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Original Research Article

Good and bad dispositions between archaea and bacteria in the human gut: New insights from metagenomic survey and co-occurrence analysis

Francesco Candeliere [a], Laura Sola [a], Stefano Raimondi [a,b], Maddalena Rossi [a,b], Alberto Amaretti [a,b,*]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy
[b] Biogest-Siteia, University of Modena and Reggio Emilia, 41125 Modena, Italy

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ABSTRACT

Archaea are an understudied component of the human microbiome. In this study, the gut archaeome and bacteriome of 60 healthy adults from different region were analyzed by whole-genome shotgun sequencing. Archaea were ubiquitously found in a wide range of abundances, reaching up to 7.2 %. The dominant archaeal phylum was Methanobacteriota, specifically the family Methanobacteriaceae, encompassing more than 50 % of Archaea in 50 samples. The previously underestimated Thermoplasmatota, mostly composed of Methanomassiliicoccaceae, dominated in 10 subjects (>50 %) and was present in all others except one. Halobacteriota, the sole other archaeal phylum, occurred in negligible concentration, except for two samples (4.6–4.8 %). This finding confirmed that the human gut archaeome is primarily composed of methanogenic organisms and among the known methanogenic pathway: i) hydrogenotrophic reduction of CO_2 is the predominant, being the genus *Methanobrevibacter* and the species *Methanobrevibacter smithii* the most abundant in the majority of the samples; ii) the second pathway, that involved Methanomassiliicoccales, was the hydrogenotrophic reduction of methyl-compounds; iii) dismutation of acetate or methyl-compounds seemed to be absent. Co-occurrence analysis allowed to unravel correlations between Archaea and Bacteria that shapes the overall structure of the microbial community, allowing to depict a clearer picture of the human gut archaeome.

1. Introduction

A dense and diverse consortium of bacteria, archaea, fungi, protozoa, and viruses inhabit the human colon, constituting the resident gut microbiota [1–4]. Bacteria are by far the most abundant and studied community within the microbiome, to the point that the bacteriome has been identified with the microbiome itself for decades. Recently, increasing information on the abundance and diversity of microbes other than bacteria have been accumulating as well, even though the role in the ecology of the gut ecosystem, the interaction with other microbes and with the host, and the effects on human health of other microbial groups remain largely unexplored. For instance, knowledge on the abundance and diversity of human-associated Archaea is still extremely limited, and little is known about their functions and health effects.

The lineages of the domain Archaea are distributed within four main clades—i.e., the Euryarchaeota and the superphyla TACK (Thaumarchaeota, Aigarchaeota, Crenarchaeota, Korarchaeota, etc.), DPANN (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, etc.), and Asgard (Lokiarchaeota, Thorarchaeota, Odinarchaeota, Heimdallarchaeota, and Helarchaeota) —some of which are mostly composed of uncultured representatives [5]. Methanogenic Archaea, which are the only known producers of biotic methane, were originally recognized as environmental microorganisms of oxygen-depleted soils and sediments and have been acknowledged as host-associated microorganisms after studies on methane production in rumen, such as the incubation of plant material with intestinal contents of ruminants by Tappeiner in 1882 [6] and the isolation and characterization of *Methanobacterium ruminantium* in 1958 [7,8]. Methanogens, that are extraordinarily well-adapted to interact with animal hosts and non-archaeal components of their microbiomes, represent the main archaeal components of the gut microbiomes in ruminants and non-ruminants, including humans [9,

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* Corresponding author. Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy.
E-mail address: alberto.amaretti@unimore.it (A. Amaretti).

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10]. The methanogens of the human microbiome are mainly represented by the lineages of Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales, Methanosarcinales [11–14], with Methanobacteriales (e.g., *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) and Methanomassiliicoccales (e.g. 'Candidatus *Methanomassiliicoccus intestinalis*' and 'Candidatus *Methanomethylophilus alvi*') being the most prevalent and abundant. Besides methanogens, halophilic Euryarcheota (e.g. *Haloferax massiliense*, within Halobacteriales), members of DPANN, and Thaumarchaeota have also been detected in the human feces, and in some cases isolated [12,14,15].

Despite the most recent advances, the human gut archaeome is still understudied, mainly due to methodological issues that have long impeded the full assessment of this community, since these organisms are generally strictly anaerobic and fastidious in terms of nutritional requirements and isolation/cultivation conditions [12,16]. Even the advent of investigation techniques relying on next generation sequencing of amplicons for the profiling of microbial complex microbial communities, that gave great impulse to the knowledge of the diversity of the bacteriome, left the archaeal community largely neglected. Most of the so-called 'universal' 16S rRNA gene primers fail to cover part of the broad archaeal diversity, as they are unable to detect certain archaeal lineages that have thus remained underestimated [16]. Shotgun metagenomics provides a non-biased approach, as it does not involve 16S rRNA gene amplification [17] but requires adequate bioinformatic pipelines and computational resources for gene-level analysis and taxonomic assignation as well as the availability of well-annotated genomes [17–19]. A catalogue of Archaea inhabiting the human gut has recently been published utilizing genomes retrieved from metagenomes in order to expand the current understanding of the human archaeome [20]. However, a better understanding of the archaea comunity, that may affect the bidirectional communication between microbiome, is required. In that study, 27 archaeal species were identified, and only 9 of them had a cultured representative.

In the present study, 60 publicly available metagenomes of gut microbiota of healthy adults belonging to five geographically different cohorts were retrieved and analyzed for the abundance and the diversity of Archaea. The shotgun sequence reads were analyzed and taxonomically classified with the k-*mer*-based algorithm Kraken 2, using Unified Human Gastrointestinal Genome (UHGG) database v2.0.1 [21]. The composition profiles of both the archaeome and the bacteriome were analyzed with FastSpar with the aim to reveal the presence of statistically significant relationships between archaeal and bacterial taxa that could lead to conserved co-occurrence networks, thus shaping the structure of the microbiomes.

2. Materials and methods

2.1. Metagenomes

60 publicly available metagenomes of gut microbiota from healthy adults were collected from NCBI Sequence Read Archive (SRA), with the accession numbers listed in Suppl. Table S1 [22]. The subjects were ascribed to 5 cohorts from 5 different countries: China (n = 17), Ethiopia (n = 11), Spain (n = 9), Sweden (n = 16), and United States (n = 7), hereinafter referred to as CHN, ETH, SPA, SWE, and USA, respectively. The selected metagenomes were sequenced through whole-genome shotgun sequencing on Illumina paired-end platforms and produced reads ranging between 100 and 150 bp in length. Before taxonomy attribution, the FASTQ files were checked for quality and primers presence with FastQC v0.11.8 [23]. Trimmomatic [24] with ILLUMINACLIP setting was utilized to remove primers from ESP cohort.

2.2. Microbial composition and diversity

Metagenomic reads were processed with Kraken2 (default parameters) [18] and Bracken (default parameters, read count threshold: 10) [25] to assess both bacterial and archaeal compositions. Output files were rearranged with kraken-biom [26] to produce a feature table for the entire dataset. Kraken2 taxonomic assignation was performed using the UHGG database v2.0.1 [21]. Feature tables were imported into QIIME2 [27] to conduct alpha (Chao1, Shannon's index, Pielou's evenness) and beta (Canberra distance) diversity analysis. The statistical significance among groups was analyzed with PERMANOVA statistical test ($P < 0.05$).

