevotella* in Indian subjects who consumed a plant-rich diet.[67] The authors demonstrated the ability of *Candida* to convert plant polysaccharides (e.g., cellulose and xylan) to arabinose, which enhances the growth of *Prevotella.* Similar deductions may be drawn in this study, as both *Candida* and *Prevotella* were dominant taxa in the Ju|'hoansi IM, but we also found positive interactions between *Aspergillus* and *Prevotella*. *Aspergillus* has similar plant polysaccharide degradation properties[68] to *Candida*, providing the necessary carbon source for bacterial growth. These results suggest a dietary-metabolite-mediated interaction between fungi and bacteria in the Ju|'hoansi IM, possibly influencing gut homeostasis.

Interestingly, specific taxa associated with infection and disease, such as *Alistipes*, *Dialister*, *Fusobacterium*, *Streptococcus*, and *Sutterella*, which were primarily identified in villages with higher antibiotic use and/or inflammation, showed positive interactions with other commensal bacteria, although the number of interactions was fewer, while interactions with fungi were limited and mainly involved *Aspergillus*. A previous study has shown that commensal bacteria can promote the virulence of potential pathogens by cross-respiration, thereby enhancing the growth yield and persistence of the pathogen.[69] This might be one factor supporting the positive interactions observed here; however, the exact mechanism(s) for this occurrence in the Ju|'hoansi is not yet known and should be elucidated in follow-up studies. Interactions of opportunistic pathogens (e.g., *Trichosporon*) with other taxa (e.g., *Faecalibacterium* and *Roseburia*) have also been observed for the BaAka, although these associations were mainly negative.[70] Nonetheless, these and our results contribute to the growing body of evidence that clinically relevant bacterial-fungal interactions exist in hunter-gatherers, which could impact host health through pathogen or inflammation control. Our results warrant further exploration to determine how bacterial-fungal interactions in the Ju|'hoansi IM enhance bacterial and fungal virulence and how antagonistic or mutualistic relationships are linked to disease.

The Ju|'hoansi fungal IM is divergent from the global IM

Thus far, the majority of studies have investigated the human mycobiome in the context of healthy vs. diseased patients, for example elucidating differences in mycobiome composition between patients with and without Crohn's disease[71] or between obese and healthy subjects.[72] The inclusion of the mycobiome in a study investigating the IM of a hunter-gatherer population is novel, and as such, the comparison of our results to the existing literature is challenging.

The healthy human fungal IM is generally lower in diversity than its bacterial counterpart and is frequently dominated by yeasts such as *Candida* and *Malassezia.*[73] *Candida* and *Malassezia* were the most abundant core fungal genera in the Ju|'hoansi IM, while *Candida* and *Cladosporium* were the only two core fungal genera common between the Ju|'hoansi and the urban and rural South African IMs. *Cladosporium* is also a common intestinal inhabitant, probably due to its abundance in air.[74] The significance of the Ju|'hoansi fungal IM in relation to diet and geographic location remains unclear but presents an interesting avenue for future research.

Both *Malassezia* and *Candida* are characterized as commensals of the human IM that can become pathogenic upon immune dysfunction.[75,76] The role of *Candida* in the human GIT has garnered some interest lately, and the research outcome has been mixed thus far.[77] *Candida* may be involved in training the immune system and preventing infections,[78] but it has also been linked to increased inflammation and candidiasis.[79] *Malassezia* may be involved in Crohn's[80] and inflammatory bowel disease.[81] However, both *Malassezia* and *Candida* are common inhabitants of the IM, irrespective of the population. Their influence on the host might, therefore, be dependent on host health. Indeed, evidence suggests that fungi such as *Candida* can disseminate from the GIT to other organs, causing life-threatening diseases in immune-compromised individuals.[82]

The Ju|'hoansi appear to have a less diverse fungal IM than what is typically reported. The Human Microbiome Project[73] reports 247 named genera, while the Ju|'hoansi have only 82. However, out of the top 15 most abundant fungal genera in Ju|'hoansi IMs, eight are not reported in the Human Microbiome Project study: *Naganishia*, *Issatchenkia*, *Stereum*, *Panellus*, *Mycena*, *Vishniacozyma*, *Neoascochyta*, and *Westerdykella*, suggesting that the Ju|'hoansi GIT is inhabited by unique fungal taxa.

Most fungal taxa inhabiting the Ju|'hoansi GIT are animal pathogens and wood or leaf saprotrophs. This includes *Amyloporia*, *Botryobasidium*, and *Wojnowiciella.*[42] *Podospora*, which is frequently found on wild animal dung,[43] is also present in the Ju|'hoansi IM. Fungal taxa prevalent in the IM seem to derive mostly from dietary and environmental factors.[83,84] The Ju|'hoansi spend most of their time outside, in close association with their environment. This might explain why their fungal IM composition is somewhat divergent from the population studied in the Human Microbiome Project,[73] which comprises participants from the United States[85] who likely subscribe to a more industrialized lifestyle than the Ju|'hoansi.

Village of primary residence equates to a significant taxonomic difference

The only variable for which IM composition was significantly different was the village of primary residence. This may result from several factors, including variable socio-economic status, dissimilar ecological conditions at village locations, the use of different water sources at each village, and family and social networks. Participant villages exhibit varying degrees of affluence, with some possessing commodities such as vegetable and fruit gardens, cattle, and even motor vehicles, while others do not. Socio-economic status is known to influence IM composition,[86] as it determines factors such as the type of food that is accessible and the level of psycho-social stress that is experienced.[87]

The geology and associated vegetation types surrounding each village also vary,[88] resulting in changes in the types of species consumed most frequently. In addition, each village has its own unfiltered water source (i.e., boreholes) that may support different microbial taxa and which might, in turn, determine the range of taxa to which residents are exposed.

