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Factors shaping the abundance and diversity of archaea in the animal gut

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Abstract

Archaea are active members of the gut microbiome, but a thorough analysis of their diversity and abundance in a wide range of animals is lacking. Here, we examined both quantitatively and qualitatively the gut archaeome of 269 species from invertebrates to primates. Archaea are present across many animals and mostly represented by four genera and one family of methanogens, but also members of Thaumarchaeota. Five major events of adaptation to the gut in the Archaea were identified. Host phylogeny, diet, and intestinal tract physiology are key factors shaping the structure and abundance of the archaeome. The abundance of methanogens is positively correlated with diet fibre content in mammals and hydrogenotrophic methyl-reducing methanogenesis (the main methanogenesis pathway in many animals) is linked to diet and methyl compounds-producing bacteria. Our results provide unprecedented insights on the intestinal archaeome and pave the way for further studies on their role in this environment.

Introduction

The intestinal microbiome plays key roles in host health^{1–8}. It is composed of bacteria, archaea, microbial eukaryotes, and viruses/phages. Research on the microbiome of many animals has unveiled features that influence the overall structure of the intestinal microbiome such as diet and the ability to fly^{9–12}. However, most of these studies have only targeted the bacterial intestinal community. It is known that host-associated archaeal methanogens produce a significant amount of methane gas in ruminants, which makes them ecologically and environmentally important¹³. In humans, archaea have been linked to various conditions of health and disease⁵. Nevertheless, archaea-centric intestinal microbiome studies have been generally conducted in a narrow group of animals such as termites, primates, humans, and ruminants^{5,14,23,15–22}.

The gut archaeome of other animals such as rats, hoatzin, pigs, seals, wallabies, kangaroos, iguanas, fish, horses, and even in the tissue of sponges was examined by independent studies using different molecular and cultural approaches^{15,24–30}. Overall, these studies reported that the most common methanogens in the gut are members of the Methanobacteriales and Methanomassiliicoccales, and that the Methanosarcinales and Methanomicrobiales are also present, although less frequently^{13,15,31,32}. Only one study addressed the distribution of intestinal methanogens in a wide variety of animals, but using methane gas detection tests³³. This study detected methanogens in a wide range of animals. It also suggested that they have been acquired early in animal evolution and were completely lost in some lineages such as the Carnivora. However, the methodology used in this study has several limitations, as it does not provide taxonomic information and cannot detect methanogenic populations with low concentrations in faeces or non-methanogenic archaea.

Here, we carried out a sequence-based analysis of the gut archaeome based on nearly 400 samples from 269 species covering a broad spectrum of animal diversity. We investigated the host range of archaea in eight animal classes, identified the major gut archaeal lineages and predicted the dominant methane metabolisms using both sequencing and quantitative approaches. We discussed the number of events of adaptation to the gut in the Archaea, including in ammonia-oxidizing Thaumarchaeota and in Bathyarchaeota, both previously rarely identified in this environment. By using a large range of metadata from the literature we define key factors structuring the abundance and composition of the gut archaeome across the animal kingdom.

Results and discussion

Archaea are present in the gut microbiome throughout the animal kingdom

We collected faeces from 269 species of animals (n samples = 391) ranging from Invertebrates to Mammals – the majority of which, except for birds, fish, and gastropods, came from captive specimens (Table S1). We used three approaches to characterize the archaeal community of these samples: *i*) quantitative PCR (qPCR) targeting total Archaea, total Bacteria, and five archaeal lineages previously found in the animal intestine (Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales, *Methanimicrococcus* and Thaumarchaeota), and *ii*) 16S rRNA gene amplicon sequencing of the Archaea only and *iii*) of the entire microbial community. We detected the presence of archaea in the gut microbiome of 175 species belonging to all eight classes of animal investigated, including 14 orders of mammals (Figure 1; Table S1).

Figure 1. Detection of archaea in animal species with three approaches. Invertebrates gather 3 classes (Insecta, Mollusca, and Malacostraca). Archaea were detected in a higher proportion of the samples when using archaea-specific primers for qPCR (78%) or amplicon sequencing (76%) with respect to the universal prokaryote primers for amplicon sequencing (44%). This difference was observed in most animal classes

(Figure 1). In addition, universal prokaryote primers also captured a lower number of ASVs (1.9 ± 2.6 ASVs per sample) with respect to the archaea-specific primers (13.6 ± 20.3 ASVs per sample) (Kruskal-Wallis $p = 1.65e^{-8}$ (Figure S1), $n = 218$). With $\sim 10,000$ prokaryotic reads per samples, the archaeal species/ASVs that represent less than 0.01% of the microbial community are likely missed, which may explain both lower proportion of archaea-positive animals and the lower archaeal alpha-diversity in the approach relying on prokaryote universal primers.

Five major events of adaptation to the gut in the Archaea

The broad taxonomic coverage of the animal hosts and the use of archaeal specific primers allowed us to identify archaeal ASVs belonging to 19 described families, 10 orders, 6 classes, and 3 phyla. 84.9% of these ASVs (94.5% of the reads) share more than 95% identity with species in the Living Tree Project (LTP, v138) database³⁴ amended with characterized candidate species, and half of the reads share more than 99% identity with known species (Table S2). Consistently, the vast majority (93.7%) of the reads are affiliated to only six genera or families (Figure 2a): *Methanobrevibacter*, *Methanosphaera* (Methanobacteriales), *Methanomethylophilaceae* (Methanomassiliicoccales), *Methanocorpusculum* (Methanomicrobiales), *Methanimicrococcus* (Methanosarcinales), *Nitrososphaeraceae* (Nitrososphaerales/Thaumarchaeota group 1.1b). These lineages also constitute more than 50% of the gut archaeome in 92% of the sampled animals and can be qualified as “dominant gut archaea”. Among them, *Methanobrevibacter*, *Methanosphaera* and *Methanomethylophilaceae* had already been extensively reported in the gut microbiota of ruminants, human and termites^{5,15,20}. Our results show that they are also the main methanogenic lineages in a much wider range of animals. *Methanobrevibacter* members are by far the most dominant methanogens in our dataset – composing over a third of the total number of reads, followed by *Methanomethylophilaceae* members which accounted for 17.5% of the total reads (Figure 2a). The two others methanogen lineages that are the most prevalent in our dataset, *Methanocorpusculum* and *Methanimicrococcus*, have been less often reported in previous studies on the animal digestive tract^{15,35}.

