Digestive tract methanodrome: Physiological roles of human microbiota-associated methanogens

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Abstract

Methanogens are the archaea most commonly found in humans, in particular in the digestive tract and are an integral part of the digestive microbiota. They are present in humans from the earliest moments of life and represent the only known source of methane production to date. They are notably detected in humans by microscopy, fluorescent *in situ* hybridization, molecular biology including PCR-sequencing, metagenomics, matrix-assisted laser desorption ionization time-of-flight mass spectrometry and culture. Methanogens present in the human digestive tract play major roles, in particular the use of hydrogen from the fermentation products of bacteria, thus promoting digestion. They are also involved in the transformation of heavy metals and in the use of trimethylamine produced by intestinal bacteria, thus preventing major health problems, in particular cardiovascular diseases. Several pieces of evidence suggest their close physical contacts with bacteria support symbiotic metabolism. Their imbalance during dysbiosis is associated with many pathologies in humans, particularly digestive tract diseases such as Crohn's disease, ulcerative colitis, diverticulosis, inflammatory bowel disease, irritable bowel syndrome, colonic polyposis, and colorectal cancer. There is a huge deficit of knowledge and partially contradictory information concerning human methanogens, so much remains to be done to fully understand their physiological role in humans. It is necessary to develop new methods for the identification and culture of methanogens from clinical samples. This will permit to isolate new methanogens species as well as their phenotypic characterization, to explore their genome by sequencing and to study the population dynamics of methanogens by specifying in particular their exact role within the complex flora associated with the mucous microbiota of human.

Keywords : Digestive tract, Human microbiota, Methanogens.

1. Introduction

Methanogenic archaea (referred to herein as methanogens) and halophilic archaea are the two sole groups of archaea that have been isolated and cultured from the human digestive tract whereas several other groups comprising of *Crenarchaeota* and *Traumarchaeota* have been sequence-detected, leaving unknown about their viability and the potential roles they may have in the physiology of the gut [1–3]. Methanogens are part of the human microbiota but are more prevalent in the digestive tract (Fig. 1). In a recent study, methanogens have been also found in colostrum and breast milk, notably *Methanobrevibacter smithii* and *Methanobrevibacter oralis* [4]. These two methanogens were found in colostrum and milk of healthy lactating mothers by culture, quantitative polymerase chain reaction (qPCR) and amplicon sequencing for the first time ever in our laboratory [4].

The important findings have been published shaked our picture of the ecology and importance of the archaea including the discovery of Thaumarchaeal ammonia oxidation, anaerobic methane oxidation [5–7], the discovery of methanogens seventh order [1,8–11] and the discovery of the methanogenic properties in *Bathyarchaeota*, a noneuryarchaeal lineage [12].

In nature, most microorganisms grow in mixed consortia. The first evidence of this consortia is methanogens which actively interact with other microorganisms was obtained from defined pure cultures, where syntrophy, mostly based on hydrogen transfer, which is the driving factor for potential benefit for both partners [5,6,13]. This syntrophy is found in biofilm, consortium or stable microbial-microbial [5].

Archaea are now recognized as members of human microbiota [1,6]. However, the archaea stay as forgotten players in the human microbiota for three main reasons: microscopic examination is non-specific; the culturing of some archaea requires an unusual atmosphere

consisting of hydrogen and carbon dioxide; and PCR-based detection requires specific primers and probes [7]. Though, several clinical microbiology teams have detected archaea including methanogens and halophilic archaea within the oral and gut microbiota with potential roles in some diseases [6,13].

In this review, we will discuss physiological roles of human microbiota-associated methanogens.

2. Methods

2. 1. Research strategy and article selection

A bibliographical search was carried out on the "Google", "Google Scholar", "PubMed" and "Web of Science" databases, using the key words: "human microbiota", "methanogenic archaea" (Fig. 2). These key words were used *in solo* and joint searches, in order to carry out a more exhaustive search as possible. We did not use a time limit in our article search, but patents and citations were excluded in our research. After reading the titles and summaries, we kept only the articles related to our subject. Then we eliminated all the duplicates.

2. 2. Setting

Bibliographic searches identified 301.846 articles on Google, Google Scholar, PubMed and Web of Science. The first selection allowed us to exclude 301.295 articles after searching titles and abstracts. Then we eliminated 458 duplicates. So, 92 articles were selected to write this review.

