Chapter 3

Archaebiotics: Archaea as Pharmabiotics for Treating Chronic Disease in Humans?

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Abstract

Recent findings highlight the role of the human gut microbiota in various disorders. For example, atherosclerosis frequently seems to be the consequence of gut microbiota–derived metabolism of some dietary components. Pharmabiotics (i.e., live/dead microbes and microbe-derived substances) and probiotics (live microorganisms with a health benefit when administered in adequate amounts) are a means to counteract these deleterious effects. Among the latter, microbes now being used or, being currently developed, are bacteria and eukaryotes (yeasts), so omitting the third domain of life—the archaea, despite their unique properties that could be of great interest to human health. Here, we promote the idea that some specific archaea are potential next-generation probiotics. This is based on an innovative example of the bioremediation of a gut microbial metabolite. Indeed, besides the fact that they are archaea (i.e. originating from a domain of life from which no pathogens of humans/animals/plants are currently known), they are rationally selected based on (i) being naturally human-hosted, (ii) having a unique metabolism not performed by other human gut microbes, (iii) depleting a deleterious atherogenic compound generated by the human gut microbiota and (iv) generating a health inert gas.

Keywords: archaea, atherosclerosis, cardiovascular disease, methanogens, *Methanomassiliicoccales*, next-generation probiotics, trimethylamine (TMA), trimethylamine oxide (TMAO), trimethylaminuria (TMAU)

1. Introduction

The human intestinal tract is populated with an important number of microbes, which until recently, were believed to account for 10-fold more than the number of human cells [1], but

now more likely being equivalent [2]. This contributes to the recent view of humans and other mammals as being metaorganisms. This microbial community is described as the human gut microbiota whose collective genomes form the gut microbiome with about 100-fold the number of human genes [3, 4]. It possesses specific functions leading one consider it as an active organ which influences multiple functions of the human host [5]. Moreover, its composition is different among individuals, resulting and being dependent on various factors: among them, one can cite the delivery mode at birth (i.e. vaginal vs. caesarean [6]), genetics [7] and environmental factors encompassing drug use and diet [8]. The human gut microbiota is typically composed of about 400 different bacterial species (designated as 'operational taxonomic units', (OTUs)) from a repertory of more than 1.000 OTUs [3, 4, 8, 9]. However, if bacteria are the main constituents of the gut microbiota, it is well known that besides viruses, the two other domains of life (i.e. archaea and eukaryota) are also normal constituents of this microbial community. Archaea represent the second most important group of microbes, in number, with a mean of 1.5% of the microbiota as recently determined from metagenomics studies on large cohorts [9] (**Figure 1**).

Among the various roles attributed to the gut microbiota, it acts as a barrier against colonization of pathogens and for the development and maintenance of the gut epithelium and its structural integrity. Also, it modulates the immune system by the continuous interplay with the host immune system. Lastly, it participates in host nutrient metabolism (which is also extended to drugs and xenobiotics) by enriching energy and compound retrieval from nutrients. It encompasses, but is not limited, to the synthesis of vitamins and the hydrolysis and fermentation of organic matter (e.g. indigestible dietary fibre), leading to the production of short-chain fatty acids (SCFA) [10]. The latter accounts for about 5–10% of the overall energy harvested by host from diet [11].

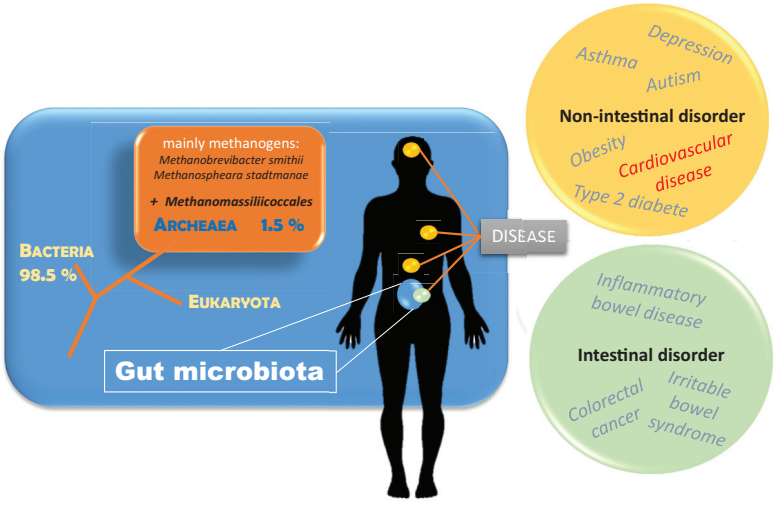

Figure 1. The human gut microbiota, its archaeal component and its role in various disorders, sometimes linked to the gastrointestinal tract (lower right circle), sometimes to other locations (upper right circle).

To date, a close link between the gut microbiota and digestive health has been reported and documented (**Figure 1**). It plays an important role in various digestive disorders [12, 13], like colon carcinogenesis [14], irritable bowel syndrome (IBS) [15] and inflammatory bowel diseases (IBD) encompassing Crohn's disease and ulcerative colitis [16]. On first thought surprising, it is also linked to non-digestive diseases encompassing type 1 and 2 diabetes (T1D and T2D) and obesity, as well as conditions such as asthma, autism and depression [10, 13]. It is also linked to cardiovascular disease (CVD) which will be described in more detail below. Based on these facts, if the microbiota or one of its components is the causing agent of disease, maintaining a healthy microbiota or restoring the microbiota from a dysbiosis state to a normobiosis state should improve health or prevent disorders. Pharmabiotics represent one of the ways by which this can be achieved [17].