Familial and social networks may also influence shared bacterial lineages. Historically, Ju|'hoansi settlement patterns and social organization were characterized by close interaction between mostly related residents who tended to live in high-density "camps" or villages.[89,90] A similar pattern of close daily interpersonal interaction between co-inhabitants of villages can still be observed today; this may explain some of the differences in IM composition observed between Ju|'hoansi villages.

The Jul'hoansi core IM is divergent from the global core IM

To compare the Ju|'hoansi core IM to those of other populations, we first considered the global core IM as a combination of the IMs of other populations. Twenty bacterial and four fungal genera were unique to the Ju|'hoansi core IM compared to the combined bacterial core IMs of the BaAka, Bantu, Papua New Guineans, and Americans and the fungal core IMs of rural and urban South African populations.[17,49,50] This includes bacteria such as *Butyrivibrio*, *Ruminobacter*, and *Rikenellaceae RC9 gut group*. *Malassezia*, *Fusarium*, *Naganishia*, and *Panellus* were not found in the core IMs of rural or urban South Africans, while they did form part of the core Ju|'hoansi IM.

We then considered each population as an individual entity and discovered that the Ju|'hoansi only shared three bacterial genera with the populations sharing similar lifestyles: *Butyrivibrio* and *Anaerovibrio* with the BaAka and *Marvinbryantia* with Papua New Guineans.

The Ju|'hoansi harbor a unique core IM compared to other populations. This could be due to genetic or environmental factors. Although the role of host genetics in shaping the IM is unclear, there are reports of ethnicity- and geography-specific variations in IM configuration, such as that among African Malawian and South American Amerindian communities.[27] Factors such as dietary preferences and cultural practices may exert a more pronounced influence on Ju|'hoansi IM composition than factors such as medical history, age, biological sex, and the degree of exposure to microbes during travel. The Ju|'hoansi culture and their relatively isolated geographic location may contribute to their unique IM composition compared to a global cohort.

Limitations of the study

The storage of specimens following sampling is known to influence DNA yield and microbial profiles.[91] While the storage of fecal samples at −20°C presents an ideal scenario, this is unrealistic in the field. We endeavored to subject samples to immediate freezing at <0°C, in combination with a preservative, which has been shown to result in the least amount of taxonomic community changes.[92]

Furthermore, sequencing 16S rRNA genes and ITS regions may result in lower taxonomic resolution, and over-estimation may occur[93,94]; however, these methods are cost effective and commonly used in microbiome research.

We wanted to incorporate a global IM comparison into this research, however, it came with certain limitations. While the comparative IMs also made use of a marker as opposed to whole-metagenome sequencing, different regions of the 16S rRNA gene and the ITS region were used. We tried to keep the data processing as close as possible to the Ju|'hoansi workflow; however, we acknowledge that inaccuracies may arise due to unidentical sequencing and processing procedures.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - DNA extraction and sequencing
  - Data pre-processing and quality control
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data pre-processing
  - Community composition and differential abundance analyses
  - Elucidation of the Ju|'hoansi core microbiome
  - Metabolic enrichment of the Ju|'hoansi IM
  - Co-occurrence network between fungi and bacteria
  - Global IM comparison

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2024.113690.

ACKNOWLEDGMENTS

We thank all our study participants and the Jul'hoansi Traditional Authority (JUTA) for providing consent for the enrollment of project participants in the study. We thank Loide Uahengo, Edgar Mowa (Namibian National Commission on Research and Technology), and Benetus Nangombe (Namibian Ministry of Health and Social Services) for issuing the relevant research permit. We thank Helvi Elago (National Heritage Council of Namibia) and Alma Nankela (Archaeology Unit, National Heritage Council of Namibia) for providing guidance and supporting documentation concerning our permit application. We thank Manda Smith and members of the Research Ethics Committee, Faculty of Health Sciences at the University of Pretoria, for providing clearance to conduct the research. We acknowledge funding generously provided by a National Geographic Society Scientific Exploration grant (no. NGS-371R-18) and the Benjamin R. Oppenheimer Trust, which supports the Oppenheimer Endowed Fellowship in Molecular Archaeology at the University of Pretoria and Oxford Brookes University. Finally, we acknowledge BioRender.com, which was used to create the graphical abstract and compile the figures.

AUTHOR CONTRIBUTIONS

Conceptualization, ethics approval, sample collection, interview conduction, R.F.R., L.O.T., and S.J.U.; DNA extraction, J.E.K.; bioinformatic analysis, M.T. and K.J.; writing, M.T., K.J., R.F.R., J.E.K., J.-B.R., S.J.U., and D.A.C.; figure creation, M.T. and K.J.; supervision, J.E.K. and R.F.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 11, 2022
Revised: October 27, 2023
Accepted: January 4, 2024