LEfSe (LDA Effect Size) [28], ANCOM [29] and ALDEx2 [30] was used to identify features characterizing the differences between cohorts.

Co-occurrence networks were calculated using the FastSpar tool [31] that allowed to compute the SparCC correlation coefficient [32] and to pinpoint correlations between bacteria and archaea. The network was visualized with Cytoscape (v. 3.9.0). The correlations were filtered according to statistical significance ($P < 0.05$) and correlation strength ($r < -0.5$ or $r > 0.5$).

3. Results

3.1. Composition of the archaeome and the bacteriome

60 publicly available metagenomes of human gut microbiomes from different geographical origin (CHN, ETH, SPA, SWE, and USA) were analyzed (Suppl Table S1). Taxonomic analysis was carried out with Kraken2, that processed 2.7 billion reads (9.2–178 million reads per sample, mean = 44.2) and yielded successful classification of 2.3 billion reads within Archaea or Bacteria domains (8.1–154 million reads per sample, mean = 38.6). A total of 28 features was recognized within the domain of Archaea and 4601 within the domain of Bacteria.

Archaea were found in all the microbiomes, always in the minority compared to Bacteria, occurring with relative amounts of whole prokaryotes ranging from 0.001 to 7.2 % (Fig. 1A). The lowest relative abundance observed in a sample for a taxonomic unit was 4 × 10-4%. Accordingly, a limit of detection of about 10^7 cells g^{-1} of feces was roughly extrapolated, assuming a magnitude of microorganism concentration in the fecal samples of 10^{11} cells g^{-1} [33]. The lowest abundance of Archaea was found in a microbiome of ETH cohort (ETH-03), while the highest in the ESP cohort (ESP-44).

The phylum Methanobacteriota dominated the archaeome of the majority of the samples, encompassing >50 % of the Archaea in 50 out of 60 microbiomes. All the Methanobacteriota belonged to the family Methanobacteriaceae. The phylum Thermoplasmatota was the dominant archaeal phylum (>50 %) in 10 microbiomes and was ubiquitously found in all the others except one. It was composed essentially of members of the family Methanomassiliicoccaceae, with members of the family Methanomethylophylaceae scarcely represented. Halobacteriota was the sole other archaeal phylum, identified in N out of 60 samples, occurring, when present, in negligible concentration, except for samples ETH-03 and USA-55 where it reached 4.8 % of the archaeome. It encompassed members of the family Haloferacaceae and Methanocorpusculaceae.

Twelve genus-level taxonomic designations were recognized, 8 of which with acknowledged nomenclature: *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera* within Methanobacteriaceae; *Methanomethylophilus* within Methanomethylophilaceae; *Methanomassiliicoccus* within Methanomassiliicoccaceae; *Haloferax* and *Halorubrum* within Haloferacaceae; *Methanocorpusculum* within Methanocorpusculaceae. Among the 28 species identified, 12 presented binomial nomenclature: *Methanobrevibacter oralis*, *Methanobrevibacter smithii*, *Methanobrevibacter smithii* A (*Candidatus Methanobrevibacter intestini* according to Chibani et al. [20]), *Methanobrevibacter woesei*, *Methanosphaera cuniculi*, *Methanosphaera stadtmanae*, *Methanomethylophilus alvi*, *Methanomassiliicoccus intestinalis*, *Methanomassiliicoccus luminyensis*, Methanomethylophilaceae UBA71 MGYG000003962 A (*Candidatus Methanoprimaticola macfarlanii* according to Chibani et al. [20]), *Haloferax massiniliensis*, and *Halorubrum lipolyticum*. Altogether, these 11 species covered a very variable share of

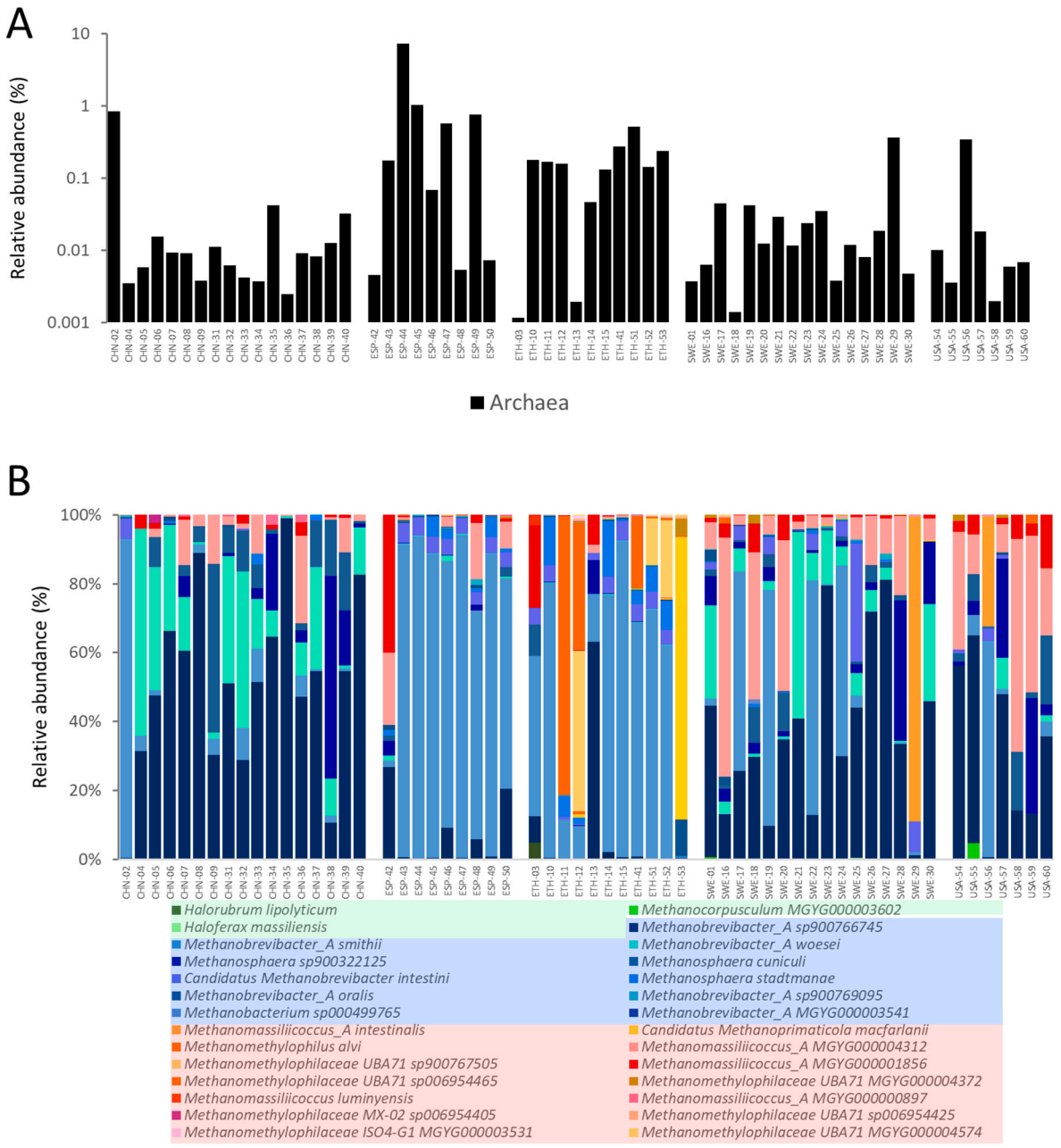

Fig. 1. Panel A: relative abundance of Archaea and Bacteria domains in 60 human gut microbiomes. Panel B: relative abundance of the species detected within the archaeome. Colors: Thermoplasmatota, red shades; Methanobacteriota, purple shades; Halobacteriota, green shades.

