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Consumption of only wild foods induces large scale, partially persistent alterations to the gut microbiome

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The gut microbiome (GM) is implicated in human health and varies among lifestyles. So-called "traditional" diets have been suggested to promote health-associated taxa. However, most studies focused only on diets including domesticated foods. Historically, humans consumed only wild foods, which might uniquely shape GM composition. We explored the impact of a wild-food-only diet on GM, particularly whether it increases the presence of health-associated and/or "old friend" taxa, and if the alterations to GM are persistent or transient. One participant collected daily fecal samples and recorded daily food consumption over an eight-week period, the middle four weeks of which he consumed only wild foods. Samples were profiled by 16S rRNA sequencing, and oligotyping and network analysis were conducted to assess microbial co-occurrence patterns. A wild-food-only diet considerably alters the composition of the GM, and the magnitude of the changes is larger than that observed in other diet interventions. No new taxa, including "old friends" appeared; instead, the proportions of already-present taxa shifted. Network analysis revealed distinct microbial co-abundance groups restructuring across dietary phases. There is a clear successional shift from the pre-, during- and post-wild-food-only diet. This analysis highlighted structural and functional shifts in microbial interactions, underscoring diet's role in shaping the gut ecosystem.

Keywords Paleodiet, Old friends, Wild foods, Gut microbiome

The gut microbiome (GM) is implicated in maintenance of human health[1,2]. The composition of this microbial community varies among human populations[3] and is strongly driven by differences in diet, lifestyle and living environment, and less by underlying host genetic differences[4]. More specifically, populations who consume food that is mass-produced, highly-processed and rich in fat and sugar, who have access to healthcare including antibiotics, and whose living and working spaces are often highly cleaned, have GM communities that are characterized by reduced taxonomic diversity and increased prevalence of certain taxa that are associated with inflammation and immune-mediated diseases, such as enterobacteria and mucus degraders[5]. Such populations are often labeled as having a "Western" or "industrialized" lifestyle, contrasting with "rural" or "traditional" lifestyles, which are associated with GM profiles characterized by wider biodiversity, and taxa with healthy functions, such as Prevotella and other bacteria that produce short chain fatty acids (SFCAs)[6].

Given the potential health benefits of a more diverse GM, several studies have explored how diet alterations might change GM profiles within individuals instead of among populations. Individuals accustomed to a "Western" diet and lifestyle who then relocate to an area with a more "traditional" way of life quickly acquire a more diverse GM with health-associated taxa[7]. The inverse is also true[8]. Even in the absence of a geographic change, changes to diet alone can cause significant alterations to the GM[9,10], including increasing biodiversity and the ratio of health-associated taxa. However, these diet intervention studies so far have generally remained within the realm of "Western" diets, which rely on foods from a restricted range of domesticated plants and animals. For most of the evolutionary history of our species, humans consumed only wild plant and animal

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foods. While the actual food items varied through time, both seasonally and over millennia, and across the many habitats in which our ancestors lived, the general composition of the diets was strikingly different from the diets consumed today, even among most "traditional" populations[11]. For example, meat from wild game has less saturated fats and more unsaturated fats than does meat from domesticated animals, while wild plants generally contain more fiber and fewer simple sugars, are are overall more variable than domesticated crops[11]. Given the link between diet and GM, and between GM and health, some have argued that returning to a diet like those of our ancestors might have health benefits. We therefore explored, in a single individual, the consequences of a wild-food-only (WF) diet on GM composition. Fecal sampling was performed daily over 8 weeks, the middle four weeks of which the participant consumed only wild food items. This is not a study of the modern PaleoDiet[12], which relies on domesticated foods and combinations of food items that would never have been available to any human ancestor in one place at one time[11]. Instead, we focused on wild (non-domesticated) foods that were available in autumn in northern Europe. Furthermore, this study isolated the effect of diet compared to other factors, such as hygiene and exposure to other people. The test subject remained living in his own house, interacting with family members, and performing other normal daily activities.

In particular, we sought to explore the following three questions. First, does adopting a wild-food diet influence GM composition and the prevalence of health-promoting species? Second, are there any old friend species that increase in abundance during the wild-food diet? Old friend species are taxa that likely have been part of the human GM in our ancestors well prior to the adoption of agriculture[13], but that are regularly not found in "Western" populations[3,14,15]. Given their long history with humans, we expect them to prefer diets like those seen in hunter-gatherer populations. Finally, do the microbiome modifications persist even after returning to a normal diet? Previous studies indicate that some elements of the GM community reverted to the previous composition, while others remained in the altered state[2].

Materials and methods

The participant, who is an experienced forager of local wild foods, collected daily stool samples during an 8-week period from 2018–09-14 until 2018–11-08. The first two weeks consisted of a normal diet, followed by four weeks of a wild-foods diet, and a further two weeks of a return to a normal diet. The wild foods were usually prepared using "primitive" technologies—they were cooked on an open, wood fire and processed using grindstones and flint flakes instead of modern kitchen utensils, with the exception of a meat grinder. Other aspects of the author's lifestyle remained unchanged; he performed his usual daily activities, continued living in his house, and interacted as usual with family members. In short, this study isolated the effects of a dietary alteration. The author is of Dutch ancestry, and at the time of the experiment was 46 yo, 1.82 m tall, and weighed approximately 76 kg. The author's weight was measured daily, and he had daily contact with a medical doctor to monitor his health and well-being during the experimental stage. All consumed food and beverage items, except water, were recorded in a daily food log. The food items in the normal diet and in the wild-food diet are listed in Supplementary Table 1 together with the correspondent average number of portions per day. Ethical evaluation of the project was conducted by the Ethics Committee of the Faculties of Humanities and Archaeology at Leiden University (Letter number 2022/23), and the specific informed consent has been obtained from the participant.

Fecal collection, DNA extraction and 16S rRNA amplicon sequencing

Fecal samples were self-collected using Fe-Col® (Alpha Laboratories Ltd, Eastleigh, United Kingdom), a disposable paper device to prevent sample contamination, and SMART eNAT® (Copan SpA, Brescia, Italy) for fecal sampling and preservation. These were sent on ice to the laboratory of the Unit of Microbiome Science and Biotechnology at the University of Bologna for further analysis. All specimens were stored at -20 °C until processing. Total microbial DNA for each fecal sample was extracted through a method combining bead-beating and column purification, as described previously[16].

The V3–V4 hypervariable regions of the 16S rRNA gene were amplified and library preparation was performed following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina) and the Nextera technology to index libraries. Indexed libraries were pooled at an equimolar concentration of 4 nM, denatured, and diluted to 5 pM prior to sequencing on an Illumina MiSeq platform with a 2×250 bp paired-end protocol (Illumina, San Diego, CA, USA). Sequencing reads were deposited in the European Nucleotide Archive (ENA) with the BioProject ID PRJEB83090.