In the early twentieth century, Eli Metchnikoff proposed the probiotic concept based on the observation that consuming host-friendly bacteria as done by Bulgarians, i.e. the bacteria found in their yogurts, led to improved health and delayed senility in this population [18]. Probiotics are defined by the World Health Organization (WHO), and the Food and Agricultural Organization (FAO), as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'. Hence, based on this definition, probiotics are microbes that can originate from sources other than food. Based on a rational design inferred from the causation of the disorder, in this chapter, we introduce a new strategic way for discovering new probiotics by highlighting the role that some archaea could play in the human gut. Indeed, one group of archaea found in humans can convert a deleterious compound of gut bacterial dietary metabolism. This bioremediation leads to methane (CH_4), which is considered to be inert for health in the human gut. Therefore, these archaea could become a source of the next generation pharmabiotics, i.e. archaebiotics, originating from the normal composition of the human gut. To date, neither probiotic nor pathogenic microorganisms have been determined to exist in the archaeal domain.

2. Diet, gut microbiota and a particularly deleterious plasma compound

Our health is intimately linked to what we eat. The diet itself may contain deleterious compounds; it may also contain initially neutral or even beneficial nutrients that may become deleterious after being metabolized by our gut microbiota. Cardiovascular disease (CVD) is representative of this two-sided effect. The example below will highlight one of the roles in gut microbiota/diet co-participation. CVD is the leading cause of death according to WHO (2012), corresponding to more than 17 million deaths every year, about one-third of all deaths. A significant part results in the development of atheromatous plaques, an accumulation of lipids and adipose tissue (in particular cholesterol), complex carbohydrates/fibrous connective tissues, minerals and cells (macrophages) on the inner layer of arterial walls. Recognized biological risks are hypertension, chronic hyperglycaemia, hyperlipidaemia and overweight/obesity. Only one fifth of CVD seems to originate from genetic factors, while environment alone, or in combination with genetics likely accounts for all other cases [19]. Among environmental factors which encompass diet, it is very difficult to determine which nutrient(s)

specifically act on atherosclerosis development [20]. However, in 2011, one compound was identified in blood that was intimately linked to CVD and whose origin was associated with various ingredients and nutrients from diet [21].

2.1. Trimethylamine oxide and atherosclerosis

In 2011, the Stanley Hazen's laboratory (Cleveland clinic, Cleveland, USA) used a systematic approach and analysed plasma metabolites from a learning cohort of 100 patients with variable CVD risks. They succeeded in determining three different molecules that were significantly present at higher levels in high-risk subjects; trimethylamine *N*-oxide (TMAO), betaine and choline were linked to CVD [21]. Interestingly, these three compounds may have the same alimentary origin that we will discuss below. Results obtained from this learning cohort were successfully used on a large validation cohort of 1876 subjects [21]. Also, an extension to 4000 subjects indicated that plasma TMAO (pTMAO) levels alone were able to predict a 2.5-fold increase of major adverse cardiac events (MACE) at 2 years [22]. Moreover, using this high-value risk factor for prognostics, the authors showed a direct implication of TMAO, or of its precursors, in the creation and development of atheromatous plaque. Studies done *in vitro* and with animal models (ApoE$^{-/-}$ mice) showed a role at the intestinal level (alteration of the cholesterol reverse transport through biliary acids); at the hepatic level (modification of the quantity and composition of the biliary acids pool) and at the arterial level (inflammation, differentiation of macrophages into cholesterol containing foam cells that bind to the atheromatous plaque). All of this contributes to atherosclerosis. Moreover, pTMAO enhances platelet hyperactivity therefore increasing the thrombosis risk [23], at least in mice.

2.2. Trimethylamine oxide and other disorders

Besides atherosclerosis, trimethylamine oxide (TMAO) has been linked to several other important disorders, which does not necessarily mean that TMAO may cause the development of the disease. Among them, chronic kidney disease (CKD) and, more specifically, the severity of renal dysfunction are strongly associated with the level of pTMAO [24]. This is likely attributable to deficiencies in glomerular filtration which leads to altered increase clearance of pTMAO (see Section 2.3.) and accumulation of TMAO in blood [25]. However, there are several lines of evidence that pTMAO itself also contributes directly to progressive renal fibrosis, at least in animals [26].

Also, type 2 diabetes (T2D) is associated with higher pTMAO levels [27], but until now, it is still unclear if this is due to a confounding action with glycaemic control or renal function. Interestingly however, various mice T2D models showed that one key liver enzyme implicated in the generation of TMA (FMO3, see Section 2.3.) was regulated by insulin [28].

Other disorders analysed so far also encompass colorectal cancer (CRC), for which data remain unclear; a study reported the building of epigenetic interaction networks from data of interactions between chemicals and genes, between diseases and genes and between proteins [29]. This approach was validated by an efficient detection of the known link between TMAO and CVD; it also revealed a stronger genetic link between TMAO and CRC as well as one

weaker with metabolic syndromes. The link between TMAO and the risk of CRC was also established in post-menopausal women [30]. However, all of these results need confirmations/validations, and more importantly, if pTMAO is linked, it is a causing agent.