the archaeome, ranging from 0.8 to 99.5 %. The archaeal taxonomic units not identified at the species level were found within the Methanomicrobia Methanomicrobiaceae (5) and Methanocorpusculaceae (1) and within the Thermoplasmatota Methanomassiliicoccaceae (3) and Methanomethylophilaceae (8).

In general, the species with the greatest prevalence also presented among the greatest abundances. *Methanobrevibacter* A sp900766745 was ubiquitously found in all the archaeomes (0.13–98.9 %, mean = 30.6 %), followed by other species which also occurred in the majority of the samples: *Methanomassiliicoccus* MGYG000004312 (0.001–69.4 %, mean = 9.3 %, n = 54), *Methanobrevibacter woesei* (0.02–60.1 %, mean = 8.4 %, n = 52), *Methanobrevibacter smithii* (0.12–93.8 %, mean = 27.6 %, n = 49), *Methanosphaera* sp900322125 (0.005–58.9 %, mean = 4.8 %, n = 45), *Methanomassiliicoccus* MGYG000001856 (0.001–40.1 %, mean = 2.6 %, n = 45), *Methanosphaera cuniculi* (0.02–49.0 %, mean = 4.1 %, n = 43), *Methanobrevibacter oralis* (0.01–10.6 %, mean = 0.5 %, n = 33), *Methanosphaera stadtmanae* (0.02–16.3 %, mean = 1.4 %, n = 32), and *Candidatus Methanobrevibacter intestini* (0.15–34.3 %, mean = 2.5 %, n = 31). The following species reached a remarkably high abundance in one or few archaeomes, even though appearing with low prevalence: *Candidatus Methanoprimaticola macfarlanii* (0.02–82 %, mean = 1.4 %, n = 14), the Methanomethylophilaceae UBA71 sp900767505 (0.03–46.6 %, mean = 1.4 %, n = 5) and UBA71 sp006954465 (0.006–37.1 %, n = 0.7 %, n = 9), *Methanomassiliicoccus intestinalis* (0.004–88.6 %, mean = 2.0 %, n = 7), and *Methanomethylophilus alvi* (0.03–81.2 %, mean = 1.7 %, n = 7).

Within the whole prokaryotic community, *Methanobrevibacter smithii* was the sole Archaea with a remarkable abundance (mean = 0.2 %, max = 6.8 %), followed by *Candidatus Methanobrevibacter intestini* (mean = 0.01 %, max = 0.39 %) and *Methanomassiliicoccus intestinalis* (mean = 0.01 %, max = 0.32 %), all the other species presenting a max abundance always <0.2 %.

Within Methanobacteriota, *Methanobrevibacter smithii, Candidatus Methanobrevibacter intestini,* and the phylogenetically related *Methanobrevibacter* sp900766745 dominated the archaeome in most of the microbiomes, with the former two species dominating in ESP and ETH cohorts and *Methanobrevibacter* sp900766745 dominating in CHN, SWE and USA. Other species of Methanobacteriaceae, such as *Methanosphaera cuniculi* and *Methanobrevibacter woesei* were frequently identified across different archaeomes, in most cases with negligible amounts even though their abundance was higher in some samples (>1 %), especially belonging to the CHN cohort. Within Thermoplasmatota, *Methanomassiliicoccus_A* MGYG000004312 was frequently identified with higher concentrations, in particular in samples from USA and SWE cohorts. Other species belonging to Methanomassiliicoccaceae or Methanomethylophilaceae were generally found in negligible concentration, except *Methanomethylophilus alvi, Methanomassiliicoccus intestinalis* and Methanomethylophilaceae UBA71 sp900767505, that reached a concentration >1 % in the archaeome of few subjects.