Bioinformatics and biostatistics analysis of GM data

All sequences were processed using a pipeline that combined PANDASeq[17] and QIIME 2[18]. After filtering the reads by length and quality, DADA2 was used to identify the amplicon sequence variants (ASVs)[19]. Taxonomic classification was performed using the VSEARCH algorithm[20] on the SILVA database (December 2017 release)[21]. Chloroplast, mitochondria, unknown, and eukaryote sequences were removed. Oligotyping[22] was then used for clustering the high-quality filtered fasta sequences from the QIIME 2 pipeline as previously illustrated by de Goffau and colleagues[23]. In particular, we used the 'Minimum Entropy Decomposition' (MED) option for sensitive partitioning of high-throughput marker gene sequences from the oligotyping software with the options -M 100 (to define the minimum abundance of an oligotype) and -V 2 (to define the maximum variation allowed in each node). The final node representative sequence of each oligotype was used for species profiling using the VSEARCH algorithm and the Genomes from Earth's Microbiomes (GEM) catalog[24] as reference database. Alpha diversity was calculated using the number of observed ASVs, the Shannon index and the Faith phylogenetic diversity index. For beta diversity, the UniFrac dissimilarities were used to construct Principal Coordinates Analysis (PCoA) plots.

Biostatistics analysis and graphical representation were performed in R using the base, vegan[25] and made4[26] packages. Data separation in the PCoA was tested using a permutation test with pseudo-F ratios (function

adonis in vegan). Kruskal–Wallis tests and Wilcoxon rank-sum test were used to assess significant differences in alpha diversity and taxon relative abundance between groups. P-values were corrected for multiple testing using the Benjamini–Hochberg procedure. A false discovery rate (FDR) ≤ 0.05 was considered statistically significant.

Network analysis

Species-level bacterial co-abundance groups (CAGs) were identified as previously described[3,27,28]. Briefly, the associations among taxa were determined using the Kendall correlation test, visualized with an heatmap and a hierarchical Ward-linkage clustering based on Spearman correlation distance metrics. The network plots were created using Cytoscape[29]. Circle sizes were proportional to species- or genus-level abundance or overabundance, and connections between nodes represented positive (gray) or negative (red) significant correlations. Keystone species were identified taking into account the topology of the network and the relative abundance of each taxon. Specifically, keystone nodes were identified by looking at the combination of the highest values of closeness centrality, between-ness centrality and degree on Cytoscape as previously described[30,31] and selecting only the taxa with a mean relative abundance > 1%.

GM across lifestyle, dietary habits and geography

GM dynamics observed in this research were compared to the results from other studies on (i) travelers in a setting with a traditional diet and lifestyle[7], (ii) people that radically changed their diet to a completely plant-based or animal-based diet[9] and (iii) "Western" or "traditional" populations[3,15,32–42].

Data from[7] and[9] were directly downloaded from the Qiita website[43], selecting the tables "55,266", "63,513" and "63,516". Each table contained the OTU abundance obtained using the QIIME pipeline with the closed-reference approach and the Greengenes database (version 13_8). Only the longitudinal samples from travelers and from people that radically changed their diet to an exclusively plant-based or animal-based diet were retained. Samples from our study were reanalyzed using the same parameters reported into the Qiita website and then merged in a new table for subsequent analyses. PCoA, genus abundance superimposition to the multidimensional space and graphical representations were obtained using vegan.

For the meta-analysis using data from previous studies on subjects from different geographical locations following different subsistence strategies, we analyzed paired-end sequences using QIIME 2[18]. The sequences were taxonomically assigned using the feature-classifier "classify-hybrid-vsearch-sklearn" option, implemented into the VSEARCH options[20] of the QIIME 2 pipeline, followed by a "q2-feature-classifier" trained on the SILVA 138.1 database[21] previously processed with RESCRIPt[44] using the developer's instructions. The resulting abundance tables, one for each study included, were merged and rarefied, resulting in a total of 966 repository-derived samples[3,15,32–42] that were then included in the analyses along with the 57 samples generated in this work. The dataset included 407 individuals from present-day tropical and subtropical hunter-gatherer groups and 24 from Inuit tribes – most of which are undergoing a rapid transition away from their traditional hunter-gatherer diet toward a more "Western" diet – 51 individuals from rural groups practicing small-scale or subsistence agriculture from Africa, South America and Papua New Guinea, 38 individuals from Native American tribes, 12 urban Nigerians and 434 urban dwellers from North America, Europe and Asia.

The PCoA analysis to produce the beta diversity plot was performed using the vegdist function in vegan, computing Bray–Curtis distances on relative abundances at the genus level, considering only genera showing more than 0.2% of relative abundance in at least three samples. Compositional data were fit onto the ordination implementing the envfit function in vegan, and only genera with FDR-corrected p-values < 0.001 were plotted.

Functional inference of GM functions

KO (KEGG ortholog) gene abundances were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software[45] by applying the default parameters, including a Nearest Sequenced Taxon Index (NSTI) value of 2. Significant differences among periods are tested by Kruskall-Wallis test and represented by box plots. For beta diversity, the Bray–Curtis dissimilarity was used to build a PCoA graph and the separation verified with a permutational test with pseudo-F ratios (function adonis, in vegan).

Food frequency data

The individual participant kept a food diary during all three dietary periods. For ease of comparison, we then converted this into frequency/day, using an average portion of food for each category eaten for each day. To define the average portion, we used a practical and consistent approach based on common portion sizes and clear descriptions in the food record. The aim was to standardise the quantity of different foods so that food intake could be accurately compared over the defined dietary periods. Specifically, for starch-rich foods such as bread, crackers, croissants, rolls, and naan, one portion was typically defined as one slice. For grains, cereals, chestnuts, acorns, burdock root, and seeds like yellow water lily seeds, a portion generally corresponded to one bowl (approximately 250–300 g). When defining portions for meat and fish, we used standard servings typically corresponding to approximately 100–150 g of cooked meat or fish. For instance, a clearly identifiable serving such as one hamburger, a fillet of plaice or cod, several slices of sausage, or a portion of venison was counted as one portion. For dairy products and eggs, portion sizes were defined based on common measurements. Specifically, one portion included one glass (approximately 250 ml) of milk, buttermilk, kefir, or chocolate milk; one standard serving of yogurt (approximately 150–200 g); one egg (either fried or boiled); or one standard slice (about 25–30 g) or small piece of cheese (such as Brie, feta, or cow cheese). Butter portions were considered based on typical amounts spread on bread or crackers (approximately one teaspoon per serving). Fruits and vegetables were measured according to common units: one portion corresponded to one medium-sized fruit (e.g., one apple, one pear, or one mandarin), a small bowl or serving of cooked or raw vegetables (approximately