Figure 2. Proposed independent events of adaptation to the gut in the domain archaea. **a)** Distribution of archaeal 16S rRNA gene sequences in the gut and other environments based on sequences obtained from the Silva database and this study. The archaeal tree is based on Borrel et al.,³⁶ enriched with DPANN lineages. Orange arrows on the tree indicate proposed events of adaptations to the gut environment, either at the base or within displayed lineages. The histogram shows the proportion of sequences from a given lineage present in either animal digestive tract (« Gut »), open natural environment (« Environment ») or built environment (« Digester »). Circle surface area represents the percentage of reads attributed to each taxon in our study including only gut-related samples. **b)** Correlation between the absolute abundance of Archaea and the absolute abundance (16S rRNA copies/gram of feces) of Bacteria (black), summed methanogen lineages (Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales, *Methanimicrococcus*; green) and Thaumarchaeota (purple), all determined by qPCR using lineage specific primers. The scale of the absolute abundance of Archaea is on panel c). Plotted samples correspond to those with amplified Archaea in Miseq, presented in panel c. **c)** Proportion of archaea corresponding to the dominant methanogen lineages (green), *Nitrososphaeraceae* (purple) and rarer taxa (light blue) in samples, based on Miseq sequencing with archaeal specific primers, according to absolute abundance of archaea in the sample (qPCR). Dots indicate the relative abundance of these three categories/lineages of archaea in each sample. Lines indicate the moving averages with a subset size of 25 samples. The dominant methanogen lineages category contains *Methanobrevibacter*, *Methanosphaera*, *Methanomethylophilaceae*, *Methanocorpusculum*, *Methanimicrococcus*. The rarest taxa category contains *Methanobacterium*, *Methanothermobacter*, *Methanomassiliicoccaceae*, *Methanosarcina*, *Methanoregulaceae*, *Methanospirillaceae*, *Methanosaeta*, *Methanocellales*, *Nitrosopumilaceae*, *Nitrosotaleaceae*, *Bathyarchaeota*, *Halobacteriales*. **d)** Phylogenetic position of dominant gut Thaumarchaeota (this study, ASV4, ASV20 and ASV21, purple) and dominant soil archaea³⁷ (DSC1 and DSC2, brown). ASV4/ASV20 are virtually similar to DSC2 representative sequence (only 1 indel in a 4/5Gs homopolymer region, which may be due to a 454-sequencing error in DSC2³⁸). ASV21 shares 99.2% identity with the DSC1 representative sequence.

The five dominant methanogen lineages in the gut have been rarely reported in open environments, as revealed by a meta-analysis of the archaeal 16S rRNA gene sequences present in the Silva database (Figure 2a). They are also often dominant in samples with the highest archaeal absolute abundance (Figure 2c). In contrast, *Nitrososphaeraceae* and rarer archaeal lineages (e.g., *Methanobacterium*, *Methanosarcina*, *Methanomassiliicoccaceae*) are more often dominating in samples with the overall lowest archaeal absolute abundance (Figure 2b and c). Sequences from the rarer archaeal lineages have been generally reported from non-gut environments such as sediments and wetland soils ^{32,39} (Figure 2a). Therefore, these lineages likely contain free-living archaea that have weaker capacity to develop in the gut and are only detectable when the most adapted archaea are absent or occur in low abundance. These lineages that are weakly associated with the gut microbiome are often the closest phylogenetic relatives to the ones that are highly abundant in the gut (Figure 2a). In addition, the dominant gut genera/families belong to orders that are often present in digestors/bioreactors, suggesting a more ancient adaptation to high resource availability in these lineages (Figure 2a). This suggests that some of the traits favouring development in the gut were already present in the last common ancestor of Methanobacteriales, Methanomassiliicoccales or *Methanosarcina*+*Methanimicroccus*, which may have facilitated the emergence of gut-specialized archaea particularly in these lineages. Considering that the dominant gut methanogen lineages are rarely detected in open environments, and that closely related lineages are rarely present in the gut, a strong specialization to the gut microbiome likely occurred at the divergence of each of the five dominant gut methanogen lineages, suggesting at least five major events of adaptation to the gut in the Archaea (Figure 2a). A few other methanogen lineages may have developed specific adaptations to certain gut conditions or specific hosts. For example, “*Candidatus* *Methanomassiliicoccus intestinalis*” was also detected in high absolute abundance in the gut of elderly people being in long-term residential care and having a disturbed gut microbiota ¹⁸. *Methanomicrobium mobile* was not detected in our samples (Figure 2a), but it can represent a large proportion of archaea in the rumen and it is almost never detected in open environments ⁴⁰, suggesting a high specialization on this gut compartment, which was not examined here.