In the bacteriome, the dominant phyla were Bacillota (former

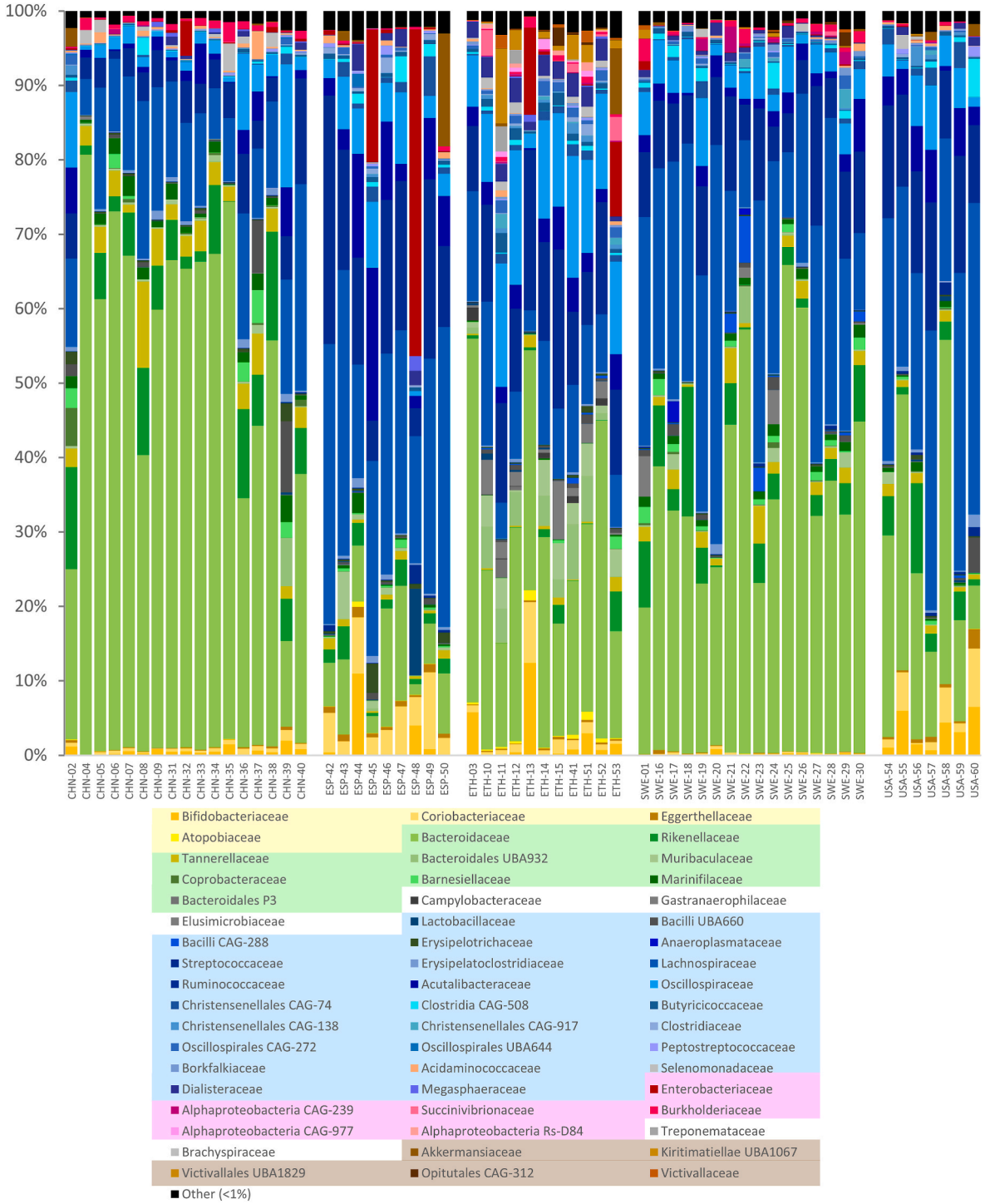

Fig. 2. Relative abundance of the families within the Bacteriome in 60 human gut microbiomes. The families that did not occur at least once with abundance >1 % were aggregated as "Other". Colors: Bacillota, blue shades; Bacteroidota, green shades; Actinobacteriota, yellow shades; Proteobacteria, pink shades; Spirochaetota and Verrucomicrobiota, grey shades.

Firmicutes) and Bacteroidota, in different ratio depending on the microbiome, followed by Proteobacteria, Actinobacteriota, and Verrucomicrobiota. The main bacterial families are outlined in the bar plot of Fig. 2.

3.2. Diversity of the archaeome and the bacteriome

Computation of alpha diversity metrics revealed that both the richness and the complexity of the archaeal community were lower than the bacterial one in all the cohorts (Suppl. Fig. 1), consistent with the higher number of features identified at all the taxonomic levels within the bacteriome. Significant difference in richness and complexity of the bacteriome was observed among the cohorts, with CHN generally presenting the lowest scores and ETH the highest. Significant differences among cohorts were observed also for the archaeome.

The beta diversity of the bacteriome and archaeome was computed utilizing Canberra metrics (Fig. 3). PERMANOVA analysis of significance revealed significant differences among cohorts ($P < 0.05$), with the cohorts mostly distributed along PCo1. SWE, ETH, USA, and CHN cohorts were separated in the four quadrants of the PCo1–PCo2 space, while ESP was more widespread, mostly laying between SWE and ETH. For the archaeome, computation of the beta diversity revealed that ETH cohort was the most distant. The other cohorts overlapped largely, nonetheless cohort grouping remained significant (PERMANOVA, $P < 0.05$).

LEfSe, ANCOM, and Aldex2 analysis was carried out in order to point out the features that characterized the archaeome and the bacteriome of the five cohorts, presenting a statistically significant differential abundance in one of them. ANCOM and ALDEx2 did not reveal any significant feature among Archaea (data not shown). On the other hand, LEfSe revealed that Methanobacteriaceae was the biomarker characterizing ESP cohort, while Methanomethylophilaceae and Methanomassiliicoccaceae characterized ETH and SWE cohorts, respectively (Fig. 4). 87 bacterial families presented a positive differential abundance in one of the cohorts (in particular in ETH cohort that encompassed 35 biomarker families), 38 out of the 87 appearing at least once >1 %. ETH

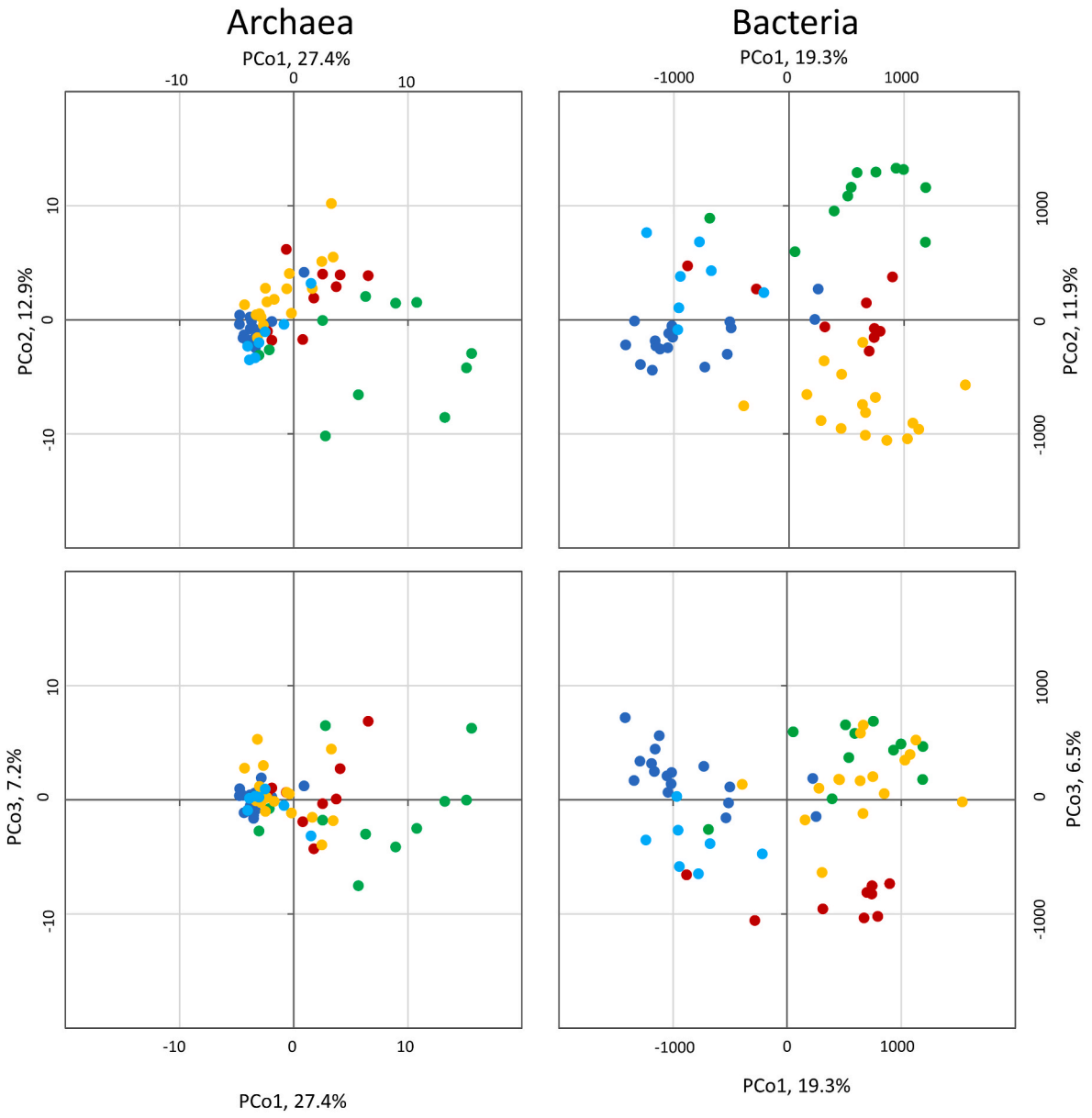

Fig. 3. PCoA plots of the beta diversity, calculated for archaeal and bacterial communities on rarefied feature tables, utilizing Canberra metrics. Colors: CHN, blue; ESP, red; ETH, green; SWE, yellow; and USA, cyan.