100–150 g), or one handful of smaller fruits and dried fruits (such as grapes, dried raspberries, or raisins). For leafy greens and wild edible plants like ground elder or nettle, one portion represented roughly one handful or a standard serving used as part of a meal. In the mushrooms and nuts category, one portion was typically defined as a handful (about 30 g) of nuts such as walnuts, hazelnuts, almonds, or peanuts, or a small bowl of mushrooms or mushroom dishes. Peanut butter was counted as one portion per typical serving spread on one slice of bread or cracker (approximately one tablespoon). Processed foods and sweets were portioned by individual units. This included one individual cookie, one stroopwafel, one serving of cake or pie, one bar or piece of chocolate, one serving of pizza (usually indicated clearly as slices or entire pizza), and similar snack items. Beverages like fruit juices and chocolate milk were portioned per glass (approximately 250 ml). For fats and condiments such as oils, vinegar, curry, mustard, and teas, portioning was defined per typical household usage. For instance, one portion represented the typical daily usage of oil in cooking (approximately one tablespoon per cooking event), vinegar or mustard used per salad or meal preparation, and one cup (approximately 250 ml) of tea or herbal infusions.

Results

Individual experience of the wild-food diet

The main staples of the wild-food diet were chestnuts and acorns, which were usually ground to make porridge. These were supplemented by a few other nuts and seeds (hazelnuts and water lily seeds), a variety of fresh greens, dried berries and fruits, and a small amount of deer meat and ocean fish. During the wild-food period, the participant lost 4 kg, with the greatest loss during the first week of the wild-food diet. Two kg were quickly regained upon returning to a normal diet. Subjectively, the participant became bored with the limited foods available to him, as there was little time to prepare more than very basic meals or to gather foods from a wider area. This likely contributed to the overall caloric reduction and weight loss. During this period, the participant kept a vlog of his experiences, which is available on Youtube[46]

Characterization of the GM during the three dietary periods

In the pre-wild-food period, the GM was dominated by taxa belonging to the three major human-associated phyla, *i.e.*, Firmicutes, Bacteroidetes and Actinobacteria. In particular, the most representative families were *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Oscillospiraceae*, *Rikenellaceae* and *Bifidobacteriaceae* (Fig. 1A), which are commonly found in healthy people living a "Western" lifestyle[47]. Beta-diversity analysis revealed a clear pattern towards segregation of the microbial communities according to the sampling period, as shown by the unweighted and weighted UniFrac distances (permutation test with pseudo F-ratio, p-value ≤ 0.001) (Fig. 1B-D). During the wild-food period, the GM configuration became significantly enriched in *Lachnospiraceae*, *Streptococcaceae*, *Erysipelatoclostridiaceae*, *Butyricicoccaceae*, and *Eggerthellaceae* and depleted in *Bifidobacteriaceae*, *Rikenellaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Dialisteraceae*, *Acutalibacteriaceae*, and *Peptostreptococcaceae* ($P < 0.05$, Kruskall-Wallis test) (Fig. 1E, see also Supplementary Table 2 for further details). Most of the modifications observed in the wild-food period returned to initial relative abundance values in the post-wild-food period, except for *Bifidobacteriaceae*, *Rikenellaceae*, *Oscillispiraceae*, and *Dialisteraceae* that remained at comparable levels to during the wild-food period ($P < 0.05$). The *Akkermansiaceae* family was even further enriched in the post-wild-food period compared to the two previous periods ($P < 0.05$). In exploring differences in alpha diversity among periods, we observed a gradual increase of biodiversity from the pre-wild-food period, to wild-food and post-wild-food periods ($P < 0.05$, Kruskall Wallis test, Fig. 1F), indicating that the intervention had an effect on the microbiome structure even after its conclusion.

These changes in the proportions of individual taxa were also mirrored by changes in clusters of co-associated bacteria, which is unsurprising given the high level of interdependence within the GM. To characterize these clusters of bacteria, we generated a heatmap based on the Kendall's tau correlation coefficients between the different 57 genera and species with a minimum relative abundance of 0.1% in at least 20% of samples. We clustered correlated bacterial species into six co-abundance groups (CAGs), indicated by different colored squares, whose relationships are represented by a Wiggum plot, where species/genus abundance is proportional to the circle diameter (Fig. 2a,c). The dominant taxa for each CAG were *Blautia* (gray), *Streptococcus* (blue), *Coprococcus comes* (green), *Erysipelatoclostridium* (yellow), *Faecalibacterium prausnitzii* (cyan) and *Ruminococcus bicirculans* (pink). The topological data analysis indicated that *Faecalibacterium prausnitzii* and *Blautia* are the two taxa with the highest combination of 1) closeness centrality (0.46 and 0.45, respectively), 2) betweenness centrality (0.04 and 0.03, respectively) and 3) degree (15 and 11 respectively), with a mean relative abundance > 1%. For these reasons they were reported as keystone taxa for the microbial community. The CAGs changed in relative abundance across the three dietary periods (Fig. 2b). The overabundance plots of the CAG members in the 3 dietary periods showed the emergence of different patterns of correlated microorganisms, which were found to be associated with the dietary periods (*i.e.*, pre-wild-food, wild-food and post-wild-food; Fig. 2d). In particular, the GM of the pre-wild-food period was characterized by a *F. prausnitzii*-centered CAG, with several co-abundant glycan degraders, such as *Bacteroides* spp. (pectin, mannan, glucan, mucin) and *Bifidobacterium* (milk oligosaccharides)[48]. One auxiliary CAG was closely correlated to these bacteria and included *R. bicirculans*, *Dialister invivus*, *Bacteroides stercoris*, *Romboutsia timonensis* and CAG-83 taxon of the *Oscillospiraceae* family, which are eclectic bacteria with different substrate propensities[49–53].

Conversely, the wild-food-type GM was found to be centered around the *Blautia* CAG, which included a plethora of well-known fiber-degrading and SCFA-producing bacteria, such as taxa within the *Coprococcus eutactus* group, *Agathobaculum butyriciproducens* group and *Lachnospira rogosae* group, as well as *Blautia*, *Anaerostipes hadrus*, *Fusicatenibacter saccharivorans* and *Lachnobacterium bovis*[54,55]. Strongly associated to this cluster, the increase in members of the *C. comes* CAG and the concomitant presence of all the other CAGs enriches the wild-food group with a wider metabolic potential than in the previous period.