3. Methods for studying human archaea in clinical microbiology

3.1. Direct microscopic examination and fluorescent *in situ* hybridization (FISH)

Direct microscopic examination of methanogens is based on auto-fluorescence. Methanogens carry factor 420, causing blue–green auto-fluorescence when they are exposed to UV light at a wavelength of 420 nm [14,15]. So, methanogens cells or colonies can be quickly identified by epifluorescence microscopy [15]. This auto-fluorescence is an interesting feature used in methanogens growth monitoring.

Methanogens can be observed microscopically using fluorescent *in situ* hybridization (FISH) incorporating an oligonucleotide probe targeting the archaea 16S rRNA gene or a specific methanogens probe targeting the methyl coenzyme-M reductase gene (*mcr*A) [16,17]. FISH is a reliable method for the visualization of methanogens in the oral mucosa [18] and the gastric mucosa [19] (Fig. 3).

3.2. Molecular approach

There are many routinely used PCR-based detection systems for archaea, one is targeting the archaeal 16S rRNA gene (forward primer: 5'- TCCAGGCCCTACGGG-3'; reverse primer: 5'- YCCGGCGTTGAMTCCAATT-3'; probe: FAM 5'-CCGTCAGAATCGTTCCAGTCAG-3') [16], another one is targeting the *mcr*A gene encoding a methyl-coenzyme-M reductase subunit (an enzyme involved in methanogenesis) (forward primer: 5'- GCTCTACGACCAGATMTGGCTTGG -3'; reverse primer: 5'-CCGTAGTACGTGAAGTCATCCAGCA -3'; probe: FAM 5'-ARGCACCKAACAMCAT GGACACWGT -3') [17] and haloarchaea-specific primers Halo 1F and Halo 1R [20]. Also, a real-time PCR protocol targeting the *rpoB* gene encoding for the beta subunit of the RNA polymerase, could be used for the detection of *Methanobrevibacter smithii* (forward primer: Ms_rpoBF, 5'-AAGGGATTTGCACCCAACAC-3'reverse primer: Ms_rpoBR, 5'-GACCACAGTTAGGACCCTCTGG-3'; probe: Ms_rpoBVIC, 5'-ATTTGGTAAGATTTGTCCGAATG-3') and *Methanosphaera stadtmanae* forward primer:

Stadt_16SF, 5'-AGGAGCGACAGCAGAATGAT-3' Reverse primer: Stadt_16SR, 5'-CAGGACGCTTCACAGTACGA-3'; probe: Stadt_16SFAM, 5'-TGAGAGGAGGTGCATGGCCG-3' [21].

3.3. Culture approach

There is different culture approach for archaea isolation from human clinical sample. For methanogens culture we use a technique which is consisting of the use of two compartments for the aerobic culture of methanogens in the presence of *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) producing hydrogen, the aerobic culture of methanogens being made possible by using antioxidants. In brief, the sample is seeded in the Hungate tube containing SAB broth supplemented with ascorbic acid, uric acid and glutathione as anti-oxydants and inoculated with *B. thetaiotaomicron* to produce hydrogen. Subculture seeded on a Petri dish containing SAB medium supplemented with agar and deposited in the upper compartment and the lower compartment contains a culture of *B. thetaiotaomicron* [22]. Recently, we developed a new technique for methanogens isolation in clinical microbiology. This new technique uses a chemical method form hydrogen production that allow a constant hydrogen production and increase a number of methanogens colonies compared to biological method [23]. In addition, we used new culture conditions to culture halophilic archaea. The culture enrichment and isolation procedures for the culture of halophilic prokaryotes were performed in a Columbia broth medium (Sigma-Aldrich), modified by adding (per Litre): $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; KCl, 2 g; $CaCl_2 \cdot 2H_2O$, 1 g; NaBr, 0.5 g; $NaHCO_3$, 0.5 g and 2 g of glucose. The pH was adjusted to 7.5 with 10 M NaOH before autoclaving. All additives were purchased from Sigma-Aldrich. Four concentrations of NaCl were used (150 g L-1, 200 g L-1 and 250 g L-1) [24].

3.4. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS has recently emerged as a rapid and cost- effective technique for the identification of bacteria, eukaryotes, and giant viruses [25–33]. To date there is only one study on the identification of archaea by MALDI TOF MS [34]. The protocol consists in mechanically lysing a suspension of methanogens colonies with glass beads in an Eppendorf tube, the supernatant obtained after the mechanical lysis being re-suspended two times in water after two centrifugations. The MALDI-TOF MS-based clustering of these archaeal organisms was consistent with their 16S rDNA sequence-based phylogeny. The obtained data proved that MALDI-TOF MS profiling could be used as a first-line technique for the identification of human archaea including halophilic and methanogens [34].