2.3. Origin of plasma trimethylamine oxide

In order to prevent these disorders, it is essential to determine the plasma origin of the above mentioned and linked molecules: betaine, choline, and more specifically, trimethylamine oxide (TMAO). People given a specific meal (eggs, see below) showed a rise of TMAO in their plasma (pTMAO). Interestingly, after broad-spectrum antibiotic treatment, leading to the eradication of as much as possible gut microbes, pTMAO disappeared after the same meal and reappeared a few weeks after the cessation of the antibiotics treatment, i.e. when the gut microbiota had recovered [22]. Therefore, pTMAO levels are dependent on the nature of the nutrients and of the gut microbiota. Nutrients are provided by a broad alimentary range of sources; and, among them, TMAO itself which is used as an osmoregulant in many marine life forms and is consequently found in some seafood. Much more studied are choline and phosphatidylcholine (PC, also referred as lecithin), which are found in eggs, red meat, fishes, cheese, and some vegetables [31, 32]. It also encompasses L-carnitine which is found mainly in red meat and recently in some energizing drinks. When ingested, a fraction of dietary choline/PC and L-carnitine reaches gut microbes which can metabolize them into trimethylamine (TMA). The metabolic activity of anaerobic organisms is still poorly understood and the bacterial enzymes which can convert these compounds into TMA have only recently been determined [33–36]. TMAO is also metabolized into TMA in the gut by bacteria. **Figure 2** indicates the different pathways that lead to TMA in the gut.

Importantly, the genes encoding these various enzymes are for the most part not specific to a bacterial lineage, and horizontal gene transfers have occurred during evolution. As a consequence, based on strictly microbial composition, i.e. 16S amplicon sequencing, this currently impairs ways to determine the levels of capability of gut microbiota to deal with these compounds in generating TMA. However, two recent papers showed that it is possible to decipher this capability from metagenomics sequencing data (sequencing of whole microbial DNA from gut/faecal microbiota) by seeking and counting reads corresponding to marker genes in these pathways [37, 38].

The discussed metabolic pathways result in TMA in the gut due to the activity of the microbiota. This forms the unique way for the accumulation of TMA in the digestive tract as TMA is not ingested by itself through the diet. In fact, this molecule has a very repulsive odour for humans as it corresponds to the odour of rotten fish, for which humans are particularly sensitive (see Section 2.4.). TMA is absorbed by the gut epithelium, diffuses and reaches the portal vein. It is next oxidized in the liver by monooxygenases, more specifically the hepatic flavin–containing monooxygenase 3 (FMO3), resulting in pTMAO. Interestingly, this enzyme is not only transcriptionally regulated by several elements encompassing sex hormones and biliary acids [39, 40] but also by insulin. pTMAO is excreted via the kidneys into urine. Indeed, TMAO has higher renal clearance than urea and creatinine in healthy humans [25]. However, as previously mentioned, impaired renal function leads to higher pTMAO levels as observed in subjects suffering from chronic kidney disease (CKD) [24].

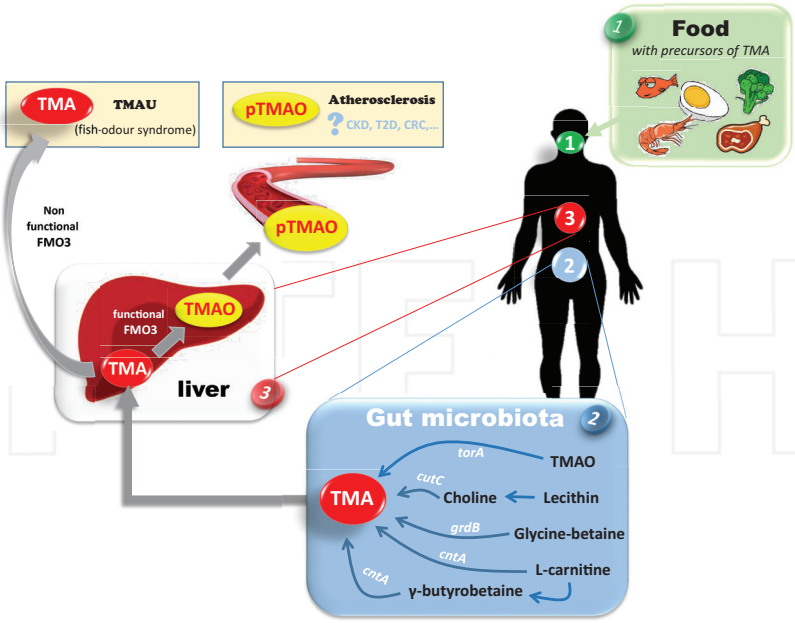

Figure 2. Origin, fate and implication of trimethylamine TMA and trimethylamine oxide TMAO in various disorders through the synthesis of TMA by the human gut microbiota. (1) various kinds of food contain nutrients like TMAO, lecithin, choline and L-carnitine. These nutrients come into contact with various gut microbes (2) that are differentially present among individuals. Some of these gut bacteria can metabolize these nutrients into TMA with dedicated enzymes whose encoding genes are indicated in italics. The TMA generated is absorbed from the gut into the portal vein from which it reaches the liver (3). Some people lack an efficient TMA oxidizing activity through the flavin-containing monooxygenase 3 (upper part, left box), either because of a genetic defect or an acquired one, through hepatic dysfunctions/down-regulation of FMO3 expression: this leads to TMA in blood, which can diffuse in any body fluid from which it is eliminated. This leads to trimethylaminuria (TMAU) or fish-odour syndrome. In a more general case, FMO3 activity is sufficient enough to oxidize TMA into TMAO, which enters the circulation (pTMAO). This compound is deleterious and leads through various mechanisms to atherosclerosis and possibly also chronic kidney disease (CKD), type 2 diabetes (T2D) and colorectal cancer (CRC) (upper central box).