REFERENCES

1. LEDERBERG, J., and MCCRAY, A.T. (2001). 'Ome Sweet 'Omics–A Genealogical Treasury of Words. Scientist *15*, 8.
2. Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. Biochem. J. *474*, 1823–1836.
3. Hansen, R., Gerasimidis, K., and Turner, S. (2019). Asthma Causation and the Gastrointestinal Microbiome and Metabolome: Might There Be a Signal, or Is it Just Noise? (Preprint at Mosby Inc.).
4. John, G.K., and Mullin, G.E. (2016). The Gut Microbiome and Obesity. Curr. Oncol. Rep. *18*, 1–7.
5. Dunne, J.L., Triplett, E.W., Gevers, D., Xavier, R., Insel, R., Danska, J., and Atkinson, M.A. (2014). The intestinal microbiome in type 1 diabetes. Clin. Exp. Immunol. *177*, 30–37.
6. Vich Vila, A., Imhann, F., Collij, V., Jankipersadsing, S.A., Gurry, T., Mujagic, Z., Kurilshikov, A., Bonder, M.J., Jiang, X., Tigchelaar, E.F., et al. (2018). Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. Sci. Transl. Med. *10*, eaap8914.
7. Amato, K.R., Jeyakumar, T., Poinar, H., and Gros, P. (2019). Shifting Climates, Foods, and Diseases: The Human Microbiome through Evolution. Bioessays *41*, 1–9.
8. Walter, J., and Ley, R. (2011). The Human Gut Microbiome: Ecology and Recent Evolutionary Changes. Annu. Rev. Microbiol. *65*, 411–429.
9. Segata, N. (2015). Gut Microbiome: Westernization and the Disappearance of Intestinal Diversity. Curr. Biol.
10. Blaser, M.J., and Falkow, S. (2009). What Are the Consequences of the Disappearing Human Microbiota? (Nature Publishing Group).
11. Gillings, M.R., and Paulsen, I.T. (2014). Microbiology of the Anthropocene. Anthropocene *5*, 1–8.
12. Schnorr, S.L., Sankaranarayanan, K., Lewis, C.M., and Warinner, C. (2016). Insights into Human Evolution from Ancient and Contemporary Microbiome Studies (Preprint at Elsevier Ltd).
13. Rosas-Plaza, S., Hernández-Terán, A., Navarro-Díaz, M., Escalante, A.E., Morales-Espinosa, R., and Cerritos, R. (2022). Human Gut Microbiome Across Different Lifestyles: From Hunter-Gatherers to Urban Populations. Front. Microbiol. *13*, 843170.
14. Rifkin, R.F., Vikram, S., Ramond, J.B., Rey-Iglesia, A., Brand, T.B., Porraz, G., Val, A., Hall, G., Woodborne, S., Le Bailly, M., et al. (2020). Multiproxy analyses of a mid-15th century Middle Iron Age Bantu-speaker palaeo-faecal specimen elucidates the configuration of the "ancestral" sub-Saharan African intestinal microbiome. Microbiome *8*, 1–23.
15. Schnorr, S.L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, E., Peano, C., Severgnini, M., et al. (2014). Gut microbiome of the Hadza hunter-gatherers. Nat. Commun. *5*, 1–12.
16. Clemente, J.C., Pehrsson, E.C., Blaser, M.J., Sandhu, K., Gao, Z., Wang, B., Magris, M., Hidalgo, G., Contreras, M., Noya-Alarcón, Ó., et al. (2015). The microbiome of uncontacted Amerindians. Sci. Adv. *1*, e1500183.
17. Gomez, A., Petrzelkova, K.J., Burns, M.B., Yeoman, C.J., Amato, K.R., Vlckova, K., Modry, D., Todd, A., Jost Robinson, C.A., Remis, M.J., et al. (2016). Gut Microbiome of Coexisting BaAka Pygmies and Bantu Reflects Gradients of Traditional Subsistence Patterns. Cell Rep. *14*, 2142–2153.
18. Girard, C., Tromas, N., Amyot, M., and Shapiro, B.J. (2017). Gut Microbiome of the Canadian Arctic Inuit.
19. Crittenden, A.N., and Schnorr, S.L. (2017). Current views on hunter-gatherer nutrition and the evolution of the human diet. Am. J. Phys. Anthropol. *162*, 84–109.
20. Pontzer, H., Wood, B.M., and Raichlen, D.A. (2018). Hunter-gatherers as models in public health. Obes. Rev. *19*, 24–35.
21. Rook, G.A.W., Raison, C.L., and Lowry, C.A. (2014). Microbial 'old friends', immunoregulation and socioeconomic status. Clin. Exp. Immunol. *177*, 1–12.
22. Thorburn, A.N., Macia, L., and Mackay, C.R. (2014). Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases. Immunity *40*, 833–842.
23. Carrera-Bastos, P., Fontes-Villalba, M., O'keefe, J., and Lindeberg, S. (2011). The western diet and lifestyle and diseases of civilization. Article in Research Reports in Clinical Cardiology.
24. Gupta, V.K., Paul, S., and Dutta, C. (2017). Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. Front. Microbiol. *8*.
25. Davenport, E.R., Sanders, J.G., Song, S.J., Amato, K.R., Clark, A.G., and Knight, R. (2017). The human microbiome in evolution. BMC Biol. *15*, 1–12.
26. De Filippo, C., Di Paola, M., Ramazzotti, M., Albanese, D., Pieraccini, G., Banci, E., Miglietta, F., Cavalieri, D., and Lionetti, P. (2017). Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. Front. Microbiol. *8*, 1979.
27. Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P., et al. (2012). Human gut microbiome viewed across age and geography. Preprint.
28. Sonnenburg, E.D., and Sonnenburg, J.L. (2014). Starving our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates. Cell Metabol. *20*, 779–786.
29. J.,S.,S., and R.B.,L.. (1992). Foragers, Genuine or Spurious?: Situating the Kalahari San in History. Curr. Anthropol. *33*.
30. Hitchcock, R. (2020). Dietary transitions of foragers in southern Africa. Nature Food *3*, 183–184.
31. Robbins, L.H., Murphy, M.L., Brook, G.A., Ivester, A.H., Campbell, A.C., Klein, R.G., Milo, R.G., Stewart, K.M., Downey, W.S., and Stevens, N.J. (2000). Archaeology, Palaeoenvironment, and Chronology of the Tsodilo Hills White Paintings Rock Shelter, Northwest Kalahari Desert, Botswana. J. Archaeol. Sci. *27*, 1085–1113.
32. Gargallo, E. (2020). Community Conservation and Land Use in Namibia: Visions, Expectations and Realities.
33. IMAMURA-HAYAKI, K. (1996). Gathering Activity among the Central Kalahari San. Afr. Stud. Monogr. *22*, 47–66. Supplementary issue.
34. Smits, S.A., Leach, J., Sonnenburg, E.D., Gonzalez, C.G., Lichtman, J.S., Reid, G., Knight, R., Manjurano, A., Changalucha, J., Elias, J.E., et al. (2017). Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. Science *357*, 802–805.
35. Denker, H., Thompson, S., and Jarvis, A. (2012). Living with Wildlife-Living with Wildlife Nyae Nyae Conservancy. The Story of Nyae Nyae-Ju/'hoansi for "The Place without Mountains, but rocky.".
36. Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2 (Preprint at Nature Publishing Group).
37. R Core Team (2021). R: A Language and Environment for Statistical Computing.
38. Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., and Callahan, B.J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome *6*, 1–14.

39. Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., and Gordon, J.I. (2007). The Human Microbiome Project. Nature *449*, 804–810.

40. Risely, A. (2020). Applying the core microbiome to understand host–microbe systems. J. Anim. Ecol. *89*, 1549–1558.

41. Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., and Kennedy, P.G. (2016). FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol *20*, 241–248.

42. Cannon, P.F., and Kirk, P.M. (2007). Fungal Families of the World (CAB International)).

43. Bell, A. (1983). Dung Fungi: An Illustrated Guide to Coprophilous Fungi in New Zealand (Victoria University Press).

44. Nagpal, R., Mainali, R., Ahmadi, S., Wang, S., Singh, R., Kavanagh, K., Kitzman, D.W., Kushugulova, A., Marotta, F., and Yadav, H. (2018). Gut Microbiome and Aging: Physiological and Mechanistic Insights (Preprint at IOS Press).

45. Bader, G.D., and Hogue, C.W.V. (2003). An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinf. *4*, 1–27.

46. Zhang, F., Aschenbrenner, D., Yoo, J.Y., and Zuo, T. (2022). The gut mycobiome in health, disease, and clinical applications in association with the gut bacterial microbiome assembly. Lancet Microbe *3*, e969–e983.

47. Ottman, N., Smidt, H., de Vos, W.M., and Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? Front. Cell. Infect. Microbiol. *2*, 104.

48. Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., Lewis, J.D., and Bushman, F.D. (2013). Archaea and Fungi of the Human Gut Microbiome: Correlations with Diet and Bacterial Residents. PLoS One *8*, e66019.

49. Martínez, I., Stegen, J.C., Maldonado-Gómez, M.X., Eren, M.A., Siba, P.M., Greenhill, A.R., and Walter, J. (2015). The Gut Microbiota of Rural Papua New Guineans: Composition, Diversity Patterns, and Ecological Processes. Cell Rep. *11*, 527–538.

50. Kabwe, M.H., Vikram, S., Mulaudzi, K., Jansson, J.K., and Makhalanyane, T.P. (2020). The gut mycobiota of rural and urban individuals is shaped by geography. BMC Microbiol. *20*, 1–12.

51. Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D.A., Hirschfield, G.M., Hold, G., Quraishi, M.N., Kinross, J., Smidt, H., Tuohy, K.M., et al. (2016). The gut microbiota and host health: a new clinical frontier. Gut *65*, 330–339.

52. Maier, T.V., Lucio, M., Lee, L.H., Verberkmoes, N.C., Brislawn, C.J., Bernhardt, J., Lamendella, R., McDermott, J.E., Bergeron, N., Heinzmann, S.S., et al. (2017). Impact of dietary resistant starch on the human gut Microbiome, Metaproteome, and Metabolome. mBio *8*.

53. Angelakis, E., Bachar, D., Yasir, M., Musso, D., Djossou, F., Gaborit, B., Brah, S., Diallo, A., Ndombe, G.M., Mediannikov, O., et al. (2019). Treponema species enrich the gut microbiota of traditional rural populations but are absent from urban individuals. New Microbes New Infect.

54. Cordain, L., Eaton, S.B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B.A., O'Keefe, J.H., and Brand-Miller, J. (2005). Origins and evolution of the Western diet: health implications for the 21st century. Am. J. Clin. Nutr. *81*, 341–354.

55. Sivaprakasam, S., Prasad, P.D., and Singh, N. (2016). Benefits of Short-Chain Fatty Acids and Their Receptors in Inflammation and Carcinogenesis (Preprint at Elsevier Inc.).

56. Lee, R.B. (1973). Ecology of Food and Nutrition Mongongo: The Ethnography of a Major Wild Food Resource.

57. Ayeni, F.A., Biagi, E., Rampelli, S., Fiori, J., Soverini, M., Audu, H.J., Cristino, S., Caporali, L., Schnorr, S.L., Carelli, V., et al. (2018). Infant and Adult Gut Microbiome and Metabolome in Rural Bassa and Urban Settlers from Nigeria. Cell Rep. *23*, 3056–3067.