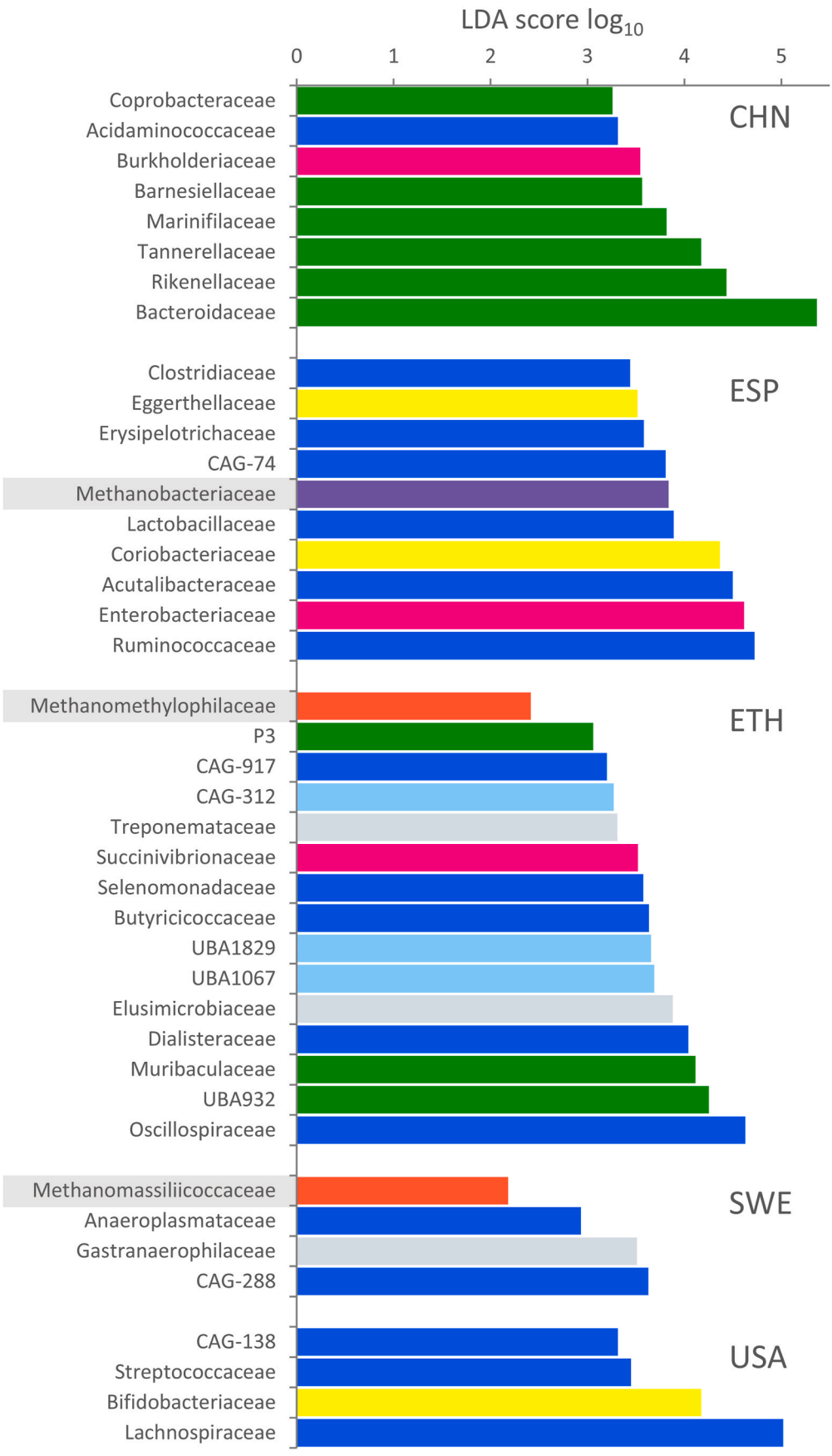

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Fig. 4. Linear discriminant analysis Effect Size (LEfSe) analysis of archaeal and bacterial families (plain and dashed bars, respectively) differentiating the microbiome cohorts, exhibiting significant differential abundance ($p < 0.05$, logarithmic LDA logarithmic score >2.0). Only bacterial families occurring at least once >1 % are reported. Archaea are highlighted. Families are colored according to the phylum: Thermoplasmatota, red; Methanobacteriota, purple; Halobacteriota, light green; Bacillota, blue; Bacteroidota, green; Actinobacteriota, yellow; Proteobacteria, pink; Spirochaetota, light grey; Verrucomicrobiota, dark grey.

cohort was characterized by some families of Bacillota (Oscillospiraceae, Dialisteraceae, Selenomonadaceae, Butyricicoccaceae and one family of Christensenellales), and by Succinivibrionaceae, Treponemataceae, Muribaculaceae, Elusimicrobiaceae, one family of Bacteroidales and 3 families of Verrucomicrobiota. CHN was characterized mostly by the Bacteroidota families (Bacteroidaceae, Marinifilaceae, Tannerellaceae, Barnesiellaceae, Rikenellaceae and Coprobacteraceae) and by Acidaminococcaceae and Burkholderiaceae, ESP by Bacillota families (Clostridiaceae, Erysipelotrichaceae, Ruminococcaceae, Lactobacillaceae, Acutalibacteraceae and one Christensenellales family) Actinobacteriota families Coriobacteriaceae and Enterobacteriaceae, and USA by Bifidobacteriaceae, Streptococcaceae, Lachnospiraceae and one family of Christensenellales.

3.3. Co-occurrence relationships between the archaeome and the bacteriome

Co-occurrence networks among archaea and bacteria were created at the family, the genus, and the species levels. Co-occurrence analysis revealed 51 significant interactions involving archaeal families ($P < 0.05$), with correlation coefficients ranging from −0.5 to −0.3 and from 0.3 to 0.58. Methanomassiliicoccaceae presented positive correlation with the families of Bacteroidota Rikenellaceae and with the Bacillota Lachnospiraceae, Ruminococcaceae, Erysipelatoclostridiaceae, and Streptococcaceae. In general, the bacterial families with a positive relationship with Methanomassiliicoccaceae presented a negative correlation with the other archaeal families Methanobacteriaceae, Methanomethylophilaceae, and/or Haloferacaceae. In addition, negative correlations with respect of Methanobacteriaceae,