Non-methanogenic lineages are components of the animal gut archaeome

The family *Nitrososphaeraceae* (Thaumarchaeota), which gathers 15% of the total reads in our study, has rarely been reported in previous gut microbiomes studies (great apes and humans) and only when archaeal specific primers coupled with high-throughput sequencing (or nested PCR) were used^{16,41,42}. Conversely, this lineage is common in terrestrial environments such as soils (Figure 2a)^{37,43–45}. The large presence of these obligate aerobes in the gut is somehow surprising, but oxygen is available in some gut sections and in proximity of the gut wall in other sections dedicated to fermentation⁴⁶ and some animal host more aerobic communities than others⁴⁷. The three thaumarchaeotal ASVs that gather the largest number of reads in our dataset (ASV4/ASV20/ASV21) are also highly distributed among animal species, ASV4 being the most widespread archaeon in our samples (present in 65 animal species from 8 classes). These three ASVs correspond to the most prevalent and abundant archaeal phylotypes (named DSC1 and DSC2) among 146 soils from various biomes³⁷ (Figure 2d). Because most animals live (eat, sleep, groom...) on soil, these dominant soil archaea may be ingested by chance, which could explain why they are present in a wide range of animal at a low absolute abundance (Figure 2d). However, a sequence closely related to ASV21 and DSC1 has also been found in the human gut⁴² (Figure 2d). Moreover, while Thaumarchaeota group I.1c are among the dominant archaea in soil^{37,48}, we only found one ASV belonging to this lineage in one sample. ASV4/ASV20 and DSC2 are closely related to several “*Candidatus Nitrosocosmicus*” species^{45,49,50}; (Figure 2d). These species can grow at ammonia concentrations (>20mM) prevailing in the gut^{51–53}, that inhibit other ammonia-oxidizing Thaumarchaeota⁴⁵. Also, one of them was cultured from a wastewater treatment plant⁴⁹, an environment that shares some characteristics with the gut. Therefore, an alternative hypothesis is that the dominant Thaumarchaeota in the animal gut can maintain in this environment, which may be beneficial for their dispersion in soils, being spread by animal faeces. The degree of adaptation and role of *Nitrososphaeraceae* in the gut remains to be elucidated.

Finally, although Bathyarchaeota were not common in our samples, most of the sequences we retrieved are closely related to a clade formed by “*Ca. Termiticorpusculum*” and “*Ca. Termitimicrobium*” (>95% id to termite sequences), two lineages recently identified in the termite gut⁵⁴. Together with sequences from anaerobic digestors and sediments, our sequences from mammal, birds, reptiles, and crayfish, form a sister clade to termite

sequences (Figure S2), suggesting that some general traits needed to maintain in the gut are shared by these Bathyarchaeota.

Specific associations between archaea and their hosts

In mammals, main factors significantly affect the beta-diversity of archaea with the following level of influence: host phylogeny > coefficient of gut differentiation > host diet > digestive tract type, regardless of the diversity measurement used – i.e., Weighted/Unweighted UniFrac, Bray-Curtis, and Jaccard (Table 1; Figure S3). Other factors such as the geographic origin of the samples and the body mass have little influence on the archaeal community structure (Supplementary text).

Beta diversity measure	Host order df = 10	Gut diff Coeff n = 23 df = 1	Diet df = 7	GIT type df = 4	Body mass df = 1	qPCR archaeal abundance df = 1	Arc:bac ratio (qpcr) df = 1	Stomach pH n = 18 df = 1	Mean retention time df = 1	Origin*
Weighted unifrac	$R^2 = 0.40$ $p = 0.001$	0.32 0.0009	0.20 0.002	0.10 0.02	0.06 0.002	0.04 0.01	0.04 0.03	ns	ns	ns
Unweighted unifrac	$R^2 = 0.33$ $p = 0.001$	0.17 0.0009	0.19 0.001	0.13 0.001	0.03 0.003	ns	ns	ns	ns	0.05 0.035
Bray-Curtis	$R^2 = 0.25$ $p = 0.001$	0.13 0.0009	0.16 0.001	0.14 0.001	0.02 0.01	0.02 0.03	0.02 0.01	ns	ns	0.05 0.018
Jaccard	$R^2 = 0.21$ $p = 0.001$	0.13 0.0009	0.14 0.001	0.11 0.001	0.02 0.02	0.02 0.02	0.02 0.02	ns	ns	0.05 0.018

Table 1. Factors that influence the Beta Diversity of archaea in Mammals. Mammals with > 2 species per order (n = 73, unless otherwise indicated) rarefied to 3000 reads per sample were subject to beta diversity analyses. * including only zoo from which more than three samples were collected, and samples from the same species were treated separately (n = 99; df = 11). Significant differences were tested for between beta diversity metrics using a permutational analysis of variance (PERMANOVA), p < 0.05 was considered significant.

The level of beta-diversity variance explained by host phylogeny is as high (or higher) as the one previously reported for bacterial communities in mammals^{9,10,47,55–58}. Specific associations between archaeal and animal host lineages are visible through the dominance of gut archaeome by Methanobacteriales/*Methanobrevibacter* in Rodentia and most Cetartiodactyla, Methanomassiliicoccales/*Methanomethylophilaceae* in *Lemuridae*, Methanomicrobiales/*Methanocorpusculum* in Perissodactyla and several Reptiles or

169 Thaumarchaeota/Nitrososphaeraceae in Gastropoda (Figure 3d). A link between archaea and
170 their host is also evident from the existence of archaeal clades associated with specific host

Figure 3. Archaeal taxonomic diversity and abundance in the animal gut. **a)** Information on animal primary diet gathered using the Elton Trait database, the Animal Diversity Website database, or from specialists who provided fecal samples. Primary diet was considered food material that made up $\geq 70\%$ of the animal's diet. **b)** Absolute abundance of archaea as determined by qPCR with archaea-targeting primers on a log scale. **c)** Observed richness (number of different ASV) of archaea. Green represents animals with low richness, black represents medium richness ~ 25 ; and red represents high richness >50 archaeal ASVs. **d)** Taxonomic diversity of archaea in the animal intestinal microbiome. Samples were rarefied to 3000 archaeal reads. **e)** Predicted methane metabolism, assigned to ASVs based on taxonomic annotation (Table S4).