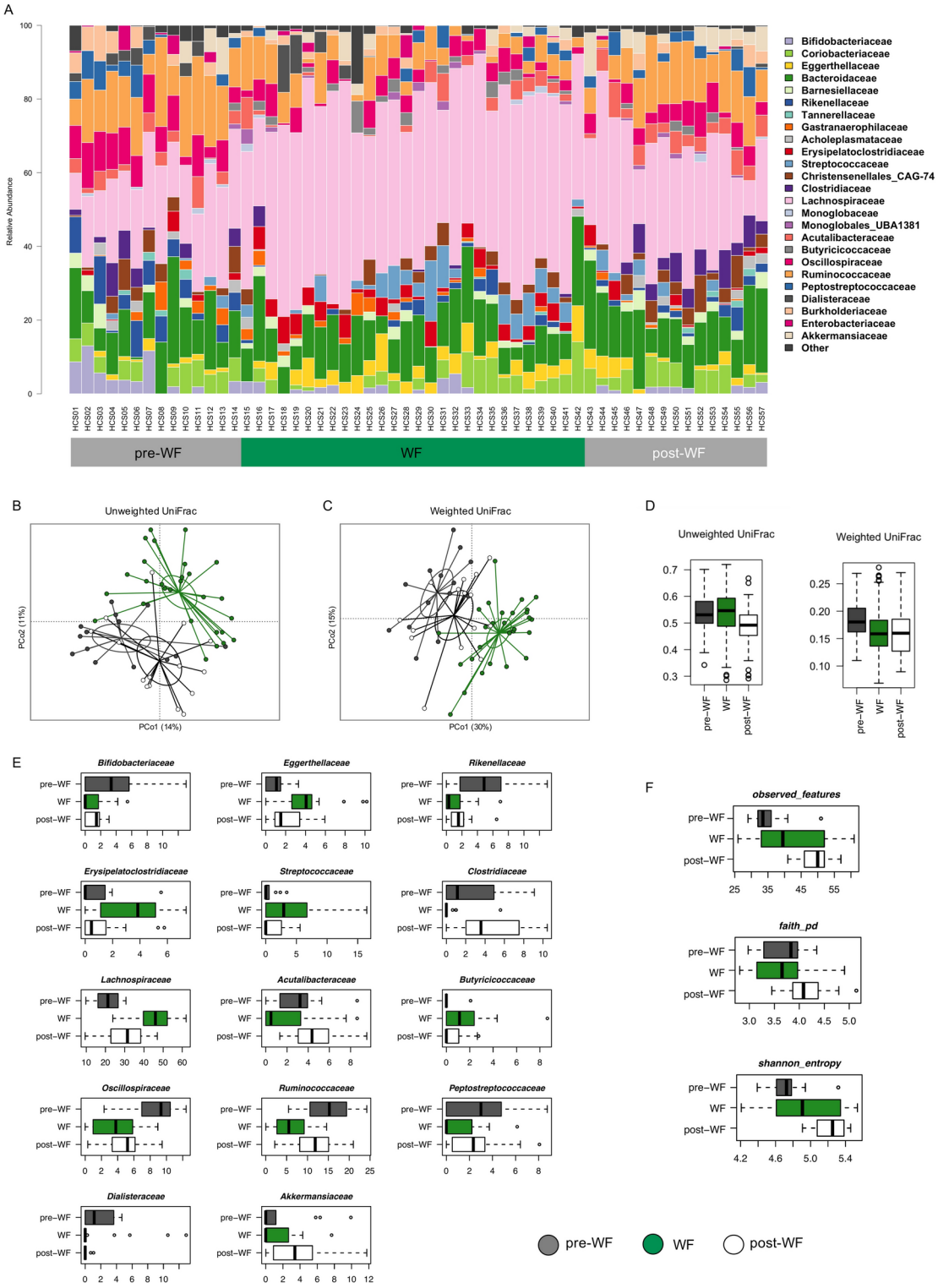

Fig. 1. Individual experience of wild-food (WF) diet is associated with a gut microbiome shift. Comparison of microbial communities between fecal samples from pre-, during- and post-wild-food periods (pre-WF, WF and post-WF in the figure), represented by barplots of the family-level relative abundances (**A**), PCoA plots based on unweighted and weighted UniFrac distances (**B**,**C**), and boxplots for intra-group distances (**D**), bacterial families (**E**), and alpha-diversity (**F**). Only bacterial families that showed a significant difference in terms of relative abundance among groups are represented ($P < 0.05$, Kruskall-Wallis test).