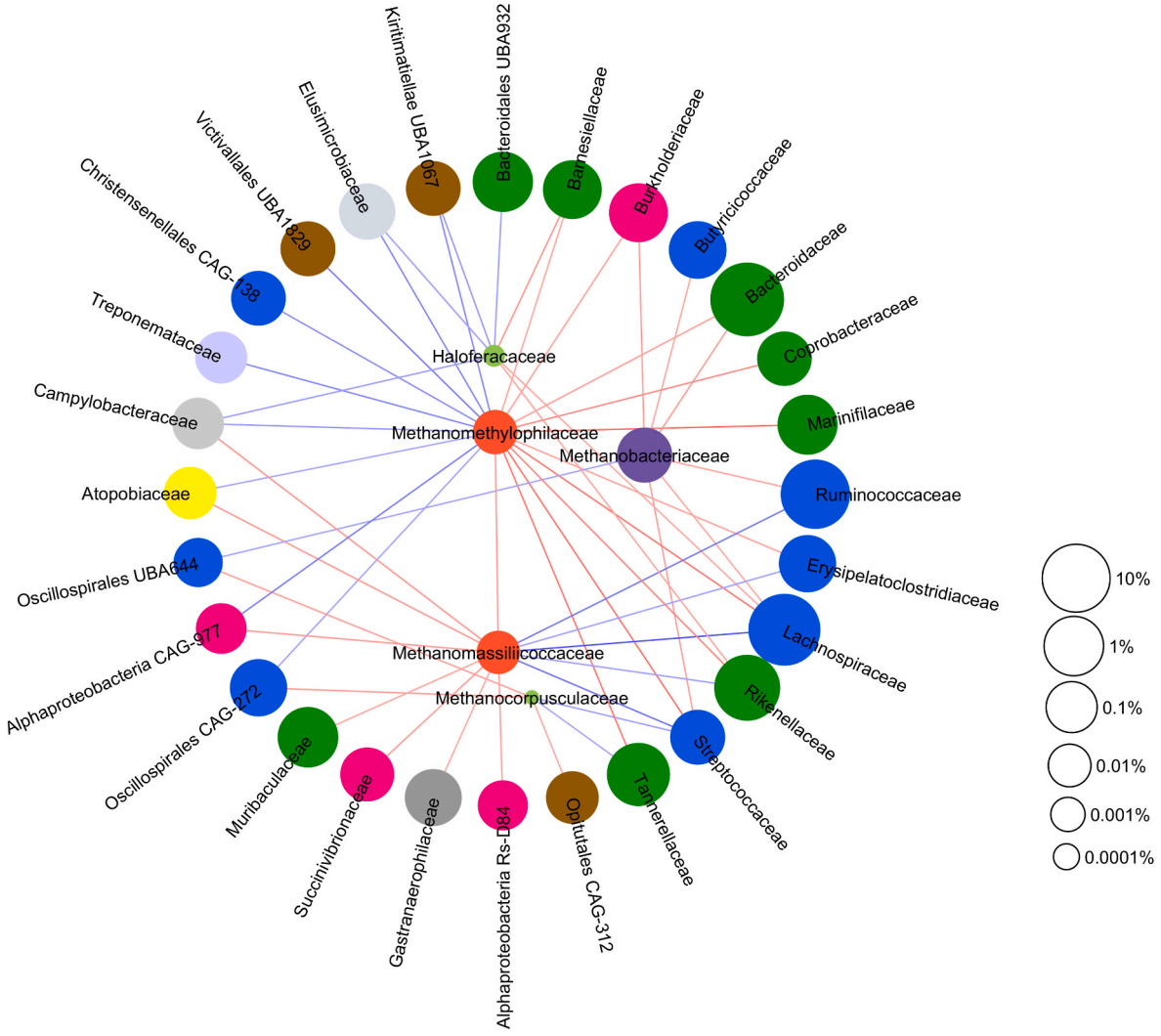

Fig. 5. Co-occurrence network of archaeal families. The diagram reports the significant co-occurrences ($P < 0.05$) with other archaeal families and with the bacterial ones occurring at least once >1 %. Edges represent co-occurrences from the most negative to the most positive, colored from the deepest red to the deepest blue, respectively. Nodes are families, sized according to their mean abundance and colored according to their phylum: Thermoplasmatota, red; Methanobacteriota, purple; Halobacteriota, light green; Bacillota, blue; Bacteroidota, green; Actinobacteriota, yellow; Proteobacteria, pink; Verrucomicrobiota, brown; Spirochaetota, light grey; Campylobacteriota, grey; Cyanobacteriota, dark grey; Elusimicrobiota, lighter grey.

Methanomethylophilaceae, and/or Haloferacaceae were found with the bacterial families Barnesiellaceae, Burkholderiaceae, Butyricicoccaceae, Bacteroidaceae, Coprobacteraceae, Marinifilaceae, and Tannerellaceae. Methanomethylophilaceae positively co-occurred with some unclassified families of Bacillota, in general negatively correlating with Methanomassiliicoccaceae. Haloferacaceae positively co-occurred with Elusimicrobiaceae, Camplylobacteraceae, and two unclassified Bacteroidota and Verrucromiocrobiota families. Methanobacteriaceae presented only one positive correlation with undefined Oscillospirales (Fig. 5).

The analysis of co-occurrences among genera revealed 102 significant interactions involving archaeal genera ($P < 0.05$), with correlation coefficients ranging from −0.5 to −0.4 and from 0.5 to 0.7. The most abundant archaeal genera, the Methanomassiliicoccaceae *Methanomassiliicoccus_A* and the Methanobacteriaceae *Methanobrevibacter_A* belonged to two different networks (Fig. 6). *Methanomassiliicoccus_A* positively co-occurred only with the genera of Bacillota *Acetivibrio_A*, *Frisingicoccus*, *Merdibacter*, *Anaerosaccharophilus*, *Stomatobaculum*, *Lactonifactor*, *Agathobacter*, *Fusicatenibacter*, *Mediterraneibacter_A*, *Monoglobales_A*, *Peptacetobacter*, and *Lachnoanaerobaculum*. *Methanomassiliicoccus_A* was in negative relationship with several unclassified families belonging to Bacillota, Bacteroidota, Methanobacteriota, and Proteobacteria phylum. *Methanomethylophilus* shared some negative correlations with *Methanomethylophilaceae ISO4-G1*, whereas *Methanomethylophilaceae UBA71* was negatively correlated with some important genera of Bacillota such as *Roseburia*. *Methanobrevibacter_A*, the most abundant archaeal genus, did not show any significant relationship with other archaeal genera or their bacterial correlation network. Instead, it exhibited three negative correlations with three unclassified genera of the Lachnospiraceae family. *Methanosphaera*, *Methanomethylophilaceae MX-02*, and *Methanocorpusculum* were also excluded from the main correlation network between archaea and bacteria, and individually presented negative relationship with some clostridial and erysipelotrichial Bacillota.

The analysis of species co-occurrences revealed that each archaeal species was involved in at least a thousand significant interactions ($P < 0.05$) with archaeal and bacterial counterparts. In total, 37032 significant interactions involving archaeal species, all with weak correlation coefficients, ranging from −0.2 to 0.3.