171 orders. *Methanobrevibacter* is the most structured genus with respect to these specific
172 associations, several *Methanobrevibacter* clades being enriched in one type of mammal host
173 (Primates, Cetartiodactyla, Perissodactyla Rodentia) (Figure 4). These results are consistent
174 with the previous report of *Methanobrevibacter* OTUs showing phylogenetic association with
175 hosts ¹⁰. In *Methanocorpusculum*, a large Perissodactyla-associated clade is sister to a
176 Cetartiodactyla-associated clade (Figure S4), suggesting that the ancestor of these two

177 archaeal clades was present in the ancestor of the Ungulata. Close relationships between
 178 Perissodactyla and Cetartiodactyla ASVs are also visible in *Methanobrevibacter* and
 179 *Methanosphaera* (Figure 4).

Figure 4. Distribution of Methanobacteriales ASVs among Mammals. The phylogenetic tree (maximum-likelihood, GTR+G4) was built with nearly full length 16S rRNA genes sequences from literature and the ASVs sequences from this study. For display purposes, the shown tree includes only the ASV representing more than 1% of the sequences per sample. The percentages on the right indicate the proportion of reads from Methanobacteriales that were annotated as *Methanobrevibacter*, *Methanosphaera*, *Methanobacterium* or *Methanothermobacter*.

In the *Methanomethylophilaceae*, there is a large Primates-associated clade containing several of the typical human-associated species (Mx-03, Mx06; ¹⁸; Figure S5). Fewer host-specific clades are observed outside mammals, except for reptile-specific clades in *Methanocorpusculum*. These clades complement the previously reported insect/termites-specific ones within *Methanobrevibacter*, *Methanomethylophilaceae* and *Methanimicrococcus* ^{20,35}) and support the hypothesis that archaea developed adaptations for specific host lineages with which they may have been associated for a long evolutionary time. In contrast, no clear host-associations are visible in Nitrososphaerales (Figure S6) which points at the absence of specialization to specific animal guts and supports the hypothesis of their low level of adaptation to the gut, as discussed above.

Host phylogeny also influences the absolute abundance and alpha diversity of archaea. Indeed, mammals have the highest absolute abundance of archaea, followed by reptiles and amphibians (Figure 5a). Birds, bony fishes, and invertebrates have instead the lowest concentrations. This global trend for archaea is mostly driven by variation in the methanogen lineages, particularly concerning the Methanobacteriales (Figure S7; Supplementary text).

Figure 5: Absolute Abundance of Archaea (red) and Bacteria (blue) determined via qPCR in a) animal classes (n = 286) and b) mammalian orders (n = 156). Animal lineages with significantly different archaeal/bacterial abundances are labeled. Wilcoxon rank sum *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

Archaea were also detected in a lower proportion of bird and fish species than in other animal classes (Figure 1; Figure S8). In Mammalia, samples belonging to Perissodactyla, Cetartiodactyla, Primates, Diprotodontia and Rodentia have the highest absolute concentration of archaea, whereas those belonging to Carnivora and Pholidota have the lowest (Figure 3b). Conversely, the abundance of bacteria is more uniform across mammalian orders (Figure 3b). Closely related groups of animals also tend to have similar levels of archaeal alpha diversity, as supported by the Moran index ($I=0.08$, $p=0.001$, $n=150$). For example, the archaeal richness is high in the members of Gastropoda and in most members of the Cingulata, Equidae (order Perissodactyla) and Bovidae (order Cetartiodactyla) within mammals (Figure 3b; Table S1). Conversely, we found comparably low levels of archaeal richness within the Aves and Actinopterygii.

Strong influence of diet on methanogen abundance and composition

Diet is another important factor affecting the gut archaeome, both in terms of alpha-diversity, beta-diversity (Table 1; Figure S9) and abundance (Figure 6).

Figure 6: Influence of host diet-type, diet-fibre content, and mean retention time on the absolute abundance of total methanogens, Thaumarchaeota and Bacteria. Abundance of **a)** total methanogens ($n=161$), **b)** Thaumarchaeota ($n=116$) and **c)** Bacteria ($n=223$) according to host diets-type. Significant differences across all groups were determined via the Kruskal-Wallis test, with $p < 0.05$ set as significant. Wilcoxon rank sum test with continuity correction was used to determine differences between diet types *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Correlation between diet-fibre content and absolute abundance of **d)** methanogens ($n=103$), **e)** Thaumarchaeota ($n=55$) and **f)** Bacteria ($n=107$) in mammal species. **g)** Dietary fibre versus averaged absolute abundance of methanogens in Primates ($n=12$). Mean retention time is significantly related to the abundance of methanogens in primates. Statistical analyses and representation

of the absolute/relative abundance of methanogens were carried out on species where archaea have been detected. **h)** Correlation between digesta mean retention time vs Averaged absolute abundance of methanogens in Primates (n = 24). Fibre consumption is significantly related to the abundance of Methanogens in primates.

Indeed, herbivorous animals have a higher number archaeal ASV than carnivorous and omnivorous animals (Figure S9). Moreover, the absolute and relative abundance of methanogens is higher in animals with a plant-based diet (e.g., leaves, fruits) than in animals feeding on meat or insects, and their abundance is intermediate in omnivorous animals (Figure 6a). This link between methanogen abundance and diet-type is further supported by the positive correlation of both the absolute and relative abundances of methanogens (but not of Thaumarchaeota, and very weakly for bacteria) with the fibre content of the diet (Figure 6d-f; Figure S10). The increase in methanogen absolute/relative abundance reaches a limit at around 200 g of crude fibre/kg of dry matter (Figure 6d; Figure S10). At lower host taxonomic level, the positive correlation also holds for Primates, for which we sampled species with contrasted average fibre intake (Figure 6g). An increased diet fibre content was previously reported to be associated with a higher expression level of methanogenesis genes in humans⁵⁹ and a greater methane production in pigs⁶⁰ and ruminants⁶¹. As the vast majority of intestinal methanogens are hydrogenotrophic, these relationships can be explained by the higher production of hydrogen from fibre/carbohydrates-rich diets (plant) than from protein/fat-rich diets (meat)⁶².