2.4. The peculiar case of trimethylaminuria

Trimethylaminuria (TMAU) is a metabolic disorder leading to a TMA/TMAO ratio in urine above 5%, indicating a deficiency in liver oxidative processing of TMA [41, 42]. This is likely an under-recognized and underdiagnosed disease. It has important psychological and social concerns for people suffering from this metabolic disorder. While TMA is partly excreted in the urine, it is also eliminated in the breath and sweat; affected people have a rotten-fish odour which is very unpleasant. Humans are very sensitive to the odour, probably a remnant of our evolution which prevents us from eating spoiled fish. This fish-odour syndrome (the other name for TMAU) is caused by a deficiency in functional FMO3. This is mainly a genetic disorder (autosomal recessive) corresponding to a rare inborn disease, with about 0.5–1.0% of the UK population being heterozygous carriers in the white UK population [43–45]. Therefore, it

can be presumed that about 1 on 40,000 subjects is affected in Great Britain. Also, temporary symptoms have been described, resulting in secondary/acquired TMAU: this is due to the regulation of the FMO3 (sex hormone dependant), transient liver deficiencies (e.g. viral hepatitis), or overload of dietary precursors of TMA by gut microbes [44, 46]

2.5. Strategy to lower plasma trimethylamine oxide

In consequence, lowering plasma trimethylamine oxide (pTMAO) levels is a goal in reaching the prevention of, at least partially, the disorders discussed above and more specifically atherosclerosis. Theoretically, three main targets may be considered: first, this can be achieved by reducing the precursors of pTMAO that are found in diet. Second, alteration of the gut microbiota composition can modify its metabolic behaviour thereby decreasing the synthesis of intestinal TMA from dietary precursors. Third, intestinal TMA that has been synthesized can be diverted from its hepatic oxidation into TMAO. If we have a more detailed look at each of these possibilities, most of them are unrealistic and/or hazardous for humans.

2.5.1. Decreasing TMAO precursors from diet

There are many concerns about trying to limit the contribution of TMAO precursors in diet. First, it seems unrealistic for several reasons. As noted previously, there are not one but several potential precursors which include lecithin, choline, L-carnitine, and TMAO. Moreover, these nutrients are found in a broad diversity of foods, which makes the formulation of ingredients and meals difficult; this has also to be considered within cultural/individual backgrounds. Also, in this peculiar case where the link between nutrients and pTMAO relies on the gut microbiota composition, which is individual specific, it is very difficult to promote some foods rather than others knowing that not all individuals would be affected in the same way.

However, the concerns over dietary TMAO precursor removal are frustrating considering the main drawback of such a proposition. First, many foods are recognized as health promoting despite the fact that they contain TMAO precursors. For example, fish consumption is recommended twice a week for people at risk for coronary heart disease due the presence of omega-3 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (American Heart Association recommendations) [47]. TMAO and choline/lecithin can be consumed in large amounts via these diets. Second, and much more important, the precursors of pTMAO are highly desirable in sufficient amounts for many other roles they play in our body, and their limitations could lead inversely to other disorders. As a prime example, such recommendations could lead to choline deficiencies, whose daily intake has been established to be between 425 and 550 mg/day, respectively, for women and men in the United States. These levels are required to avoid the induction of major cognitive complications and liver disease such as hepatic steatosis [48–50].

2.5.2. Decreasing intestinal TMA synthesis

Decreasing TMA synthesis in the gut by the microbiota would result in less TMA reaching the liver where it is converted into deleterious pTMAO. This goal may be achieved by several ways,

for example, the use of medication, prebiotics, probiotics and faecal microbiota transplantation (FMT). To our knowledge, there is no evidence, to date, of a probiotic with activity that is capable of reducing TMA synthesis in the gut; but, the use of probiotics would be a fine strategy due to its potential for large-scale use. FMT is also very promising as it is used for several disorders that are associated by a dysbiosis of the gut microbiota and could be of the highest interest in this case. However, this technique is still in its infancy and long-term consequence/maintenance of a supposed 'good' microbiota is unknown. In fact, it seems that such maintenance is rather an 'ecological agreement' between the recipient (its physiology/genetics/diet/gut microbiota) and the faecal microbial transplant, leading to a mixed microbiota of host/donor. It cannot be assumed that microbes leading to TMA will be eradicated or at least drastically lowered. Therefore, such an intervention may have to be conducted frequently and at a large scale. This approach relies on limited donors, and there is an absence of a biotechnological way to amplify such complex microbiota. Also, there is still a lack of an efficient safety procedure concerning the donor at the moment of transplantation due to the absence of an efficient way to preserve the faecal microbiota; increasing donors and repeating the process could lead to an increase of the risk of transmission of infectious diseases by viruses or pathogenic bacteria.

Prebiotics is another way to support beneficial microbes of the gut. It can be noted that compliance to a Mediterranean diet (which is known to decrease CVD risks) leads to lower pTMAO levels [51], lending support to the fact that some elements of this kind of diet may contain beneficial factors for this purpose. Interestingly, it has been shown recently that resveratrol, a natural phytoalexin found notably in grapes and i.e. the Mediterranean diet has a prebiotic effect; it remodels the gut microbiota in a beneficial way (encompassing an increase of bile acids by *de novo* synthesis in the liver) and leads to a decrease of pTMAO [52].