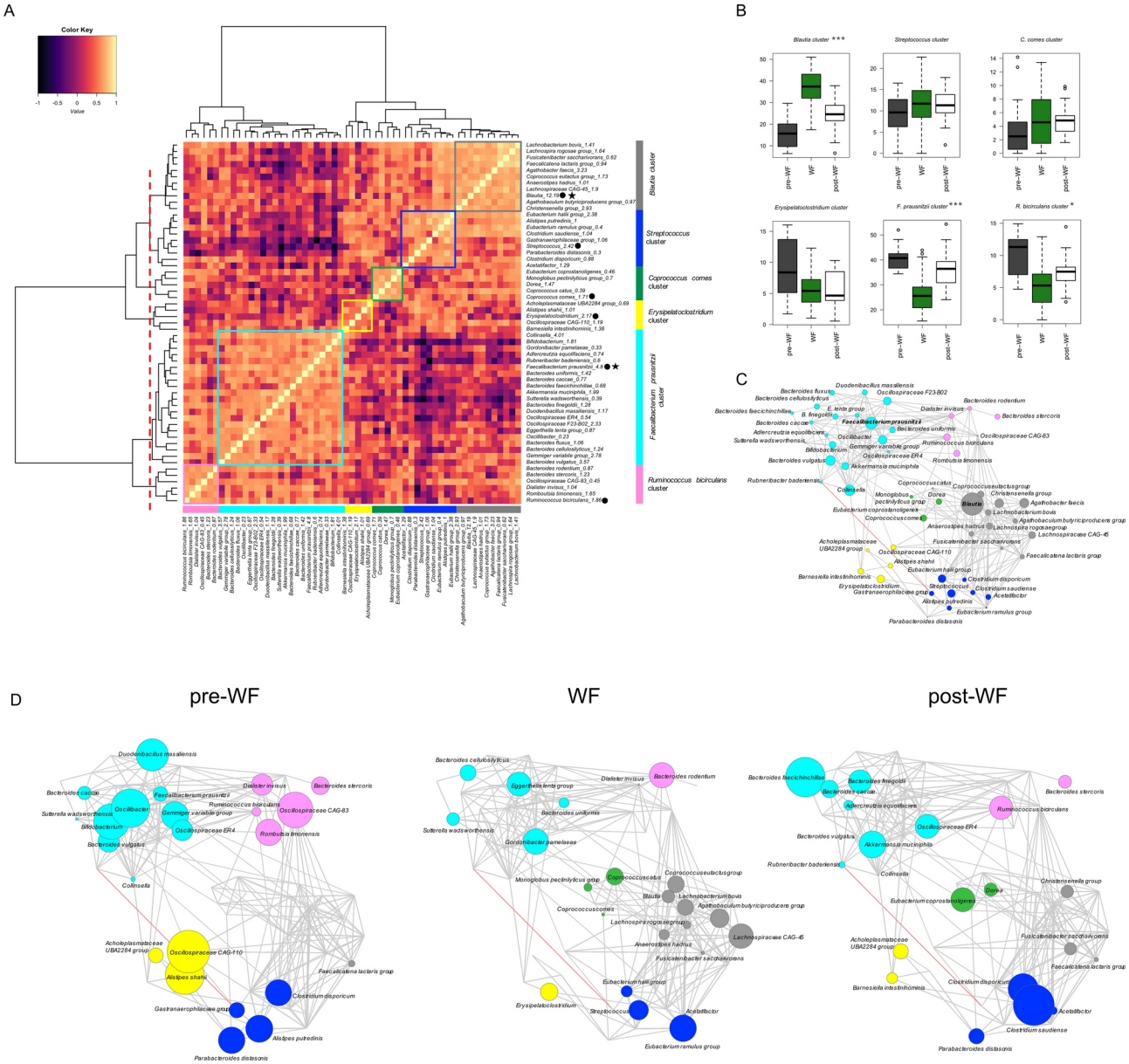

Fig. 2. Co-abundance analysis highlights distinct bacterial networks characterizing the three dietary periods. (**A**) A network heatmap based on Kendall's correlation coefficient and gut microbiome data was generated using the most abundant taxa at the genus or species level across all samples. The most dominant clusters identified are highlighted by different colored boxes and were confirmed by permutation tests with pseudo-F ratios ($P < 0.05$, adonis of the R package vegan). One setting was used for cluster analysis (red dashed lines), which identified six clusters. The *F. prausnitzii* cluster is highlighted in cyan, the *R. bicirculans* cluster in pink, the *Erysipelatoclostridium* cluster in yellow, *C. comes* in green, *Streptococcus* in blue and *Blautia* in gray. The main representative taxa of each cluster are marked with a dot. The keystone taxa for the network structure, as highlighted by the network analysis of betweenness centrality, closeness centrality and degree, are marked with a star. The mean relative abundances for each taxon in the overall cohort are reported next to the taxon name. (**B**) Cumulative relative abundance of the different groups of taxa among the three periods (* $P < 0.001$, *** $P < 0.00001$; Kruskall-Wallis test). (**C**) Bacterial network scheme. Only significant Kendall's tau correlation coefficients were considered. The leading taxa in each network are highlighted. A positive correlation is shown with a gray line and a negative correlation with a red line. Disc size is proportional to the mean relative abundance in the whole cohort. (**D**) Network plots corresponding to the three periods from the whole cohort analysis, in which disc sizes indicate genus/species over-abundance compared to the average relative abundance in the whole cohort. Samples from pre-, during- and post-wild-food period are indicated in the figure as pre-WF, WF and post-WF respectively.

As expected, the post-wild-food period was characterized by an intermediate configuration between the pre-wild-food and the wild-food periods, suggesting a reapproach, although not complete, to the initial profile. Indeed, we observed a strong increase of members of the *F. prausnitzii* CAG, such as *Bacteroides* spp., to values comparable with the pre-wild-food period, together with the resilience of some members of the *Blautia* and *C. comes* CAGs. Notably, this period was also characterized by a higher abundance of the mucin degrader *Akkermansia muciniphila*, than the previous two periods.

Unlike the previous CAGs, the *Streptococcus* and the *Erysipelatoclostridium* CAGs did not show appreciable variations among the different dietary regimes, but only some relevant associations of specific members to each period. For instance, higher proportions of the proteolytic and animal fat-degrading taxa, such as *Alistipes shahii*, *Alistipes putredinis* and *Clostridium disporicum* were representative of the pre-wild-food period, whereas higher abundances of the SCFA producers *Eubacterium hallii*, *Eubacterium ramulus*, *Streptococcus* and *Erysipelatoclostridium* were characteristic of the wild-food period[9].

Collectively, the wild-food consumption caused a deep modification of the GM structure, but the GM nevertheless maintained a relevant level of inertia to partially return to the initial configuration when the participant resumed a normal diet. However, there were some traits that differentiated the GM between the pre- and post-wild-food periods: (i) some members of the *Blautia* and *C. comes* CAGs remained at higher abundances respect to the pre-wild-food period; (ii) the post-wild-food microbiome was characterized by new traits respect to the initial period, *e.g.*, the higher abundance of *A. muciniphila*.

Putative functional changes corresponding to the observed taxonomic variations were obtained by inferred metagenomics. Given the changes in individual taxa and CAGs, some of the functional aspects of the GM were altered during the wild-food period as well. The GM of the wild-food period showed a higher propensity for starch and atrazine degradation, and phenylalanine, tyrosine and tryptophan biosynthesis, compared to the other periods, as indicated by functional assignment of GM genes using the PICRUSt2 tool ($P < 0.05$, Kruskall-Wallis test) (Fig. 3). These changes seemed to mirror the changes to the diet that included a very heavy reliance on starch-rich nuts (acorns and chestnuts) and reduced consumption of animal products (limited meat and fish, and no poultry, dairy or eggs), which may have increased the need for amino acid biosynthesis. Phenylalanine and tyrosine are both common in milk, eggs, and some meat products, while tryptophan is common in eggs and meats. These food items were limited during the wild-food period. While atrazine has been banned in the EU since 2004, this herbicide is highly persistent in groundwater[56]. The location where the participant acquired the wild leafy greens included field borders and previous agricultural land. Herbicide-degrading microorganisms could be possibly acquired through the ingestion of food sources endowed with these specific microbiome components, allowing their adaptation to environments under xenobiotic threat of anthropic origin[57].

Diet-induced successional changes and "old friends"

The patterns observed above do not represent a stochastic change to the GM, but instead reflect a distinct successional pattern from pre-wild-food, to wild-food, to post-wild-food diets. The weighted proportion of species maintained, gained and lost in the temporal succession of paired samples revealed a constant ratio of species shared or newly acquired between two consecutive timepoints, while the proportion of species lost decreased slightly but significantly ($P < 0.05$, Wilcoxon test) in the wild-food and post-wild-food periods with respect to the pre-wild-food period (Fig. 4). This result indicates distinct successional dynamics of the GM after the dietary modifications, with the GM keeping more diversity during and after the wild-food intervention. In particular, we found an increase in the number of persistent species during and after the wild-food period (*i.e.*, species present in at least 70% of samples for each group). The pre-wild-food period was characterized by the constant presence of *F. prausnitzii*, *Collinsella*, *Bacteroides vulgatus*, *Blautia*, *Gemmiger variabile* group, *Christensenella* group, *Oscillispiraceae F23-B02*, *Oscillospiraceae CAG-110*, whereas the WF period was characterized by *Blautia*, *Christensenella* group, *Oscillospiraceae F23-B02*, *Bacteroides cellulosilyticus*, *Eggerthella lenta* group, *Erysipelatoclostridium, Lachnospiraceae CAG-45*, *C. eutactus* group and *E. hallii* group. Finally, the post-wild-food was characterized by a higher number of persistent species, with the constant presence of *F. prausnitzii*, *Oscillospiraceae*, *Collinsella*, *B. vulgatus*, *G. variabile* group, *Blautia*, *Christensenella* group, *Dorea*, *Barnesiella intestinihominis*, *Agathobacter faecis*, *Ruminococcus bromii* group, *A. muciniphila*, *R. bicirculans* and *Adlercreutzia equolifaciens*. Notably, the list of the resilient taxa of this latter period included both some taxa characteristic of the pre-wild-food group, consistent with the resumption of a normal diet, but also completely new taxa that emerged after the wild-food diet (see the paragraph above).