3.5. *In vitro* susceptibility testing

Most antibiotics used to fight bacteria are *in vitro* inactive against methanogens [35]. Indeed, intestinal *Methanobrevibacter smithii* (*M. smithii*) isolates are highly resistant to beta-lactams, aminoglycosides, glycopeptides, lincosamides and fluoroquinolones and susceptible to metronidazole, fusidic acid, rifampicin, bacitracine and squalamine [35–38]. Lovastatine is a pro-drug, which needs to be metabolized by anaerobes before being active against *M. smithii* [39]. The *in vitro* susceptibility of methanogens to chloramphenicol is variable: *M. smithii, Methanobrevibacter oralis*(*M. oralis*) and *Methanomasillicoccus luminyensis*(*M. luminyensis*) encodes a chloramphenicol O-acetyltransferase and exhibit minimum inhibitory concentration (MIC) up to 25 mg/L, in contrast to *Methanosphaera stadtmanae* (*M. stadtmanae*) which exhibits MIC of 4 mg/L [36].

4. Role of Human-associated methanogens

4.1. Direct physical contact might support symbiotic metabolism

Methanogens in different human microbiota are in close contact with bacteria. This association between methanogens and bacteria has been used to develop a method of cultivating methanogens in co-culture with *B. thetaiotamicron* which produces hydrogen and therefore allows the production of methane by methanogens by consuming hydrogen [22].

Methanobrevibacter massilensis was co-cultivated for the first time in the oral cavity with the bacterium *Pyramidobacter piscolens* [40] and a recent article has shown Syntrophy via Interspecies H_2 Transfer between *Christensenella* and *Methanobrevibacter* genus [41].

4.2. Methanogens and carbohydrates degradation

The human diet contains numerous oligosaccharides and polysaccharides (complex carbohydrates) correlates with the evidence that the digestive microbiota contains microbial species diversity that have evolved in a very large number and variety of enzymes to assimilate (break down) these molecules to simple sugars [1,42–46]. In this sugar environment, methanogens stand out by their almost complete lack of enzymes for carbohydrate assimilation. Thus, the genomes of *M. smithii*, *M. oralis*, *M. stadmanaea*, *Methanobrevibacter arboriphilicus* (*M. arboriphilus*) and *Methanobrevibacter millerae* (*M. millerae*) do not encode enzyme for the breakdown of glycosidic bonds, while *M. luminyensis* only encodes two enzymes involved in its own intracellular trehalose cycle. Although these methanogens have little capacity to breakdown external glycans to monosaccharides, their genomes do contain the enzymes that can link monosaccharides to a variety of acceptors to build glycoconjugates. It has been reported that the methanogens are unable to recycle the carbohydrate structures that they assemble [47].

4.3. Methane release and H_2 consumption

Methanogens such as *M. smithi* and *M. stadtmanae* have able to remove hydrogen excess from the gut in the case hydrogen accumulation in human gut reduces the energy and the efficiency of microbial processes [48]. The gut methanogens metabolize the hydrogen generated during the fermentation of carbohydrates into methane and promotes more ATP synthesis in anaerobic bacteria in the gut microbiota and subsequently promotes resident bacterial population growth including opportunistic pathogens. This evidence suggests that methanogens, through their methane production, can have a directly positive impact in human intestinal transit[48].

In an anaerobic respiration, methanogens oxidize carbon such as CO_2 as a terminal electron acceptor. Thus, methanogens are common in habitats that are poor in other electron acceptors, (O_2, NO3, Fe^{3+} and SO_4^{2-}). Methanogens as strict anaerobes have long been classified to be limited to anoxic habitats. However, recent studies have shown that some methanogens are able to produce methane in oxygenated soils [49] and even in human microbiota [18]. Methanogens were classified into three biochemical groups based on the substrates using for hydrogen production: hydrogenotrophic, aceticlastic and methylotrophic [6,41,49] (Fig. 4). The most group described in human microbiota is hydrogenotrophic methanogens who oxidize H_2, formate or a few simple alcohols and reduce CO_2 to CH_4 [1,6,41].