Finally, medication is another way by which the gut microbiota may be remodelled. At the extreme, the use of antibiotics provides an efficient way to this goal, it is sometimes used to treat TMAU patients [42]. This approach proves that TMA synthesis can be efficiently but temporarily curtailed; it seems, however, unreasonable to propose the large-scale use of broad spectrum antibiotics for this purpose, or its repeated use in affected individuals. Ideally, the use of a drug or a combination of drugs would have a more specific action, either by targeting bacteria from groups that are known to potentially produce TMA or better by targeting the bacterial enzymes involved in TMA synthesis. A very promising drug has been recently described, 3,3-dimethyl-1-butanol (DMB) [53]. This compound is a substrate analogue of choline that inhibits TMA-lyases, the bacterial enzyme family that converts several substrates into TMA. It is not only active against the synthesis of TMA from choline, but also from L-carnitine, at least in some bacterial groups and does not seem to have any lethal effect on these bacteria. Therefore, its use should not lead to dramatic changes in the microbiota constitution. Also, interestingly, DMB is naturally found in components of the Mediterranean diet (olive oils, red wines) [54]. It remains however to be determined to which extent DMB use will decrease pTMAO. In addition, another drug was described a few years ago, already known to have clinically cardioprotective effects, supposedly by lowering L-carnitine content in the body [55]. This compound, meldonium (also referred as mildronate, formally 3-(2,2,2-trimethylhydrazinium) propionate dehydrate), has been shown to lower pTMAO [56] which may be due partly on the prevention of bacterial L-carnitine use [57].

2.5.3. Preventing TMA oxidation into TMAO by the liver

Once TMA has been generated by the microbiota in the gut, one can conceivably try to lower its oxidation into pTMAO by host FMO3 enzymes in the liver. Among various solutions, one could focus on the inhibition of FMO3. However, this would (i) likely lead to deleterious effects by affecting the oxidation/detoxification of other compounds and (ii) allow TMA to enter the general circulation at high amounts; symptoms similar to TMAU would appear, i.e. a rotten fish odour emanating from the subjects. Therefore, alternatives need to be found that lower TMA availability in the gut. While it seems unrealistic to inhibit specifically its absorption by acting on epithelial transporters, charcoal is sometimes efficiently used by TMAU patients [42], which leads to a decrease of symptoms by increasing TMA excretion in faeces. Therefore, this likely lowers TMA in body fluids in these patients, and it can reasonably be assumed that TMA reaching the liver would be lowered in non-TMAU patients, and that, in consequence, pTMAO would be lowered. However, it is unclear if this can be applied as a long-term strategy without lowering the absorption of other important intestinal compounds and that could lead to deficiencies.

In summary, it would be of great interest to have a specific agent acting only on intestinal TMA. This could be achieved by a biological system able to convert TMA into inert molecules in a complex environment (the gut and its microbiota) and without danger to humans. While no bacterial species seems suitable, one recently identified group of archaea seems to provide all these properties.

3. Human naturally hosted archaea that prevent plasma trimethylamine oxide formation

Archaea belong to one of the three domains of life [58]. They are unicellular microorganisms distinct from bacteria and eukaryotic cells in regard to evolution and some of their cellular/molecular processes. For example, their cell membrane has a different composition compared to bacteria and eukaryotes [59]. In fact, archaea can be characterized as cells looking like bacteria but having cellular, molecular and genetics characteristics of eukaryotes but with some differences. One archaeal group recently identified (*Lokiarchaeota*) has been proposed to be at the origin of eukaryote evolutionary divergence, and so, perhaps, a direct ancestor of our own cells [60]. Also, the domain *Archaea* contains microbes with a unique mechanism by which they derive energy not found anywhere else in other life forms, i.e. methanogenesis [61]. Some methanogenic archaea are found as natural components of the gut microbiota [62], where they are principal methane producers. So far also, no pathogens are known among the archaea, neither in humans nor in any other animal or plants.

3.1. A recently discovered archaeal lineage primarily recovered from human stools that thrived in various environments

About a decade ago, we revealed the existence of unknown archaea in the faecal microbiota of some humans [63, 64], with clues that they were methanogens even if belonging to a lineage

(*Thermoplasmata*) with no other known methanogens. Up to now, only one member has been isolated in pure culture. *Methanomassiliicoccus (Mmc.) luminyensis* strain B10 was isolated by the joint efforts of two laboratories [65], which lead to the creation of a new methanogenic archaeal order named *Methanomassiliicoccales* [66]. Also, we have currently a highly enriched culture with another strain from this order which shows only 89% nucleotide identity for its 16S rRNA compared to *Mmc. luminyensis* B10. It has been named *Ca*. Methanomethylophilus alvus Mx1201. These two strains were obtained from human faecal samples. Phylogenetic data based on a DNA survey revealed that this group of methanogens is found in various ecosystems [67, 68]. These anaerobic environments encompass notably seafloor, wetlands, biogas fermenters, etc. but also the gastrointestinal tract of a broad variety of animals. Among them, one can cite insects like termites [69], ruminants [68, 70–73], and humans [62–65, 74]. Very interestingly, three clusters of *Methanomassiliicoccales* have been identified [67, 68], reflecting the environmental source of sampling. One large cluster of neighbouring 16S and/or *mcrA* (a molecular marker of methanogens) sequences corresponds to members almost exclusively retrieved from gastrointestinal tracts. *Ca*. Methanomethylophilus alvus Mx1201 belongs to this cluster, also referred as the host-associated- or Gastro-Intestinal Tract (GIT) clade. A second cluster contains members retrieved from GIT as well as anaerobic environments like soils or freshwater lakes, etc. and is therefore named the mixed cluster, to which *Methanomassiliicoccus* spp. belongs. Finally, the third clade is formed of members exclusively recovered from nondigestive environments, at least until now [67, 68].