4. Discussion

The ecological functions of archaeal and their interactions with other bacterial taxa in the gut ecosystem have not been fully investigated so far. The knowledge of the role of these prokaryotes in carbon metabolism and nutrient cycling would provide a crucial contribution to understand the ecological role of these gut taxa, to assess their role in health and disease, and to promote development interventions targeted to a health promoting microbiota. The archaeome and bacteriome of the 60 healthy adults with different origin were studied, using a whole metagenome sequencing approach. Archaea were found in all the metagenomes herein analyzed. The Archaea represented a small portion of the intestinal microorganisms, in the vast majority laying below 1 % of the microbiome. This result is consistent with the mean of 1.2 % of

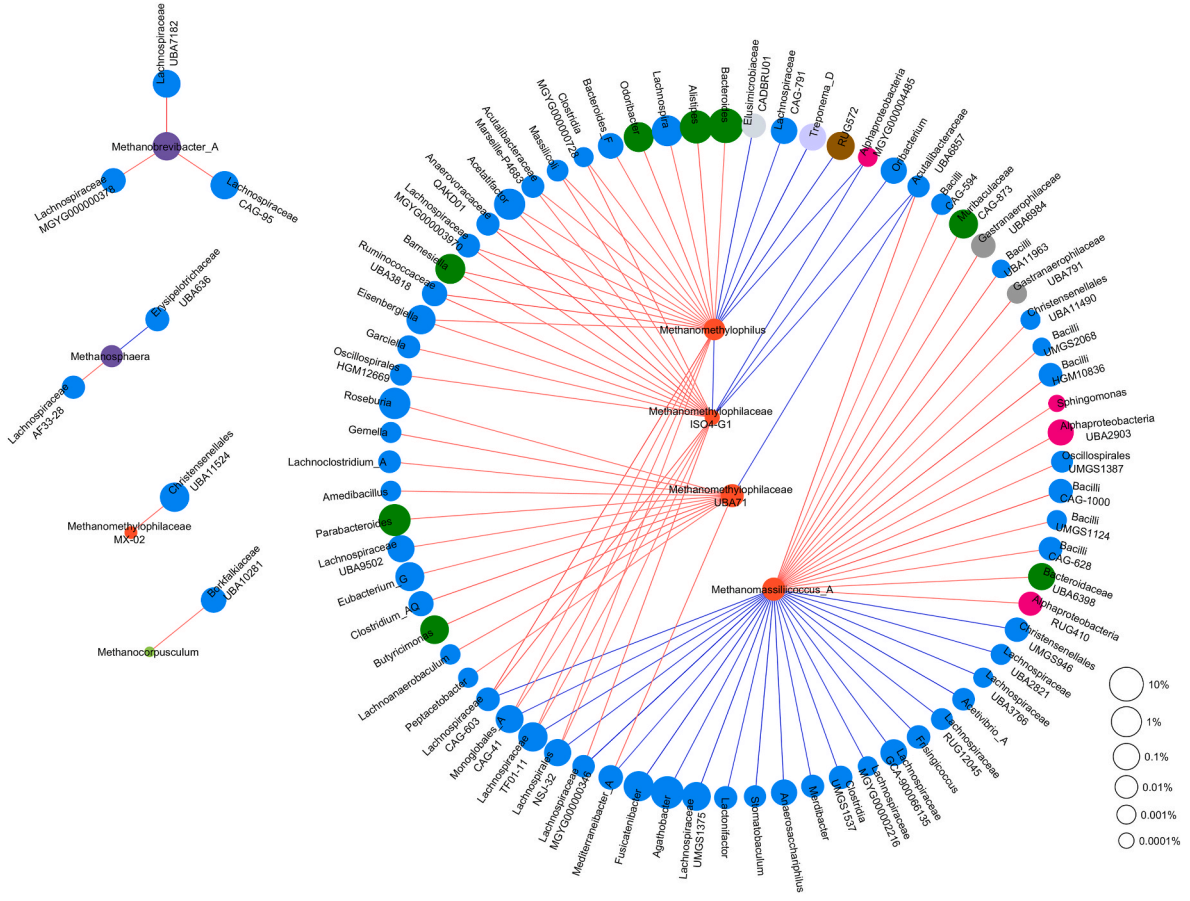

Fig. 6. Co-occurrence network of the main archaeal genera. The diagram reports the significant co-occurrences ($P < 0.05$) with other archaeal genera and with the bacterial ones occurring at least once >1 %. Edges represent co-occurrences from the most negative to the most positive, colored from the deepest red to the deepest blue, respectively. Nodes are genera, sized according to their mean abundance and colored according to their phylum: Thermoplasmatota, red; Methanobacteriota, purple; Halobacteriota, light green; Bacillota, blue; Bacteroidota, green; Actinobacteriota, yellow; Proteobacteria, pink; Verrucomicrobiota, brown; Spirochaetota, light grey; Campylobacteriota, grey; Cyanobacteriota, dark grey; Elusimicrobiota, lighter grey.

Archaea in the human gut microbiome reported by Chibani et al. [20]. In this elegant and comprehensive study several metagenomes have been analyzed through MAGs assembly, resulting in the identification of 18 different species, 11 of which with a recognized taxonomic definition, and 4 with a proposed one. In the present study, the archaeal community was investigated using the Kraken2, that bypasses genome assembly and provides allowed a different investigation of archaea, providing new insights into gut microbes ascribed to this domain. As a whole, 28 species features were identified in the 60 metagenomes, among which 12 presented a taxonomic designation, including two with Candidatus status, and a higher number of unassigned ones.

As expected, the archaeome was less abundant the bacteriome. The reliance of archaea on bacteria degrading complex compounds and providing them with substrates is at the basis of such low abundances in the intestine and other environments. Despite their abundance was often quite low, they reached a remarkable concentration in some subjects, with peculiar prokaryotic profile of gut microbes, anyhow consistent with a health status. Since most of the archaea identified are methanogenic, including these subjects, it seems that a high production of methane is not detrimental for the health status, albeit it has been associated to obesity. There is not sufficient information, both from metadata and from functional profiling of Archaea and bacteria, to understand whether the dominant population of methanogens is related to methane emission/flatulence.

The archaeome was not only less abundant but also less rich and complex than the Bacteriome. Richness and evenness indices were lower for Archaea compared with Bacteria, with the few archaeal taxa being not evenly distributed and the archaeomes being dominated by one or two taxa, in most cases *Methanobrevibacter*. Previous studies utilizing the amplification of ribosomal sequences report Methanobacteriota as the prevalent phylum, the genus *Methanobrevibacter* encompassing the vast majority (70–90 %) of the signatures [12,34]. In the present study, *Methanobrevibacter* as the major intestinal archaeal genus, with *M. smithii* as main species. The genus *Methanobrevibacter* was identified in all the metagenomes and encompassed 7 different species. Three of them, *M. smithii, Candidatus M. intestini,* and the phylogenetically related *Methanobrevibacter* sp900766745, dominated the archaeome in most of the microbiomes, with abundances that differed depending on the cohort, reaching up to 93.7, 34.3, and 98.9 %, respectively.