4.4. Heavy metal transformation

Heavy metals or metalloids are transformed into methylated derivatives, which are more toxic compounds [50]. *M. smithii* and *M. stadtmanae* were shown to be able to produce more trimethyl-bismuth by bismuth reduction produced in human feces[50,51]. This volatile bismuth is produced in human feces and has toxic effects not only on human cells but also on bacteria such as *B. thetaiotaomicron* [50,51].

4.5. Methanogens as probiotics

206 The possible use of methanogens as probiotics has received particular attention since the
207 recent discovery that some methanogens can use trimethylamine as a substrate for
208 methanogenesis [52]. This trimethylamine is produced by intestinal bacteria from food
209 ingredients and an abnormal level of trimethylamine in the blood correlates with a very high
210 risk of cardiovascular disease [53,54]. In a recent study, Brugère *et al* demonstrated that *M.*
211 *luminyensis* is able to use hydrogen to reduce trimethylamine to methane during its growth
212 [52]. According to the same authors, *Candidatus* Methanomethylophilus alvus and
213 *Candidatus* Methanomassiliicoccus intestinalis are also able to use hydrogen to reduce
214 trimethylamine to methane during their growth [52]. The level of trimethylamine in the
215 human body is therefore modulated by the composition of the intestinal microbiota and in
216 particular by the quantity of these methanogen species [52]. On this basis they were able to
217 demonstrate that the use of these different species of methanogens as a probiotic in
218 individuals with a hereditary defect in flavin-containing monooxygenase 3 would be an
219 alternative to the treatment of metabolic disorders linked to a high level of trimethylamine
220 [52].

221 The share of methanogens, in particular *M. smithii* has also been demonstrated in severe acute
222 malnutrition [55]. In this recent study, Million *et al* showed the complete absence of *M.*
223 *smithii* in children suffering from severe acute malnutrition compared to healthy children
224 [55]. Considering the fact that methanogens represent 10% of anaerobic microorganisms in
225 the gut and that the species *M. smithii* is the most represented with a prevalence of up to
226 97.5% [21,56,57]; considering their essential role in the digestion of food by using the
227 hydrogen produced by the fermented products of the bacteria thus facilitating the absorption
228 of food; considering the fact that malnutrition could be the consequence of a food
229 malabsorption syndrome and the total depletion of *M. smithii* observed only in severe acute
230 malnourished in the study carried out by Million *et al*, an exogenous intake of this strain in

children suffering from severe acute malnutrition would be an alternative to be taken into consideration in view of the predominant role played by this species in food digestion.

5. Human associated methanogens and human diseases

5.1. Methanogens associated with dysbiosis

Methanogens are the only archaea groups involved in the dysbiosis of the human microbiota, especially the intestinal, oral, sinus, vaginal and urinary microbiota. The methanogens involved in dysbiosis of the intestinal microbiota are mainly *M. smithii* (most involved), *M. stadtmanae* and *M. luminyensis* [58]. There is a decrease in methanogens during Crohn's disease, ulcerative colitis, malnutrition [55,59,60] and an increase during diverticulosis, inflammatory bowel disease, irritable bowel syndrome, constipation, obesity, colorectal cancer and colonic polyposis [61–76].

There are three methanogens involved in dysbiosis of the oral microbiota, namely *M. oralis* (most involved), *Methanobrevibacter massiliense* (*M. massiliens*) and *M. smithii* [77]. They have been found in periodontal dysbiosis such as periodontitis, peri-implantitis and gingivitis [77–83]. In a recent study conducted in our laboratory, Sogodogo *et al* found *M. oralis*, *M. smithii* and *M. massiliense* in sinus abscesses, more precisely in refractory maxillary sinusitis [84].

M. smithii is so far the only archaea implicated in vaginal dysbiosis including vaginosis [85,86]. The role of archaea in dysbiosis of the urinary microbiota was studied in a recent study by Grine e*t al* [85]. In this study, the authors used a polyphasic approach (PCR-sequencing and culture) to highlight *M. smithii* as the only archaea involved in urinary tract infection [85].