Still little is known about these archaea, except for the fact that they are methanogens with a peculiar metabolism [72, 75, 76]. Their biology is mainly known from genomic studies with recent new data (**Table 1**). Also, microbiological studies have been possible on enrichment

Name	Clade	Host/ Environment	Isolate[$]	TMA use[*]	References
***Mmc. luminyensis* B10**	Mixed	Human	Y	Y (G-AE)	Gorlas et al. [93]; Borrel et al. [67, 84]; Brugère et al. [80]
***Ca*. Mmp. alvus Mx1201**	Digestive	Human	-	Y (G-EE)	Borrel et al. [75]
***Ca*. Mmc. intestinalis Issoire Mx1**	Mixed	Human	-	Y (G)	Borrel et al. [67, 84]
***Ca*. Methanoplasma termitum**	Digestive	Termite	-	N (G-EE)	Lang et al. [77]
RumEn MG1–RumEn MG2	Digestive	Bovine	-	Y (G)	Sollinger et al. [68]
ISO4-G1	Digestive	Sheep	-	Y (G)	Kelly et al. [94]
ISO4-H5	Digestive	Sheep	-	Y (G)	Li et al. [95]
***Ca*. Mmp. sp. 1R26**	Digestive	Bovine	-	Y (G)	Noel et al. [96]

Notes: [$]Y refers to a *Methanomassiliicoccales* strain that has been obtained in pure culture.

[*]Y and N signify, respectively, that the strain can use (Y) or not use (N) TMA as a methanogenesis substrate, based on genomic evidence (G), experimental evidence with enriched culture (EE) or axenic culture (AE).

Table 1. Available genomic data from *Methanomassiliicoccales* (end of year 2016).

cultures with the isolate *Mmc. luminyensis* B10 [65]. However, the highly enriched culture of *Ca*. Methanomethylophilus alvus Mx1201 from the host-associated clade has already helped to characterize one potential health-related metabolic property.

3.2. TMA remediation into methane by some *Methanomassiliicoccales*

3.2.1. Original methanogens with unique biological property

The biological features, mainly deciphered from genomic data, have revealed several uncommon points. Among them, it is likely that they possess two cellular membranes [77], whose composition is typical of other archaea; their phospholipids are indeed composed of L-glycerol (instead of D-glycerol as observed in bacterial and eukaryotic phospholipids) and of isoprenoid side-branch chains (instead of fatty acids as in the two other domains of life). They are linked to L-glycerol by an ether bond, instead of an ester bond. However, the cell membranes of *Mmc. luminyensis* are somewhat different from known archaeal membranes. There are chemically important nuances; some tetraether lipids (typically two L-glycerols linked by isoprenoid chains at both sides and thus not forming a two-layer membrane) are composed of one butanetriol or one pentanetriol replacing one L-glycerol [78]. Also, the processes that drive energy acquisition from methanogenesis (electron-transport and proton extraction outside the cell to be used as a proton motive force linked to ATP synthesis) are different from other methanogens [76, 77, 79] and are not currently fully understood.

However, the other important feature is their ability to synthesize and incorporate a specific unusual amino-acid, pyrrolysine (IUAB nomenclature: Pyl, O) during the translation of mRNAs [76, 80]. This incorporation is mediated by a dedicated tRNA, whose particularity, besides its size, is to recognize the *amber* codon UAG, which is usually one of the three nonsense codons (**Figure 3**). This '22nd amino-acid' is restricted to specific organisms (methanogens of the taxonomic family *Methanosarcinaceae* and about 20 different bacterial species) and to specific proteins, the methyl transferases that can capture methyl groups in methylamines (i.e. monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA)). The reader can find more information about this amino acid and the mechanisms underlying its incorporation into proteins, at least in bacteria, in various excellent articles and reviews [81–83].

3.2.2. Genomic data indicating a TMA remediation in some but not all members of Methanomassiliicoccales

Analysis of the genome of *Ca*. Methanomethylophilus alvus strain Mx1201 [75] and *Ca*. Methanomassiliicoccus intestinalis strain Issoire Mx01 [84] revealed that these *Methanomassiliicoccales* could use methylamines as substrates for their methanogenesis. Comparative genomic analysis with the genome of *Mmc. luminyensis* revealed that it should also be the case for this unique isolate available at this time [76]. Indeed, all the genes necessary for this metabolism were present, sometimes at more than one copy each. Here, we will focus on the genome of *Ca*. Methanomethylophilus alvus strain Mx1201 as it specifically illustrates this point. As shown in **Figure 3**, the genes encoding methyltransferases and associated proteins, together with the genes necessary for methane release (methyl-coM reductase (MCR)) are present; this encompasses genes for the use of MMA