However, the level of H₂ produced from fibre degradation also depends on which bacteria are involved, Clostridiales being known to producing more H₂ than Bacteroides during fibre degradation⁶³. Thus, other than diet, methanogens are also influenced by the composition of the bacteria degrading it. In humans, cellulolytic *Ruminococcaceae* (Clostridiales, Firmicutes) spp. have been reported to be present in the gut of methane producers, while cellulolytic *Bacteroides* spp. prevail in non-methane producers⁶³, and methanogens are enriched in subjects with Firmicutes/*Ruminococcaceae* enterotype⁶⁴. We found that eight *Ruminococcaceae* OTUs (including six from uncharacterized genera) co-occur with methanogens, and -more generally- 19 out of the 30 bacterial OTUs positively associated with methanogens belong to Clostridiales and only four to Bacteroidales (Table S3, Supplementary text). Other than benefiting from fibre degradation, methanogens can also favour it by stimulating microbes involved in its degradation. Indeed, the presence of

methanogens in cocultures has been shown to increase the level of extracellular polysaccharide-degrading enzymes of *Ruminococcus flavefaciens*⁶⁵.

The abundance of hydrogenotrophic methyl-reducing methanogen lineages (i.e., Methanomassiliicoccales and *Methanimicrococcus*) is less influenced by fibre content than lineages that include hydrogenotrophic CO₂-reducing methanogens (i.e., Methanobacteriales and Methanomicrobiales; Figure S11). Moreover, hydrogenotrophic methyl-reducing methanogens represent a lower proportion of the methanogens in herbivorous animals than in animals having another type of diet ($p = 0.003$). As methyl-reducing methanogens depend on different methyl-compounds (e.g. methanol, methylamines) for their energy metabolism and because they can utilize hydrogen at lower concentration than CO₂-reducing methanogens⁶⁶, their distribution may be more affected by the availability of methyl-compounds than by fibre content. One of these methyl-compounds, methanol, is produced by the bacterial degradation of pectin⁶⁷. This metabolism was shown to occur in the animal gut (e.g., human, pigs, lemurs, ruminants) as revealed by the identification of bacteria with a methylesterase activity^{68,69} and by the increase in methanol concentrations in response to pectin consumption^{70–72}. Our data show that the ratio of hydrogenotrophic methyl-reducing to CO₂-reducing methanogens is higher in frugivorous species than in herbivorous ones ($p = 0.005$), which is likely related to large amounts of pectin in fruits. This support a previous hypothesis that the high relative abundance of *Methanosphaera stadtmanae* (an obligate methanol-reducing methanogen) in orangutan is related to their high fruit consumption⁷³.

We also found a high relative abundance of hydrogenotrophic methyl-reducing methanogens in most of the sampled Primates (Figure 3e), and particularly in *Lemuridae*, which may be related to the presence of fruits in their diet (Table S1). This relationship is further substantiated by the association between an archaeal OTU closely related to *Methanomethylophilaceae* sp. Mx06¹⁸ and a bacterial OTU closely related to *Lachnospira pectinoschiza* (OTUarc_11; OTUbac_2345; Table S3). This bacterium grows mainly on pectin, producing methanol as a by-product of its degradation⁷⁴, and *Methanomethylophilaceae* sp. Mx06 has the genetic potential to grow by reducing methanol and methylamines with H₂¹⁸. A similar link may exist in humans, as both *Lachnospira pectinoschiza* and Methanomassiliicoccales abundance increases with age^{75,76}. Moreover, *Methanomethylophilaceae* sp. Mx06 is the dominant archaeon in the gut of Yanomami Amerindians^{18,77}, whose diet is largely composed of fruits⁷⁸.

270 As *Methanomassiliicoccales* and *Methanimicrococcus* may also grow on other methyl-
 271 compounds than methanol, such as trimethylamine, they might be influenced by other types
 272 of diet. *Methanomethylophilaceae* OTUarc_11 is also correlated with an OTUs closely related
 273 to *Sarcina* sp. (OTUbac_4310; Table S3) that can produce trimethylamine ⁷⁹. A similar
 274 correlation between *Methanomethylophilaceae* and *Sarcina* was previously reported in the
 275 human gut ⁸⁰. Precursors of trimethylamine (i.e. glycine-betaine, carnitine and choline) are
 276 present in various diets ^{81–83} and pectin is not limited to fruit but is also a constituent of the
 277 plant cell wall ⁸⁴, which therefore do not limit the presence of hydrogenotrophic methyl-
 278 reducing methanogens to frugivorous animals. In our dataset, hydrogenotrophic methyl-
 279 reducing methanogens constitute almost 40% of the overall methanogen reads (Figure 7a;
 280 Table S4) and represent a large fraction of the methanogens in many animals (Figure 3e;
 281 Supplementary text). This contrasts with many non-host environments (e.g. sediments, peat