5.2. Methanogens associated with abscesses

There are only three studies carried out on the role of archaea in human abscesses and all these studies were done in our laboratory [87–89]. In a study conducted by Drancourt *et al*, *M. oralis* was isolated and cultured from a brain abscess [87]. In this study, the authors carried out a polyphasic approach (PCR-sequencing, metagenomics and culture) on brain abscess samples. They were able to detect 8/18 positive samples by qPCR, 28/32 positive samples by metagenomics and succeeded in isolating by culture the *M. oralis* in a brain abscess sample taken from a 51-year-old woman with a history of dental avulsion of one month [87]. *M. smithii* was detected by PCR specific for 16S RNA and *mcrA* genes in a paravertebral muscle abscess in a 41-year-old man suffering from lumbar swelling with night sweats and chronic fever [88]. In a study by Nkamga *et al*, *M. oralis* was detected by PCR-specific sequencing of the 16S RNA and *mcrA* genes in a brain abscess sample from a 30-year-old woman suffering from persistent headaches for four days and a left temporal abscess [89].

6. Conclusions and perspectives

Archaea are an integral part of the human microbiota and are found in humans from the first day of birth [1,19]. There are three phyla found in humans including *Euryarchaeota* (the most important), *Crenarchaeota* and *Thaumarchaeota* [1] and methanogens are the most common archaea found in humans [1,15]. Since their first isolation in humans more than five decades ago, only ten species of archaea have been cultured in humans and all these species belong to the phylum *Euryarchaeota* (eight methanogens and two halophilic archaea) [1–3,6,23,90]. This fact is linked to their fastidious culture which requires anaerobiosis control and the supply of hydrogen [23,91,92]. There is therefore a need to optimize current methods of isolating and culturing archaea in order to be able to isolate new species. It would also be very

useful to conduct studies to better understand their role in human pathologies as well as their possible use as probiotics [52].

There is a real enormous deficit of knowledge and partially contradictory information concerning human methanogens, it is helpful to develop a methodology and standard operating procedures allowing detection, quantification and characterization of methanogens in clinical samples.

The development of new methods of identification and culture of these particular microorganisms from clinical samples is therefore necessary. This will allow to isolate new species and characterize them phenotypically, to explore their genome by sequencing and study population dynamics in particular specify their exact role within the complex flora associated with the mucous microbiota of human.

Conflicts of interest

There is no conflict of interest

Highlights

Methanogens are very important for humans and contribute to human well-being by playing major physiological roles. However, their involvement in certain pathologies related to dysbiosis has been mentioned in several works.

The current review emphasizes the physiological roles of human microbiota-associated methanogens.