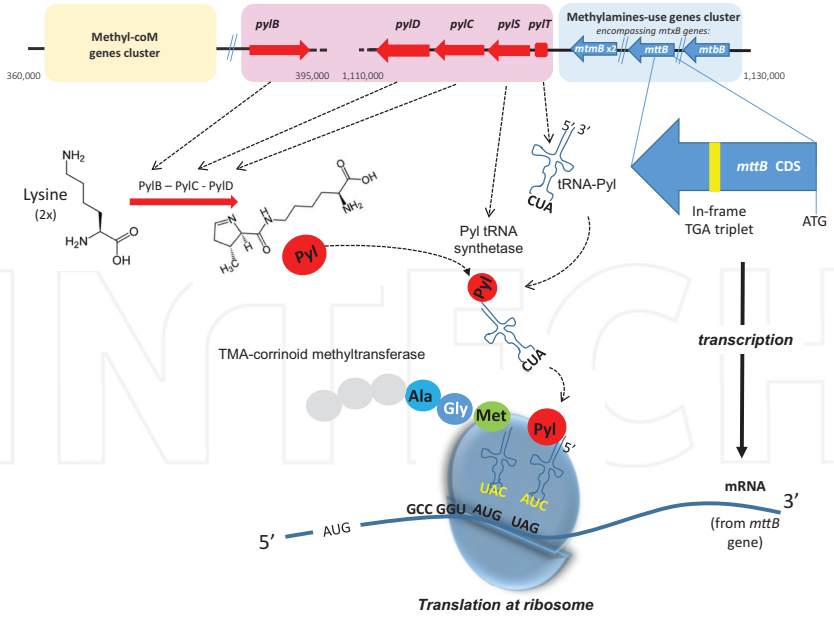

Figure 3. Synthesis mechanism of functional, pyrrolysine-containing TMA-corrinoid methyltransferase. The genetic loci of important genes involved in H_2-dependant methanogenesis using TMA as a substrate are given in the upper part of the figure, taking *Ca*. Methanomethylophilus alvus Mx1201 as an example. Genes involved in the synthesis and translational use of Pyl are indicated by arrows in the upper central box. It encompasses *pylB* which is genetically linked to the genes encoding the methyl-coM reductase (MCR, upper left box), an essential enzyme of methanogenesis necessary for the ultimate reaction in any methanogenic pathway. It also encompasses four other genes that neighbour genes implicated in the use of methylamines (MMA, DMA and TMA; upper right box). Among them, only genes encoding methylamines-corrinoid methyltransferases are shown (*mtxB*), with a focus (right part) made on the *mttB* CDS (coding DNA sequence) which encodes TMA-corrinoid methyltransferase. A TGA triplet is found in its reading frame. Pyrrolysine originates from two lysines using three enzymes encoded by *pylB, C* and *D*. A specific Pyl-tRNA bearing a CUA anticodon is synthesized by transcription of *pylT*. It is further charged with pyrrolysine by specific enzymatic activity of a dedicated AA-tRNA synthetase encoded by the *pylS* gene. After transcription of the *mttB* gene, the corresponding mRNA is translated on ribosomes (lower part of the figure). The *amber* codon UAG, instead of being interpreted as a translational stop, is instead recognized by Pyl-tRNA, which leads to the continuation of translation and the incorporation of Pyl into the TMA-corrinoid methyltransferase, which is necessary for its biological activity.

(*mtm* genes), DMA (*mtb* genes) and TMA (*mtt* genes). Also, the genes for the use of methanol for methanogenesis (*mta* genes) are present. At the current stage of our investigations, data seem to indicate that this species is limited to four possible substrates, namely methanol, MMA, DMA and TMA, at least one of them being essential for its life. In fact, all other potential pathways for methanogenesis are absent, as genomes sequenced to date show a complete lack of genes involved in methanogenesis from CO_2 + H_2 (hydrogenotrophic methanogenesis) and from acetate (aceticlastic methanogenesis pathways) [68, 76, 77]. Considering methylamines, their use is dependant of the ability of the organism to encode Pyl: as previously mentioned, this is the case, at least for *Ca*. Methanomethylophilus alvus, *Mmc. luminyensis* and *Ca*. Mmc. intestinalis. However, the example of *Ca*. Methanomethylophilus alvus is very interesting; instead being grouped in one

operon as typically found in other Pyl-coding organisms, the *pyl* genes necessary for synthesis and translational incorporation of Pyl (encompassing the gene encoding the *amber* suppressive tRNA) are scattered among two different loci. The gene *pylB* which encodes one enzyme necessary for the biosynthesis of Pyl is found in the vicinity of the genes encoding the essential enzyme of methanogenesis, MCR, while all other *pyl* genes are found in the same locus for genes encoding methyltransferases for the use of MMA, DMA and TMA. This can be interpreted as an evolution which has favoured the linkage of methanogenesis (*mcr* operon), Pyl encoding property and the use of methylamines. This highlights the possible importance of this organism, which lives in the human gut, having the ability to use methylamines.

However, the genetic properties for the use of TMA for methanogenesis are not shared by all *Methanomassiliicoccales* in humans [38]. Currently, available sequence data of *Methanomassiliicoccales* genomes (end of year 2016) also reveal that one termite-hosted *Methanomassiliicoccales* does not carry the *mtt* genes for TMA use [77] (**Table 1**): using enrichment cultures, it showed no growth on TMA or DMA (in presence of H_2, see below) but was able to grow on methanol and MMA (+H_2), as predicted from genomic data [77]. So, one question remains: are there some *Methanomassiliicoccales*, preferably isolated from human GITs for which TMA depletion into methane is demonstrated?