58. Marlowe, F. (2002). Why the Hadza Are Still Hunter-Gatherers, pp. 247–275.

59. Lee, R.B. (1968). What Hunters Do for a Living, or, How to Make Out on Scarce Resources. Man the Hunter, 30–48.

60. Louis, P., Hold, G.L., and Flint, H.J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. Nat. Rev. Microbiol. *12*, 661–672.

61. Ge, J., Han, T.-J., Liu, J., Li, J.-S., Zhang, X.-H., Wang, Y., Li, Q.-Y., Zhu, Q., and Yang, C.-M. (2015). Meat intake and risk of inflammatory bowel disease: A meta-analysis. Turk. J. Gastroenterol. *26*, 492–499.

62. Jarvis, J.P., Scheinfeldt, L.B., Soi, S., Lambert, C., Omberg, L., Ferwerda, B., Froment, A., Bodo, J.M., Beggs, W., Hoffman, G., et al. (2012). Patterns of Ancestry, Signatures of Natural Selection, and Genetic Association with Stature in Western African Pygmies. PLoS Genet. *8*, e1002641.

63. Sam, Q.H., Chang, M.W., and Chai, L.Y.A. (2017). The Fungal Mycobiome and Its Interaction with Gut Bacteria in the Host. Int. J. Mol. Sci. *18*, 330.

64. Maas, E., Penders, J., and Venema, K. (2023). Fungal-Bacterial Interactions in the Human Gut of Healthy Individuals. Journal of Fungi *9*, 139.

65. Paterson, M.J., Oh, S., and Underhill, D.M. (2017). Host–microbe interactions: commensal fungi in the gut. Curr. Opin. Microbiol. *40*, 131–137.

66. Pérez, J.C. (2021). The interplay between gut bacteria and the yeast Candida albicans. Gut Microb. *13*.

67. Pareek, S., Kurakawa, T., Das, B., Motooka, D., Nakaya, S., Rongsen-Chandola, T., Goyal, N., Kayama, H., Dodd, D., Okumura, R., et al. (2019). Comparison of Japanese and Indian intestinal microbiota shows diet-dependent interaction between bacteria and fungi. npj Biofilms and Microbiomes *5*, 1–13.

68. de Vries, R.P., and Visser, J. (2001). Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiol. Mol. Biol. Rev. *65*, 497–522.

69. Stacy, A., Fleming, D., Lamont, R.J., Rumbaugh, K.P., and Whiteley, M. (2016). A commensal bacterium promotes virulence of an opportunistic pathogen via cross-respiration. mBio *7*.

70. Sharma, A.K., Davison, S., Pafco, B., Clayton, J.B., Rothman, J.M., McLennan, M.R., Cibot, M., Fuh, T., Vodicka, R., Robinson, C.J., et al. (2022). The primate gut mycobiome-bacteriome interface is impacted by environmental and subsistence factors. npj Biofilms and Microbiomes *8*, 1–11.

71. Li, Q., Wang, C., Tang, C., He, Q., Li, N., and Li, J. (2014). Dysbiosis of Gut Fungal Microbiota is Associated With Mucosal Inflammation in Crohn's Disease. J. Clin. Gastroenterol. *48*, 513.

72. The Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. Nature *486*, 207–214.

73. Nash, A.K., Auchtung, T.A., Wong, M.C., Smith, D.P., Gesell, J.R., Ross, M.C., Stewart, C.J., Metcalf, G.A., Muzny, D.M., Gibbs, R.A., et al. (2017). The gut mycobiome of the Human Microbiome Project healthy cohort. Microbiome *5*, 153.

74. Suhr, M.J., and Hallen-Adams, H.E. (2015). The human gut mycobiome: Pitfalls and potentials-a mycologist's perspective. Mycologia *107*, 1057–1073.

75. Kumamoto, C.A., Gresnigt, M.S., and Hube, B. (2020). The gut, the bad and the harmless: Candida albicans as a commensal and opportunistic pathogen in the intestine. Curr. Opin. Microbiol. *56*, 7–15.

76. Dawson, T.L. (2019). Malassezia: The Forbidden Kingdom Opens. Cell Host Microbe *25*, 345–347.

77. Musumeci, S., Coen, M., Leidi, A., and Schrenzel, J. (2022). The human gut mycobiome and the specific role of Candida albicans: where do we stand, as clinicians? Clin. Microbiol. Infection *28*, 58–63.

78. Quintin, J., Saeed, S., Martens, J.H.A., Giamarellos-Bourboulis, E.J., Ifrim, D.C., Logie, C., Jacobs, L., Jansen, T., Kullberg, B.-J., Wijmenga, C., et al. (2012). Candida Albicans Infection Affords Protection against Reinfection via Functional Reprogramming of Monocytes.

79. Pappas, P.G., Lionakis, M.S., Arendrup, M.C., Ostrosky-Zeichner, L., and Kullberg, B.J. (2018). Invasive candidiasis. Nat. Rev. Dis. Prim. *4*, 1–20.

80. Limon, J.J., Tang, J., Li, D., Wolf, A.J., Michelsen, K.S., Funari, V., Gargus, M., Nguyen, C., Sharma, P., Maymi, V.I., et al. (2019). Malassezia Is Associated with Crohn's Disease and Exacerbates Colitis in Mouse Models. Cell Host Microbe *25*, 377–388.

81. Yang, Q., Ouyang, J., Pi, D., Feng, L., and Yang, J. (2022). Malassezia in Inflammatory Bowel Disease: Accomplice of Evoking Tumorigenesis. Front. Immunol. *13*, 846469.