Despite these considerable changes to the GM communities during the dietary shifts, no old-friend taxa, *e.g.*, *Treponema*, *Prevotella* and *Succinivibrio*[3,14,15], increased during or after the wild-food diet. The GM changes almost exclusively involved the taxa already present into the microbial community, without the addition of new taxa. The most relevant aspect of the GM rearrangement during the transition from a normal to a wild-food diet was the switch of keystone species (*i.e.*, the most important taxa in defining the microbiome structure as highlighted by network analysis – see Methods for how we identified keystone taxa), from *F. prausnitzii* to *Blautia*. This supports the emerging interest in the *Blautia* genus, which has recently been proposed as a next-generation probiotic candidate, also due to its role in ameliorating inflammatory and metabolic diseases[54,58]. These changes were also associated with an overall rearrangement of butyrate-producing bacteria, from a configuration dominated by *F. prausnitzii* to a configuration where the contribution of *A. hadrus* and *E. hallii* were more relevant.

GM shift caused by wild-food diet is larger than in other dietary perturbations

Previous studies have explored the changes in GM structure in individuals commonly consuming a Western, industrial diet after adopting a new, more traditional diet while traveling[7], or shifting entirely to a plant-only or animal-only diet[9]. Interestingly, when compared to these, the shift in beta diversity between the initial "Western"

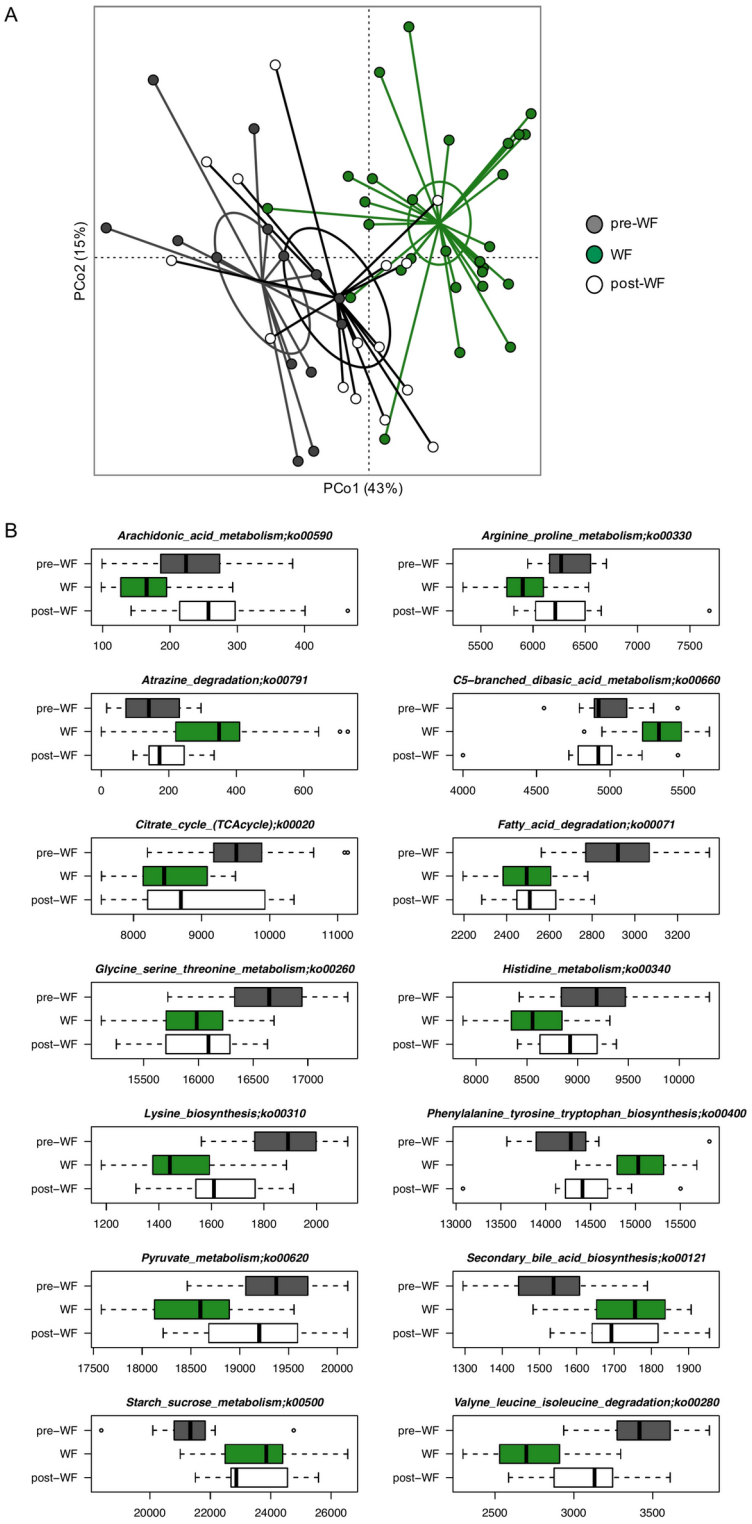

Fig. 3. Functional characterization from inferred metagenomes allowed identifying specific traits associated with the wild food experience. (**A**) PCoA using Bray–Curtis distances based on functional abundance of KO genes and (**B**) boxplots showing significant differences ($P < 0.05$; Kruskall-Wallis test) at pathway level of the KEGG orthology database, as inferred by PICRUSt2. Samples from pre-, during- and post-wild-food period are indicated in the figure as pre-WF, WF and post-WF respectively.