Figure 7: Main methanogenesis pathways in the animal gut. **a)** Proportion of the total archaeal reads that are assigned to taxa with a predicted CO_2 -dependent hydrogenotrophic methanogenesis ($\text{H}_2 + \text{CO}_2$; blue) or methyl-dependent hydrogenotrophic methanogenesis ($\text{CH}_3\text{-R} + \text{H}_2$; orange) pathway. *Methanosarcina* spp. can have diverse methanogenesis pathways (the two above-mentioned pathways and the methyl-dismutation (or methylotrophic) and acetoclastic pathways). **b)** Diagram of the most favourable methanogenic metabolisms depending on methanol concentration (C(methanol) in mol/l) and hydrogen partial pressure (p(H₂) in bar). Coloured area in the map indicate ranges of C(methanol) and p(H₂) for which either $\text{CH}_3\text{-R}$ dismutation, $\text{CH}_3\text{-R} + \text{CO}_2$ or $\text{CO}_2 + \text{H}_2$ is the most favourable pathway, i.e. concentrations and pressure ranges for which the associated ΔG_{cat} expressed in kJ/mol CH₄ is the most negative. ΔG_{cat} values were calculated for T = 298 K, pH = 7 and p(CO₂) = p(CH₄) = 10⁻¹ bar, when the difference in ΔG_{cat} between two or three catabolisms was less than 10 kJ/mol CH₄, catabolisms were then considered to be equally favourable. This corresponds to central coloured areas in the diagram where either two or three metabolisms are shown as equally favourable. The dotted line indicates particular values of C(methanol) and p(H₂) for which all three catabolisms have exactly the same ΔG_{cat} . Ranges of C(methanol) and p(H₂) found in the literature for either gut or marine sediments environments were also mapped on the graph: dots correspond to mean values and bars indicate minimal and maximal values. See Material and Methods section for the references on the studies providing the concentrations.

bog), where hydrogenotrophic methyl-reducing methanogens constitute a minor fraction of the overall methanogens^{39,85}. It also reinforces the hypothesis that the gut environment is particularly propitious for this kind of methanogenesis, which could have led to the transition from methylotrophic and CO₂-reducing methanogenesis to methyl-reducing methanogenesis in *Methanimicrococcus* and *Methanosphaera*, respectively³⁵. Other methane metabolisms (based on dismutation of methyl-compounds or acetate) are almost absent from the animal gut and may occur in the few *Methanosarcina* members (0.6 % of the total reads) identified in our dataset (Figure 7a). The few data on methanol concentration and H₂ partial pressure in the gut highly contrast with those observed in marine sediments where dismutation of methyl-compounds is prevalent (Figure 7b). Gut conditions with relatively high partial pressure of hydrogen are generally more favourable for CO₂-reducing hydrogenotrophic methanogenesis but can vary to be equally favourable for methyl-reducing hydrogenotrophic methanogenesis or even for dismutation of methyl-compounds (Figure 7b). Why methanogens having this latter metabolism are not more prevalent in the gut is unclear, it might be related to the conditions generally favouring other metabolisms but this could also be related to the lower affinity of their enzymes for methanol and H₂ than methyl-reducing methanogens⁴⁶.

The influence of diet is further substantiated by the link between the archaea present in the gut microbiota of both predators and their preys. Indeed, within *Methanobrevibacter*, sequences of insectivorous mammals from distinct orders (Carnivora, Pilosa, Pholidota, Cingulata, Afrotheria) are clustered with sequences from insect clades (Figure 4) reported in the literature²⁰. Except for cane toad (Urodela), no sequences from non-mammal insectivores are present in these clades. Moreover, a similar phylogenetic clustering of insectivores and insect-derived archaeal sequences was not observed for other known insect clades outside of *Methanobrevibacter* (even if a few non-mammal insectivores are present in the *Methanimicrococcus* insect clade). This suggests that *Methanobrevibacter* may also develop in the gut of insectivorous mammals while other insect-associated methanogens are mostly transients.

Impact of digestive tract physiology

Both the coefficient of gut differentiation⁸⁶ (i.e., proportion of the gut dedicated to fermentation) and where the fermentation takes place (e.g. foregut, hindgut, caecum) explain

part of the variance in of the beta-diversity (Table 1). In addition, many ASVs are almost ubiquitous in the ruminant Cetartiodactyla (paraphyletic, Ruminantia and Tylopoda), but mostly absent from non-ruminant Cetartiodactyla or other animals, highlighting possible cross influence of gut physiology and host-phylogeny (Figure S12). Whether these archaea found in faeces originate from the rumen compartment or can colonize more largely the gut of these animal is currently unknown. The total abundance of methanogens is positively correlated with gut differentiation coefficient in mammals ($R^2 = 0.33$, $p = 0.0036$, $n = 25$), while there was no correlation with abundance of Thaumarchaeota and Bacteria (Figure S13).

It was previously reported that digesta mean retention time (MRT) is positively correlated with methane emission in herbivorous mammals⁸⁷. Also, a positive relationships between methanogen abundance and MRT was reported in humans⁸⁸. When considering only Primate species, we also highlight a positive correlation between MRT and methanogen (or total archaea) abundance (Figure 6h). However, we found only a weak positive correlation between MRT and methanogen (or total archaea) abundance in all animals ($R^2 = 0.11$; $p = 0.02$, $n = 69$). However, the distribution of the values suggests that the abundance of methanogens is mostly influenced by the lower range of MRT values. Indeed, there is a stronger positive correlation ($R^2 = 0.33$; $p = 4.3e-06$, $n = 54$) for MRT values ranging from 0.5 to 50 h and significantly less archaea in animals with an MRT < 20h than animals with an MRT > 20h (Kruskal-Wallis $p = 0.0008$, $n = 69$). Diet type and MRT are generally related since digestion of fibre-rich diet relies on microbial fermentation (“allo-enzymatic” digestion) which requires longer MRT than digestion of protein/soluble carbohydrate-rich diets that are processed at high rate by animal enzymes (“auto-enzymatic” digestion)^{89,90}. An exception is the extreme case of the giant panda, an herbivore with a short MRT (8 h) that is a clear outlier in the relationships between fibre content and both methanogen absolute/relative abundance (Figure S10). Conversely, carnivorous reptiles have a long MRT, which can exceed a week for some large snakes⁹¹. Among them, boa constrictor and reticulated python have a high abundance of methanogens compared to other carnivorous animals which suggests that a long MRT can allow a substantial development of methanogens on meat diet. An increase in the relative abundance of Firmicutes, potential partners of methanogens, has also previously been recorded in Burmese pythons during digestion⁹². However, while long MRT may facilitate methanogen development on meat-diet, it should be stressed that most carnivorous reptiles have a low abundance of methanogens. Outside of Mammals and Reptiles, most birds,

fish, amphibian, and invertebrates have generally a low concentration of archaea and especially of methanogens (Figure 5). Many flying birds feeding on plant materials use only readily digestible components of their diet, and rapidly expel recalcitrant cell-wall constituent without significant microbial fermentation⁹³. This was suggested to be an adaptation to improve flight power by decreasing the body mass⁹³. It is thus likely that the short transit time and the low level of plant fermentation have a negative impact on methanogen abundance in birds. In addition to low concentrations of methanogens in Carnivora, bird and fish, we found no clear archaeal clades associated with these animals (with few exceptions, like a small Carnivora-associated clade in *Methanosphaera*; Figure 4), suggesting that no lineage of methanogens developed strong adaptations to these hosts.