82. Alonso-Monge, R., Gresnigt, M.S., Román, E., Hube, B., and Pla, J.s. (2021). Candida albicans colonization of the gastrointestinal tract: A double-edged sword. PLoS Pathog. *17*, e1009710.

83. Kong, H.H., and Morris, A. (2017). The Emerging Importance and Challenges of the Human Mycobiome, pp. 310–312.

84. Hallen-Adams, H.E., and Suhr, M.J. (2016). Fungi in the Healthy Human Gastrointestinal Tract, pp. 352–358.

85. Aagaard, K., Petrosino, J., Keitel, W., Watson, M., Katancik, J., Garcia, N., Patel, S., Cutting, M., Madden, T., Hamilton, H., et al. (2013). The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. Faseb. J. *27*, 1012.

86. Bowyer, R.C.E., Jackson, M.A., Roy, C.I.L., Lochlainn, M.N., Spector, T.D., Dowd, J.B., and Steves, C.J. (2019). Socioeconomic Status and the Gut Microbiome: A TwinsUK Cohort Study. Microorganisms *7*, 17.

87. Amato, K.R., Arrieta, M.-C., Azad, M.B., Bailey, M.T., Broussard, J.L., Bruggeling, C.E., Claud, E.C., Costello, E.K., Davenport, E.R., Dutilh, B.E., et al. (2021). The human gut microbiome and health inequities. Proc. Natl. Acad. Sci. USA *118*, 2017947118.

88. Ward, J.,D., and R.,S.. (1992). Pan Types and Geology: Some Observations from Bushmanland.

89. Draper, P. (1973). Crowding among Hunter-Gatherers: The !Kung Bushmen. Science *182*, 301–303.

90. Marshall, L. (1960). !Kung Bushman Bands. Africa *30*, 325–355.

91. Ezzy, A.C., Hagstrom, A.D., George, C., Hamlin, A.S., Pereg, L., Murphy, A.J., and Winter, G. (2019). Storage and handling of human faecal samples affect the gut microbiome composition: A feasibility study. J. Microbiol. Methods *164*, 105668.

92. Song, S.J., Amir, A., Metcalf, J.L., Amato, K.R., Xu, Z.Z., Humphrey, G., and Knight, R. (2016). Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. mSystems *1*, 21–37.

93. Mizrahi-Man, O., Davenport, E.R., and Gilad, Y. (2013). Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads: Evaluation of Effective Study Designs. PLoS One *8*, e53608.

94. Bailén, M., Bressa, C., Larrosa, M., and González-Soltero, R. (2020). Bioinformatic strategies to address limitations of 16rRNA short-read amplicons from different sequencing platforms. J. Microbiol. Methods *169*, 105811.

95. Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods *13*, 581–583.

96. Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. PeerJ *4*, e2584.

97. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glockner, F.O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. *41*, D590–D596.

98. Nilsson, R.H., Larsson, K.-H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., et al. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. *47*, D259–D264.

99. McMurdie, P.J., and Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One *8*, e61217.

100. Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., et al. (2019). Welcome to the {tidyverse. J. Open Source Softw. *4*, 1686.

101. Teunisse, G.M. (2022). Fantaxtic - Nested Bar Plots for Phyloseq Data (Preprint).

102. Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., et al. (2020). vegan: Community Ecology Package. R package version 2.5-6. 2019. Preprint.

103. Gloor, G.B., Macklaim, J.M., and Fernandes, A.D. (2016). Displaying Variation in Large Datasets: Plotting a Visual Summary of Effect Sizes. J. Comput. Graph Stat. *25*, 971–979.

104. Fernandes, A.D., Reid, J.N.S., Macklaim, J.M., McMurrough, T.A., Edgell, D.R., and Gloor, G.B. (2014). Unifying the analysis of high-throughput sequencing datasets: Characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome *2*, 1–13.

105. Fernandes, A.D., Macklaim, J.M., Linn, T.G., Reid, G., and Gloor, G.B. (2013). ANOVA-Like Differential Expression (ALDEx) Analysis for Mixed Population RNA-Seq. PLoS One *8*, e67019.

106. Mikryukov, V. (2022). metagMisc: Miscellaneous Functions for Metagenomic Analysis (Preprint).

107. Lahti, L., and Shetty, S. (2017). Tools for Microbiome Analysis in R.

108. Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504.

109. Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., Huttenhower, C., and Langille, M.G.I. (2020). PICRUSt2 for prediction of metagenome functions. Nat. Biotechnol. *38*, 685–688.

110. Doncheva, N.T., Assenov, Y., Domingues, F.S., and Albrecht, M. (2012). Topological analysis and interactive visualization of biological networks and protein structures. Nat. Protoc. *7*, 670–685.

111. Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics.

112. Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). circlize implements and enhances circular visualization in R. Bioinformatics *30*, 2811–2812.

113. Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (Springer-Verlag).