3.2.3. Experimental evidence that TMA is depleted by some human gut members in presence of H_2

In addition to experiments that have been conducted with enriched, nonpure cultures, we have been able to show that the isolate from the mixed cluster *Mmp. luminyensis* was able to use methanol as well as MMA and TMA, in presence of H_2, to generate methane [80]. Recently, experiments were also conducted with *Ca*. Methanomethylophilus alvus in enrichment cultures [38]. The results clearly indicated that this strain, obtained from a human faecal sample, is able to use TMA in the presence of H_2. Importantly, H_2 is a gas which is generated in large amounts in the gut by the fermentative metabolism of gut microbes. In order to keep fermentations of high yield, without catabolic repression, it needs to be efficiently removed by hydrogenotrophs, i.e. mainly hydrogenotrophic methanogens, sulphate reducers and bacteria performing reductive acetogenesis. Also, analyses of faecal TMA levels have very recently revealed significant differences of the presence or the absence of *Methanomassiliicoccales* [38]: In fact, in the Irish ELDERMET cohort consisted of older subjects (>64 years of age) [85, 86], subjects had significantly lower TMA concentrations in their stools when harbouring *Methanomassiliicoccales* possessing the genetic behaviour to deplete TMA compared with subjects not carrying *Methanomassiliicoccales*. The significance of this observation was increased when the level of potentially TMA using *Methanomassiliicoccales* was above 10^8 cells/gram of stool than below [38].

4. Conclusion: archaebiotics, next-generation probiotics?

Through these preliminary data, it is very tempting to propose the use of strains like *Ca*. Methanomethylophilus alvus Mx1201 as next-generation probiotics (**Figure 4**). That is, a periodic ingestion of an archaeal strain in a form and dosage that remains to be determined

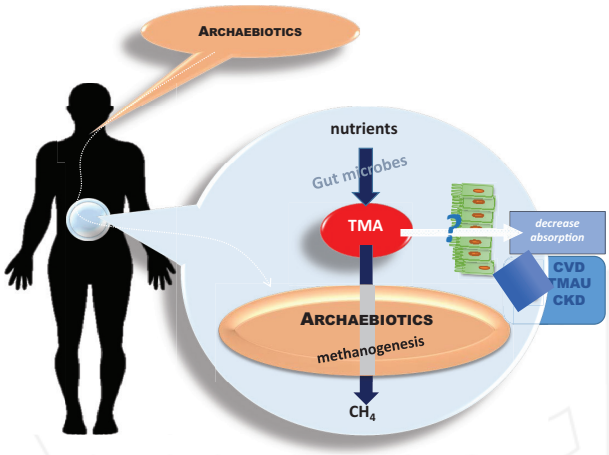

Figure 4. Principle and indications for the use of *Methanomassiliicoccales* as an archaebiotic for depletion of atherogenic trimethylamine TMA. Using the unique property of TMA use by some *Methanomassiliicoccales* and knowing the mechanisms by which pTMAO originates, it is proposed using the human-hosted strain *Ca*. Methanomethylophilus alvus Mx1201 and other rationally selected strains to lower TMA generated by gut microbes directly in the gut/before its absorption. This bioremediation would lead to methane which is considered as a heath-inert intestinal gas. This would, either limit circulating TMA, therefore lowering symptoms of trimethylaminuria, or limit pTMAO levels, therefore preventing, at a minimum, atherosclerosis and chronic kidney disease.

with the aim of preventing at least CVD by limiting the yield of pTMAO. The mechanism relies on the remediation of gut TMA synthesized by gut microbes into a gas (methane) considered as inert to human physiology. Interestingly, this action should also be effective in lowering the symptoms of TMAU. To our knowledge, this is, therefore, a very innovative probiotic design considering that

- Archaea, for which no pathogens are known, had not been considered for probiotics use until now.
- The microorganisms that fit this use are directly selected from human gut microbial inhabitants. They are more prevalent in older people when considering Western countries [38, 64, 74, 87], but they are also highly prevalent in any age group in one Amazonian tribe, the Yanomami hunter-gatherers in Venezuela [88].
- Their selection is based on the knowledge of the mechanisms underlying the pathology and is therefore rationally selected first using simple *in vitro* tests (here, capability of TMA usage).

Importantly, despite lacking definitive proof, it has to be noted that the use of TMA by some human-hosted *Methanomassiliicoccales* is very likely the metabolism they naturally perform in the gut environment. The distribution pattern of the *pyl* genes in the genome of *Ca*. Methanomethylophilus alvus Mx1201 supports this hypothesis (see above). In fact, it is also suspected that this metabolism is the reason for their presence in such an environment; i.e. they occupy an ecological niche that other gut members cannot occupy, and they are highly adapted to this environment. Considering

its importance to resisting this host-derived antimicrobial component, the presence of a bile salt hydrolysis gene in *Ca*. Methanomethylophilus alvus Mx1201 from a bacterial lateral gene transfer (LGT) also supports this hypothesis. It should also be considered that this activity has a possible role in CVD prevention by lowering circulating cholesterol [89].

In any case, many more experiments are needed to address the question that some *Methanomas siliicoccales* may be efficiently and safely used in humans. *In vitro* experiments in systems mimicking the gut microbial environment [90, 91] which has been shown to efficiently support methanogens [92] should help to determine the metabolic behaviour of these archaea as well their interactions with other gut microbes. The isolation of a digestive clade member (for example, *Ca*. Methanomethylophilus alvus Mx1201) is therefore an essential and necessary step, before animal and clinical tests.

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