The genus *Methanomassiliicoccus*, together with the closely related *Methanomassiliicoccus* A, was also identified in all the 60 metagenomes and encompassed 5 different species, with the sole *M. intestinalis* and *M. luminyensis* possessing a designated species status within this genus in the context of the human microbiome. In the present survey, *M. intestinalis* and *M. luminyensis* were frequently detected albeit their presence was relatively low, particularly in the case of *M. luminyensis* that was generally negligible (<0.0001 %). Previous studies have already identified, cultured and sequenced *M. intestinalis* and *M. luminyensis* [35–37], the latter being ascribed to the "free living" clade of Methanomassiliicoccales, occurring also in environmental samples [38]. With respect to other minor components of the intestinal archaeome, this study points out the presence of Methanobacteriaceae (e.g., *M. stadtmanae*, *M. cuniculi*, *M. oralis*, *M. woesei*), Methanomethylophilaceae (e.g., *M. alvi*) and Methanomassiliicoccaceae, and Haloferacaceae (e.g. *H. massiniliensis* and *H. lipolyticum*). It is remarkable that, due to the low general amounts of the archaea within the archaeome, only *M. smithii* presented a remarkable abundance among the prokaryotes inhabiting the gut (mean = 0.2 %, max = 6.8 %), comparable to that of abundant bacterial species. The other most important species, such as *Candidatus M. intestini*, *Methanobrevibacter* sp900766745, and *M. intestinalis*, in general were individually one magnitude less abundant than M. smithii.

Methane generation in archaea is known to occur through one of the following methanogenic pathways: i) hydrogenotrophic reduction of methyl compounds; ii) hydrogenotrophic reduction of CO_2; iii) dismutation of acetate or methyl-compounds [9]. According to the composition herein described, the gut archaeome of the analyzed subjects is composed essentially of methanogenic organisms. Hydrogenotrophic CO_2-reducing methanogenesis, carried out by Methanobacteriaceae such as *M. smithii,* is the predominant methanogenic pathway in all the samples by virtue of the remarkable abundance of Methanobacteriaceae, followed by hydrogenotrophic methyl-reducing methanogenesis, carried out by Methanomassiliicoccales [36,39]. In virtue of its high abundance within microbiomes, the hydrogenotrophic *M. smithii,* together with the closely related species, is expected to be the main methane producer and to exert the main metabolic impact in the intestinal ecology. The lack of *Methanosarcina* in the present data set confirms that other methanogenic pathways involving dismutation of acetate or methyl compounds are negligible [9]. Halobacteria, that were found in negligible amount in the present study, were the sole non-methanogenic archaea.

The archaeal and the bacterial communities herein analyzed were found to group geographically, since the cohorts were significant according to PERMANOVA analysis of beta-diversity, presented several biomarkers with significant differential abundance, and significantly differed also with regards to the alpha-diversity metrics. Despite the low numerosity of the subjects per cohort could have affected this result, these observations are in agreement with previous studies that described the archaeal community in native Africans as richer and more complex than in western and westernized populations [15] and, in general, reported that archaeome composition depends on of the diet, lifestyle and correlates with demographic and geographic parameters [20]. Therefore, it was investigated whether significant co-occurrences among bacteria and archaea could be on the basis of the differentiation among cohorts. The analysis of co-occurrences revealed that the main hydrogenotrophic methyl-reducing methanogens belonging to Thermoplasmatota *Methanomassiliicoccus*_A and some Methanomethylophilaceae were in negative relationship with each other and established two networks with opposite behaviors with respect to some of main bacterial groups. Methanomassiliicoccaceae was in positive relationships with Ruminococcaceae, Lachnospiraceae, Streptococcaceae, Rikenellaceae and Erysipelatoclostridiaceae, that negatively correlated with Methanomethylophilaceae. On the other end, Atopobiaceae, Campylobacteriaceae and members of Oscillospirales and Alphaproteobacteria correlated positively with Methanomethylophilaceae and negatively with Methanomassiliicoccaceae. Interestingly, the hydrogenotrophic CO_2-reducing Archaea (mainly Methanobacteriota, with Methanobacteriaceae and Methanobrevibacter as main representatives) were not in direct relationship with other Archaea. At the family level, the co-occurrence network of Methanobacteriaceae, was similar to that of Methanomethylophilaceae, opposing to that of Methanomassiliicoccaceae. Nonetheless, at the genus level, *Methanobrevibacter* A lay in a separate network with negative correlation with some Lachnospiraceae, without any connection with either *Methanomassiliicoccus* A or the Methanomethylophilaceae.

The trophic networks laying at the basis of these co-occurrences between archaea and bacteria and shaping the overall structure of the microbial community remain to be clarified. Interspecies H_2 transfer is expected to be at the basis of trophic relationships involving hydrogenotrophic methanogens [40]. Hydrogen production is widespread among intestinal bacteria, in particular Cloastridiales and Bacteroidetes, that harbor hydrogen:ferredoxin oxidoreductases, participating in the oxidative tract of central carbohydrates catabolism and involved in H2 release to regenerate reducing power [41]. The wide distribution of hydrogen:ferredoxin oxidoreductases among abundant bacterial groups such as Bacteroidetes and clostridial Firmicutes could explain the fact that the hydrogenotrophic *Methanobrevibacter* was not found co-occurring with specific bacterial types.

Methyl-reducing methanogens are known to thrive on methyl-compounds such as trimethylamine [11,20,39], a compound produced by bacterial species such as *Escherichia coli*, *Clostridium* spp. and *Dorea formicigenerans*, that are ubiquitously found in Mammalia [20,42].

Trimethylamine is a bacterial metabolite of great health impact, being involved in the development of cardiovascular diseases, but, despite the great efforts, the trimethylamine-producing community has only been partially defined, encompassing clostridia of the former Cluster XIVa now classified as Lachnospiraceae [43]. Furthermore, extensive anaerobic subculturing of human feces identified no single commensal bacterium capable of L-carnitine to trimethylamine transformation, except the Clostridiales *Emergencia timonensis* [44]. Unfortunately, such available information is not sufficient to hypothesize that trimethylamine or any other methyl compound is the pivot of a trophic relationship between specific bacteria and archaea, thus corroborating the co-occurrences herein observed.

Associations between the diversity of gut-associated archaea with several demographic and geographic patterns, evidently arising from differences in diet and lifestyle affecting the whole structure of the microbiota, have been initially proposed in previous studies [20,34] and are presumably at the basis for the differences among the cohorts herein observed. For instance, *Methanobrevibacter* was positively associated with diets rich in carbohydrates, but negatively with diets high in amino acids, protein, and fatty acids [8,34] and was also observed at a large scale, also among multiple animal species [9]. Nevertheless, it remains challenging to establish significant associations between archaeal communities and human lifestyles or diseases. This is primarily due to the limited available information on the human gut archaeome and the lack of comprehensive metadata to enable sufficiently robust statistical analysis [20].

The data herein presented offer numerous ideas for further studies on the intestinal Archaea, including their functions in the gut environment, the trophic relationships that they establish with bacteria and dietary compounds, and their interactions with the host and its health status.

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CRediT authorship contribution statement

Francesco Candeliere: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Laura Sola:** Investigation, Methodology, Software, Writing – review & editing. **Stefano Raimondi:** Data curation, Investigation, Methodology, Software, Writing – review & editing. **Maddalena Rossi:** Resources, Supervision, Writing – review & editing. **Alberto Amaretti:** Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

All authors declare that the research was conducted in the absence of any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.12.007.

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