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
DNA/RNA Shield-Fecal Collection Tube	Zymo Research	Cat. No. R1101
PowerLyzer® PowerSoil® Kit	Qiagen	Cat. No./ID: 12855-50
Deposited data		
BaAka and Bantu intestinal microbiome data	MG-RAST	Accession number: 16608
Papua New Guinea and United States intestinal microbiome data	MG-RAST	Accession number: 4576511.3–4576572.3
Rural and Urban South African intestinal microbiome data	NCBI	Accession number: PRJNA589500
Ju|'hoansi intestinal microbiome	NCBI	Accession number: PRJNA1029329
Software and algorithms		
QIIME2	https://docs.qiime2.org/2021.2/	https://doi.org/10.1038/s41587-019-0209-9
SILVA-138-99 database	https://www.arb-silva.de/	PMID: 23193283
UNITE version 8 dynamic database	https://unite.ut.ee/	PMID: 30371820
R-4.2.1	https://cran.utstat.utoronto.ca/bin/windows/base/	N/A
Decontam	https://www.bioconductor.org/packages/release/bioc/html/decontam.html	https://doi.org/10.1101/221499
Phyloseq	https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html	PMID: 23630581
Tidyverse	https://cran.r-project.org/web/packages/tidyverse/index.html	https://tidyverse.tidyverse.org/articles/paper.html
Fantaxtic	https://github.com/gmteunisse/fantaxtic	N/A
Vegan	https://cran.r-project.org/web/packages/vegan/vegan.pdf	N/A
ALDEx2	https://bioconductor.org/packages/release/bioc/html/ALDEx2.html	PMID: 23843979
Cytoscape	https://cytoscape.org/download.html	PMID: 14597658
NetworkAnalyzer	https://apps.cytoscape.org/apps/networkanalyzer	https://doi.org/10.1038/nprot.2012.004
MCODE	https://apps.cytoscape.org/apps/mcode	PMID: 12525261
PICRUST2	https://huttenhower.sph.harvard.edu/picrust/	https://doi.org/10.1038/s41587-020-0548-6
ComplexHeatmap	https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html	PMID: 27207943
circlize	https://cran.r-project.org/web/packages/circlize/index.html	PMID: 24930139
ggplot2	https://cran.r-project.org/web/packages/ggplot2/index.html	https://doi.org/10.1080/15366367.201901565254
metagMisc	https://github.com/vmikk/metagMisc	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact and corresponding author, Riaan F. Rifkin (riaanrifkin@gmail.com).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

- All raw sequencing data have been uploaded to the NCBI under accession number PRJNA1029329.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The four Ju|'hoansi villages from which our fecal samples are derived are located 18 km–28 km (x = 23.3 km) from Tsumkwe, the primary village in the Otjozondjupa Region. Following informed consent, samples were acquired from an equal number of adult self-identified males (n = 20) and females (n = 20) ranging from 19 years to 69 years of age (median = 38 years). To make age a categorical variable, age was divided into two groups based on the median age of the participants.

These were collected in July 2019, during the winter (dry) season (i.e., from May to November), when foraging is less important. In winter, the Jul'hoansi subsists mainly by purchasing food from the various stores in Tsumkwe, including starches (e.g., maize, rice, and macaroni) and meat (i.e., beef and goat).

Research participants were recruited with the assistance of our co-researcher, research facilitator, and interpreter, Leon ≠Oma Tsamkxao, who is fluent in Jul'hoansi, Afrikaans, and English, and written informed consent was obtained from all participants.

Along with samples, metadata was collected to document (1) the ages of research participants, (2) their former use of antibiotic treatment for tuberculosis, (3) self-identified biological sex (i.e., male or female), (4) whether diarrhea is or had been experienced following the consumption of certain foods, 5) whether participants have ever experienced an intestinal infection, (6) their former or current use of malaria medication, (7) their exposure to local, regional and international travel, and (8) the villages of primary residence of each research participant.

All participants provided informed consent for publication of study results of the collected biomaterials, agreeing that all information required for the study (i.e., their location, biological sex, age, and medical history), except for their names, could be disclosed in this study. Ethical clearance for this research was obtained from the Research Ethics Committee, Faculty of Health Sciences at the University of Pretoria. All the research methods occurred in accordance with the Declaration of Helsinki.

METHOD DETAILS

DNA extraction and sequencing

Fecal samples were collected in collection tubes containing 9 mL DNA/RNA ShieldTM (Zymo Research Corp, Irvine, CA, USA) and stored at 4°C. After homogenizing the samples through vortexing, ~1 mL was transferred to a clean 2 mL tube, centrifuged for 5 min at 10,000 x g, and the supernatant was removed. The average weight of the resulting pellets was 125 mg, which was subsequently resuspended in 750 μL bead solution from the DNeasy PowerLyzer PowerSoil Kit (Qiagen GmbH, Hilden, Germany). The DNA isolation was performed according to the manufacturer's protocol, with the following adaptations: two rounds of bead beating (1 min at 4,000 rpm, PowerLyzerTM, Mo Bio Laboratories, Inc., Carlsbad, CA, USA) followed by 5 min incubation on ice, subsequently, bead beating tubes were centrifuged for 5 min. After the addition of Solution C6 (elution buffer), the spin columns were incubated at room temperature for 5 min before centrifugation.

Paired-end (2 x 300 bp) sequencing of the isolated DNA (V3-V4 16S rRNA for bacteria and ITS1 and ITS2 for fungi) was performed at Applied Biological Materials Inc., Richmond, B.C., Canada, using the MiSeq platform (Illumina, San Diego, CA, USA). Two controls were used in this study. CON-CTRL contained the DNA/RNA ShieldTM used to preserve the samples, while KIT-CTRL comprised the contents of the DNeasy PowerLyzer PowerSoil Kit.