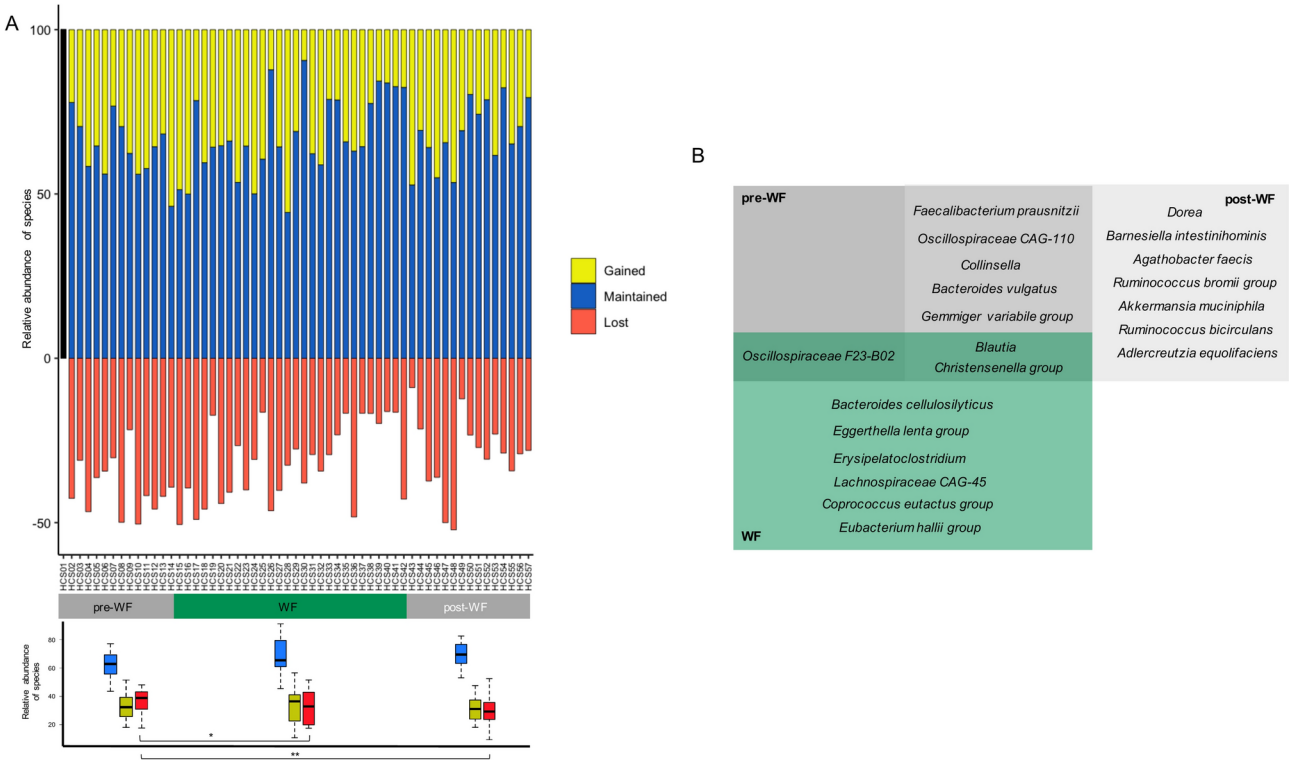

Fig. 4. Longitudinal succession of bacterial species through the three dietary periods. (**A**) Weighted proportion of species maintained, gained, and lost in the downstream member of a pair of neighboring samples (* $P < 0.05$, ** $P < 0.01$; Kruskall-Wallis test). The black bar represents the first sample. (**B**) List of species belonging to the core gut microbiome of pre-, during- and post-wild-food periods (pre-WF, WF and post-WF in the figure). The core microbiome was defined as species present in 70% or more of samples for each group.

diet and a wild-food-only diet was significantly larger (Fig. 5A-C, $P<0.001$ permutation test with pseudo F-ratio and Kruskall-Wallis test). Furthermore, we compared the GM configurations, specifically the genus-level relative abundance profiles, of our entire study to published data from hunter-gatherer, rural agriculturalist and urban-industrial communities[3,15,32–42]. The PCoA of Bray–Curtis distances showed a clear separation between traditional and urban-industrial GMs, consistent across the different studies (Fig. 5D). In addition, the samples from our study nested within the other urban-industrial populations, in an intermediate position between the majority of the urban-industrial samples and the GM from Native Americans and rural agriculturists. Together, our analysis highlighted how the changes in diet during the wild-food period instigated a rearrangement of the individual GM that did not alter in depth the microbiome structure, but acted more on the present species changing their relative abundance.

Discussion

Our study reveals three main conclusions. First, the adoption of a diet completely lacking in domesticated foods considerably alters the human GM, and these changes are much greater than those seen in other dietary perturbations. This was surprising, given the lack of other changes to the individual's lifestyle, including the environment and the general exposome, during this period. However, the adoption of a wild-food diet did not entirely alter a "Western" GM configuration to a "traditional" GM configuration, but represented a new and different arrangement of taxa. This suggests that despite considerable changes to the GM communities and relative frequencies, there remains strong inertia in the overall system, perhaps reflecting the portion of GM that is influenced by other lifestyle factors. There is some evidence for a rearrangement of health-associated taxa, including an increase in *Blautia* and the corresponding reduction in *F. prausnitzii* and *Bifidobacterium*, the latter known to be associated with the consumption of dairy foods[3]. The increase in *Blautia*, which has been linked to anti-inflammatory and metabolic benefits[59], may indicate a potential health-promoting effect of the wild-food diet, though further research is needed to confirm these associations. Together, these observations seem to corroborate the adaptive nature of the human GM, able to rearrange its compositional and functional layout keeping the homeostatic balance of the human holobiont in response to dietary shifts. Indeed, according to our findings, functional attributes of the GM community did change during the wild-food period, probably in response to changes in macronutrient profiles of the diet. Both the WF and the pre- and post-WF diets contained relatively small amounts of animal protein. However, the other micronutrients, including fat sources and sources of carbohydrates differed considerably, and the pre- and post-WF diets contained large amounts of dairy and