It was proposed that some animals, including birds, rely relatively little on their gut microbiota⁹⁴. In addition, bacteria recovered from birds show little host specificity and do not display phylosymbiotic patterns with their host or correlation with diet, differently from what has been generally observed in most mammals gut microbiota⁹. In our dataset, the low abundance of bacteria in the gut microbiota of bird supports the hypothesis of Hammer et al.,⁹⁴ and extend the observations of Song et al.,⁹ on the particularity of the gut microbiota of these animals. However, we found that concentrations of faecal bacteria in other animals proposed to rely less on their gut microbiota, such as Carnivora species⁹⁴, are as high as in other mammals (Figure 5b).

Conclusions

Our work provides first key insights into the lifestyle and role of intestinal archaea across a diverse range of animal hosts. Increased sampling efforts, time-series analyses, and metagenomic investigation will help to answer standing questions about the impact of geography, captivity, residency, and adaptations of intestinal archaea throughout the animal phylogeny.

Material and Methods

Sample collection and DNA extraction

A majority of animal faecal samples were donated from various zoological institutions in France (Table S1). Fresh faecal samples (n = 392) were stored at -20°C until DNA extraction. Total DNA was extracted using a modified QIAamp PowerFecal DNA Kit (Hilden, Germany)

protocol. Cells were lysed using the Fastprep (MP Biomedicals) cell homogenizer 'faecal sample' default setting in the lysis buffer provided in the PowerFecal DNA kit. For subsequent analyses, genomic DNA was diluted ten times, to limit the effect of PCR inhibitors.

Quantitative PCR

Total bacteria, total archaea, and specific archaeal lineages (Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales, *Methanimicrococcus*, Thaumarchaeota) were quantified using quantitative PCR with lineage specific primers (Table S5). qPCR was performed on a qTower3 Touch device (Analytik Jena GmbH) using SensiFAST SYBR® & Fluorescein Kit (Bioline, Paris, France). For each run, a standard curve was prepared using a 10-fold serial dilution (10^9 to 10^1 copies/ μ l) of a plasmid containing a 16S rRNA. Plasmids containing a partial archaeal or bacterial 16S rRNA gene were generated through cloning PCR amplified 16S rRNA gene of the groups into *E. coli*. Bacterial 16S rRNA genes were amplified from a faeces sample using the B-27F-YM/B-1492R primer set^{95,96}. Archaeal 16S rRNA genes were amplified from *Methanimicrococcus blatticola*, *Methanocorpusculum aggregans*, *Methanomethylophilus alvus*, *Methanosphaera stadtmanae* and *Nitrososphaera viennensis* using the A-21F/A-1386R primer set^{97,98}. PCR products were cloned with a pGEM-T vector according to the manufacturer's instructions (Promega, Charbonnières-les-Bains, France). The accuracy of the plasmid construction was confirmed through sequencing and all plasmids were diluted to 10^9 copies/ μ l, aliquoted and stored at -20°C. The accuracy of the qPCR assay was confirmed through melting curve analysis. All quantifications were performed twice in independent runs. The final concentration of all the microbial was averaged between replicates and normalized as copies of 16S rRNA gene per gram of faeces.

16S rRNA gene amplicon sequencing

Archaeal 16S rRNA genes were amplified in two steps (Nested-PCR; Table S6) to allow the inclusion of a larger range of samples. Prokaryotic 16S rRNA genes were directly amplified with Illumina tagged primer pairs (Table S6). Sequencing was performed on an Illumina MiSeq platform (Biofidal, Vaulx-en-Velin, France) according to the Illumina protocols for PE 2x300 bp, and resulted in more than 21 million reads and more than 16.7 million reads for the prokaryotic and archaea specific sequencing, respectively.

Microbial Diversity Analyses

Reads were processed and assigned to amplicon sequence variants (ASVs) using the DADA2 software (v1.12.1) in R (v3.6.0). Briefly, reads were trimmed and quality-filtered using the standard parameters - maximum expected errors for forward and reverse reads = 2, quality score = 2, and trimming length = 273 and 170 base pairs for forward and reverse reads, respectively. Forward and reverse reads were merged with a 20 base pair overlap, ASVs were generated, and chimeras were discarded. ASV annotation was performed using the Silva 16S rRNA database (v132). Assignment of ASVs to a main type of methane metabolism (hydrogenotrophic CO₂-reducing, hydrogenotrophic CH₃-reducing, acetoclastic and methylotrophic (methyl-dismutation)), was done based on their taxonomic affiliation, since all members of almost all methanogen genera/families have the same dominant type of methane metabolism (Table S4). *Methanosarcina* is the main exception, as species from this group can have one or several types of methane metabolisms. All ASVs that were not annotated as archaea were removed from the archaeal-specific primer generated sequences, and ASVs annotated as archaea or bacteria were kept from the universal primer generated sequences. Samples from the same species were merged by summing ASV abundances. These approaches resulted in 1307 archaeal ASVs from the archaea specific primers, as well as 140 archaeal ASVs and 19,145 bacterial ASVs from the universal primers. To estimate the novelty of the archaeal ASVs (obtained with the archaea-specific primers), we compared them using BLAST to 16S rRNA genes of isolated archaea retrieved from the SILVA Living Tree Project LTP database⁹⁹ plus additional sequences of candidate species belonging to Methanomassiliicoccales and Thaumarchaeota. For diversity analyses, rarefaction was performed to normalize sequencing depth to 3,000 reads, leading to 1,253 archaeal ASVs. Bacterial ASVs were normalized to a sequencing depth of 12,000 reads per sample. Observed richness (alpha diversity) was estimated and all beta diversity analyses were performed using the 'phyloseq' package in R (v1.30.0). Subsequent statistical analyses were performed using the base Rstudio 'stats' package (v3.6.0) as well as the R package 'vegan' (v2.5-6). To test for significant differences using the various beta diversity metrics (Table 1) a permutational multivariate analysis of variation (PERMANOVA) from the R package 'vegan' (function adonis) was used. A pairwise Wilcoxon rank sum test with continuity correction from the R package 'stats' (function pairwise.wilcox.test) was used to determine differences between the absolute abundance of archaea and bacteria in animal diet types, as well as between animal