Data pre-processing and quality control

Raw paired-end 16S and forward ITS reads were imported into QIIME2-2021.2.[36] Quality control with DADA2,[95] including denoising, dereplication, and filtering of chimeras, yielded 4,184 and 1,271 ASVs for 16S and ITS data, respectively. The 3′ ends of the 16S forward reads were truncated to a length of 292 bp, and 25 bp were trimmed from the 5′ end. The 3′ ends of the 16S reverse reads were truncated to a length of 250 bp, and 25 bp were trimmed from the 5′ end. ITS forward reads were truncated to a length of 297 bp at their 3′ ends, and 26 bp were trimmed from the 5′ end. The rest of the parameters were set to default. As initial ITS taxonomic classification of the ASVs resulted in the identification of very few taxa, ITS reads were first clustered at 98% sequence similarity using qiime vsearch[96] closed-reference clustering and then re-classified, which resulted in 167 OTUs.

16S taxonomic classification was performed by extracting V3-V4 regions from the SILVA-138-99 database[97] using q2 feature-classifier extract-reads based on the primer sequences used to amplify the 16S data. A naïve-Bayes classifier was then trained on the extracted SILVA sequences and full-length UNITE version 8 dynamic sequences[98] for 16S and ITS data, respectively. The classifiers were then used to taxonomically classify the respective datasets using the qiime fit-classifier naïve-Bayes plug-in.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data pre-processing

Following import into R-4.2.1,[37] the taxonomic, counts, and metadata tables were imported as phyloseq objects.[99] Six samples were identified as outliers due to either insufficient ASV/OTU count or very low diversity and removed from the analysis. Since analyzing the interaction between the fungal and bacterial IM necessitated equal sample sizes between the two groups, if an outlier was removed from one dataset, it was also removed from the other. The data was then inspected for contamination using decontam[38] at a prevalence threshold of 0.1. Decontam determines the likelihood of an ASV/OTU being a contaminant based on the prevalence of that ASV/OTU between controls and true samples. The control samples were subsequently removed. Phyloseq objects were converted to relative abundance and used in downstream analyses. Tidyverse[100] was instrumental in dataset manipulation. Relative abundance was visualized with Fantaxtic.[101]

Community composition and differential abundance analyses

Community composition was analyzed using the vegan package.[102] The Kruskal–Wallis test was used to test α-diversity if there were only two-factor levels, and the Dunn test was used for more than two-factor levels. β-diversity was tested with ANOSIM.

Differential abundance was tested using ALDEx2[103–105] by first filtering the data at a prevalence threshold of 0.1 using metagMisc[106] and then aggregating it to genus level. Two groups were compared at a time with mc.samples set to 16. Mc.samples was also set to 128 with no effect on results. All other ALDEx2 parameters were used at their default values. Genera were considered differentially abundant if their Benjamini-Hochberg corrected p values for Welch's t test were <0.1.

Elucidation of the Jul'hoansi core microbiome

The medium and soft core microbiomes were analyzed by selecting all ASVs/OTUs at a prevalence of 70% and 50%, respectively, and a detection threshold of 0.1%.[107] Relative abundances for ASVs/OTUs belonging to the same genus were collated and plotted as heatmaps with heatmap.2[107] in R. To investigate the soft core microbiome between Ju|'hoansi villages, the data was first divided into villages, then aggregated to genus level. Cytoscape[108] was used to generate the core microbiome network.

Metabolic enrichment of the Jul'hoansi IM

To obtain functional profiles of the Ju|'hoansi IM, data were exported from QIIME2-2021.2[36] and filtered to include only taxa that were prevalent in at least two individuals with a count of more than two reads, which resulted in 485 ASVs. This was used as input into the PICRUST2[109] full pipeline with default settings. Since PICRUST2 does not provide accurate functional enrichment of fungal data, we performed fungal functional prediction with FUNGuild.[41]

Differentially abundant pathways between multiple groups were assessed with the aldex.glm module in ALDEx2. Effect sizes were plotted for each village with the corrected Benjamini-Hochberg corrected p values.

Co-occurrence network between fungi and bacteria

A co-occurrence network was generated comprising consistently detected and highly abundant ASVs (16S) and OTUs (ITS) across all villages: the community data were filtered using only ASVs and OTUs with a relative abundance >0.5%. This filtering step resulted in a core community of 834 bacterial ASVs and 91 fungal OTUs. Spearman correlations were calculated between all ASVs and OTUs in the filtered dataset (absolute abundances) with Benjamini-Hochberg FDR p value correction. Significant relationships with a correlation coefficient (ρ) $\geq \pm 0.7$ and $p < 0.01$ were selected and translated into a network in Cytoscape.[108] The topological properties of the network were subsequently analyzed with the NetworkAnalyzer[110] tool. Modular structures and groups of highly interconnected nodes were identified using the MCODE[45] application with standard parameters. Taxa with the highest degree (>20) and betweenness centrality (>0.02) values were considered keystone taxa as determined by scatterplots.

Global IM comparison

To compare the Ju|'hoansi core IM with that of a global cohort, we downloaded quality-controlled sequences from MG-RAST using the accession numbers 4576511.3–4576572.3[49] (PNG and USA) and 16608[17] (BaAka and Bantu). The South African fungal IM was downloaded from the NCBI using accession number PRJNA589500.[50] In each case, the most processed data available were downloaded and further processed using the same workflow as the Ju|'hoansi data. This was done to minimize variability in workflow both between the respective authors and us and between the global populations and the Ju|'hoansi. The core microbiome of the global cohort was analyzed at the same detection and prevalence threshold as the Ju|'hoansi (0.1% and 50%, respectively) and compared. The global IM was first considered collectively and then as individual populations. Heatmaps were constructed using ComplexHeatmap,[111,112] and the IM network was constructed in Cytoscape.[108]

Ggplot2 was instrumental in figure creation.[113]