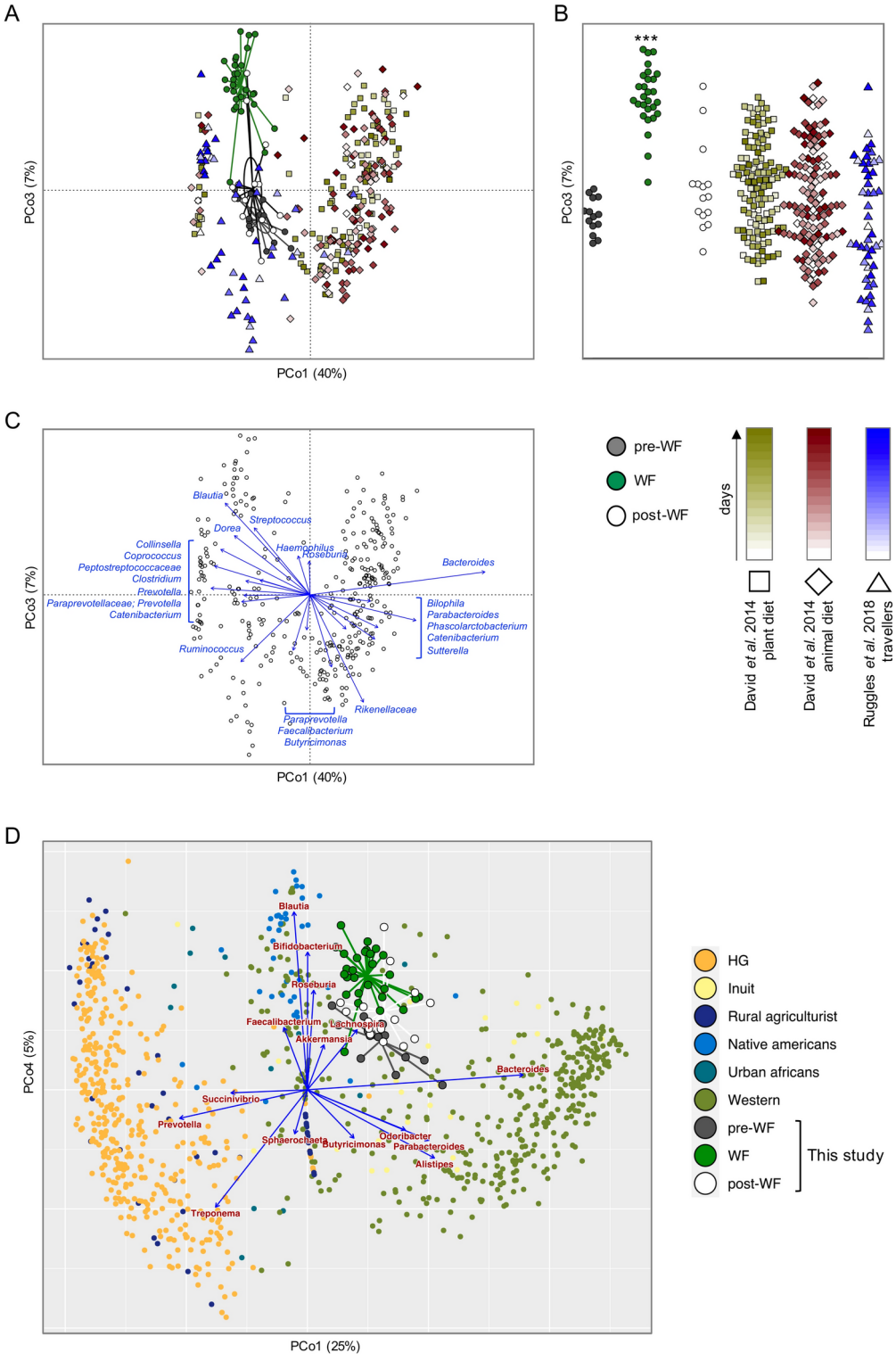

Fig. 5. Comparison with previous studies on gut microbiome variation related to diet and lifestyle. (**A**) PCoA based on Bray–Curtis distances of genus-level classification using data from (i) this study, (ii) travelers in a setting with a traditional diet and lifestyle[7] and (ii) people who radically changed their diet to a completely plant-based or animal-based diet[9]. (**B**) PCoA coordinates on the PCo3 axis discriminated microbiome configurations of the WF period from the rest of the samples ($P < 0.001$, Kruskall-Wallis test). (**C**) Superimposition of genus-level relative abundance on the same PCoA of Fig. 5A reveals the most important taxa leading to the observed separation on the microbiome space ($P < 0.001$). (**D**) PCoA based on Bray–Curtis distances of genus-level classification using data from this study and other works on gut microbiome characterization in populations with different geographic origin and lifestyle[3,15,32–42]. Blue arrows represent genus-level relative abundance superimposition on the PCoA space ($P < 0.001$, permutation test). Samples from pre-, during- and post-wild-food period are indicated in the figure as pre-WF, WF and post-WF respectively.

eggs. These differences in macronutrient composition may have driven some of the observed microbiome alterations, underscoring the strong diet-microbiome relationship.

Second, despite the striking changes to the GM, there is no evidence that old friend taxa increased in abundance during the wild-food period. These taxa were missing in the pre-wild-food period and did not appear during the dietary alteration. Instead, the changes we observed were limited to abundances of already-present taxa. This suggests that wild foods did not introduce new bacterial species into the GM community, despite the use of open-fire cooking and grindstones instead of modern kitchen utensils. This finding suggests that the contemporary GM, even under significant dietary modifications, may be constrained in its ability to reacquire lost ancestral microbial taxa. A more immersive interaction with the wild environment beyond only cooking might be necessary to facilitate microbiome shifts[60]. However, even this may not be sufficient due to the anthropization and modernization of environments which have led to reduced biodiversity and changes to environmental microbiome compositions[61,62].

Finally the adoption of a wild-food-only diet can induce persistent reorganization of the GM community. This altered GM profile in the post-wild-food period may reflect the flexibility of certain GM communities, and that there is a range of variation in taxa that are equally effective at assisting with digestion of normal diets. The persistence of these changes suggests that short-term dietary interventions may have prolonged effects on GM composition, though longer follow-up studies are required to determine the duration of these alterations.

These three main observations emerge clearly in our network analysis, which reveals the intricate interactions between microorganisms. It appears that changes in diet and associated lifestyle trigger a reconfiguration of microbial structure, as if the existing cooperative and competitive relationships drive a collective shift in configuration. However, our study design is limited, focusing on one individual for only a month. Furthermore, the potential effects of the participant's change in mood during the wild-foods diet on the GM remains unexplored, together with genetic predisposition and dietary history before such a study. Despite this, the degree of change associated with a wild-food-only diet was striking in its magnitude, even when compared to other severe dietary interventions such as in[9]. This suggests that wild and domesticated foods have widely divergent properties that can be better utilized by specific GM layouts. Future research should aim to investigate the metabolic and immunological consequences of these microbiome changes in a broader population, as well as their potential implications for long-term health.

Data availability

The sequences generated in the current study are available in the ENA repository under the project ID PRJEB83090.

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Author contributions

M.C. and A.G.H. designed the study, D.P. organised the experiment, M.B. extracted the DNA and performed the sequencing, S.R. and M.F. performed the bioinformatic analysis, S.R. and A.G.H. wrote the main text of the manuscript, S.T. revised and integrated the manuscript. All authors revised and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics

Ethical evaluation of the project was conducted by the Ethics Committee of the Faculties of Humanities and Archaeology at Leiden University (Letter number 2022/23). All methods were carried out in accordance with relevant guidelines and regulations.

Additional information

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