classes. Linear regressions from the R package 'stats' (function lm) were used to determine the relationships between the abundance (log-transformed) of methanogens, thaumarchaeota and bacteria, and mean retention time (MRT) and dietary fibre consumption. Significance cut-off was $p < 0.05$ for all analyses.

Placement of ASVs within Reference 16S rRNA gene trees

All archaeal ASVs were filtered on a per sample basis, to keep only ASVs representing at least 1% of the total number of reads of the sample. Reference sequences > 1200 bp with a quality >95% were obtained from the Silva SSU 138 database¹⁰⁰, RDP database¹⁰¹, and an in-house dataset. Redundancy was removed from reference sequences with a 98% or 97% sequence identity threshold using the VSEARCH software¹⁰². For each archaeal order, long reference sequences were combined with the ASV sequences and were aligned using the G-IN-SI algorithm in MAFFT¹⁰³. Phylogenetic trees were generated using the GTR+G4+I model in the IQTREE software¹⁰⁴. The distribution of ASVs host orders were mapped using ITOL¹⁰⁵. Reference sequences were ultimately removed from the tree to only keep the ASVs sequences.

Co-occurrence of Archaea and Bacteria

To identify co-occurrence signal between archaea and bacteria across Mammalia, Reptilia, and Aves, we integrated the sequences from both the Universal and Archaea specific 16S rRNA gene amplicon sequencing. Only bacterial reads were selected from the Universal 16S rRNA gene amplicon sequencing for this analysis. We used VSEARCH¹⁰² to cluster ASVs into OTUs at 97% in order to reduce the size of the dataset and to filter out truly low abundance lineages of microbes. Then, to merge these datasets in a way that accurately represented the microbial community in terms of relative abundance between archaea and bacteria, we normalized the two datasets both in terms of sequence depth and in terms of archaea-bacterial ratios - information which was gathered through qPCR data. OTUs that were present in less than 10% of the animal classes – Mammalia, Aves, and Reptilia independently- were removed. Following this, we implemented both the SPIEC-EASI (Spiec.Easi package v1.1.0,¹⁰⁶) and the SparCC algorithms¹⁰⁷ (part of the Spiec.Easi package (v1.1.0)) in Rstudio (v3.6.0) to determine co-occurrence trends between archaea and bacteria. Networks were calculated with 1000 iterations. The output from these analyses were filtered using a 0.5 minimum threshold of

edge stability (SPIEC-EASI) (Table S3) and a p -value ≤ 0.05 (SparCC), independently. Only the co-occurrence patterns identified by both algorithms were further analysed.

Investigation of archaea distribution in the gut and other environment

All archaeal 16S rRNA gene sequences from Silva database⁹⁹ longer than 800 bp and with more than 80% sequence quality, alignment quality and pintail quality were downloaded. Sequences from metagenomes were removed because their environmental origin was not clearly indicated. The annotation of each sequence was retrieved from GenBank and used to classify them as “Gut”, “Environmental” or “Human built” origin. Sequences from sponge, animal environments (e.g. nest) or polluted sites (e.g. dump) were not included. The relative abundance of each category was mapped on a tree of archaea built with genomic sequences used in Borrel et al.,³⁶ as well as additional DPANN sequences not present in this study.

Gibbs free energies of methanogenic pathways

The following chemical reactions were considered for methanogenic catabolisms:

CH₃-R dismutation:

CH₃-R + CO₂:

CO₂ + H₂:

For each catabolism Gibbs free energy (ΔG_{cat}) calculations were performed using the R package CHNOSZ¹⁰⁸ considering C(methanol) between 10⁻³ and 10⁻⁷ mol/l, p(H₂) between 1 and 10⁻⁷ bar, T = 298 K, pH = 7 and p(CO₂) = p(CH₄) = 10⁻¹ bar.

Origin of the metadata

Animal metadata were collected from various literature sources and online databases. Diet information for mammals and birds were downloaded from the EltonTraits database¹⁰⁹, and information for other animal diets were annotated using the Animal Diversity Web database (Museum of Zoology, University of Michigan, <https://animaldiversity.org/>). Information on body weight were also gathered on this website. Information about coefficients of gut differentiation, pH, diet fibre content, and intestinal tract structure and mean retention time

were gather from ^{3,86,110–114}. Information on methanol, H₂ CO₂ and CH₄ in the gut and in marine sediments originate from ^{18,46,115–123}.

Data Availability

Data have been deposited in GenBank under the bioproject PRJNAXXXX.

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Author contributions

C.M.T., G.B. and S.G. conceived the study. C.M.T. did the experiments and E.D. the thermodynamic calculations. C.M.T. and G.B. analyzed the data and wrote the manuscript with the input of S.G.

Competing interests

The authors declare no competing interests.

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