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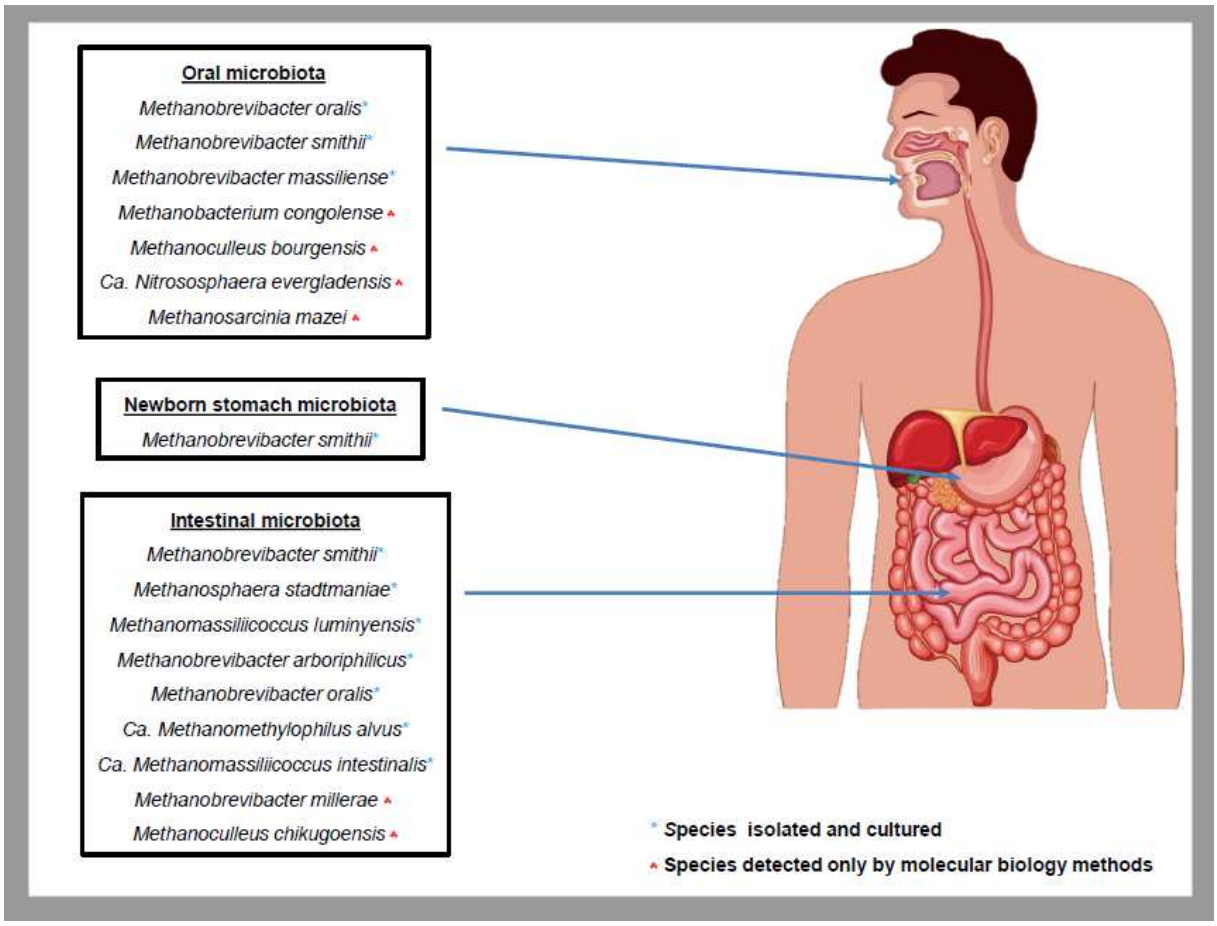
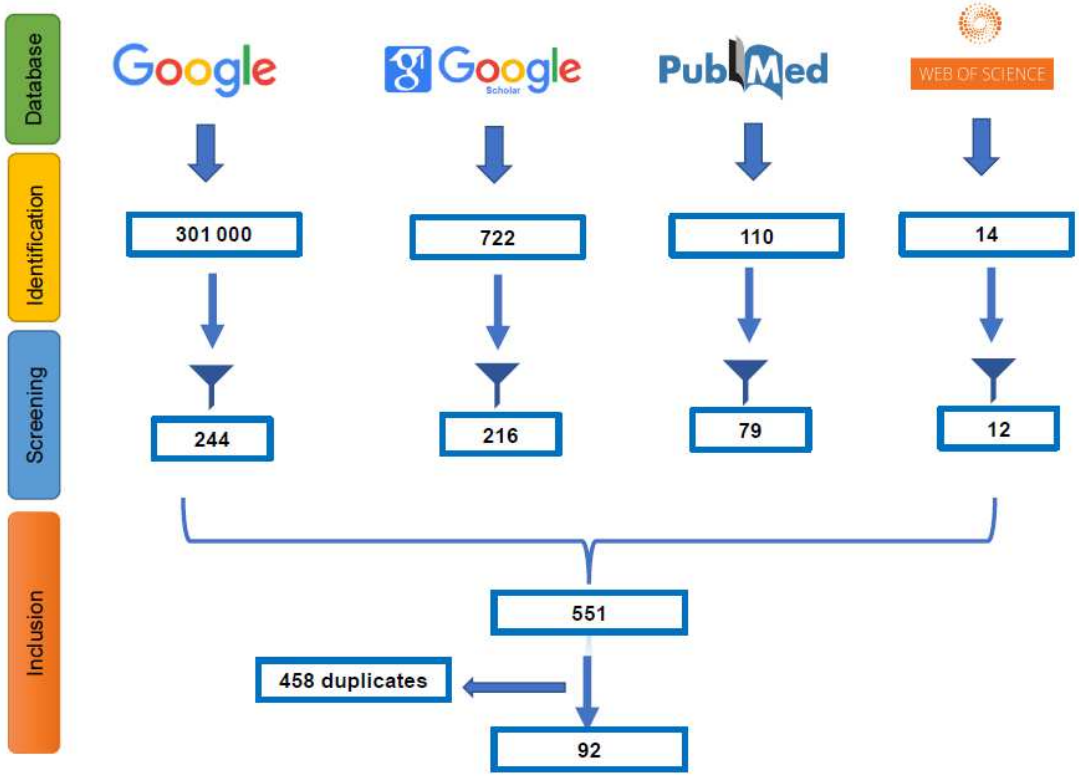
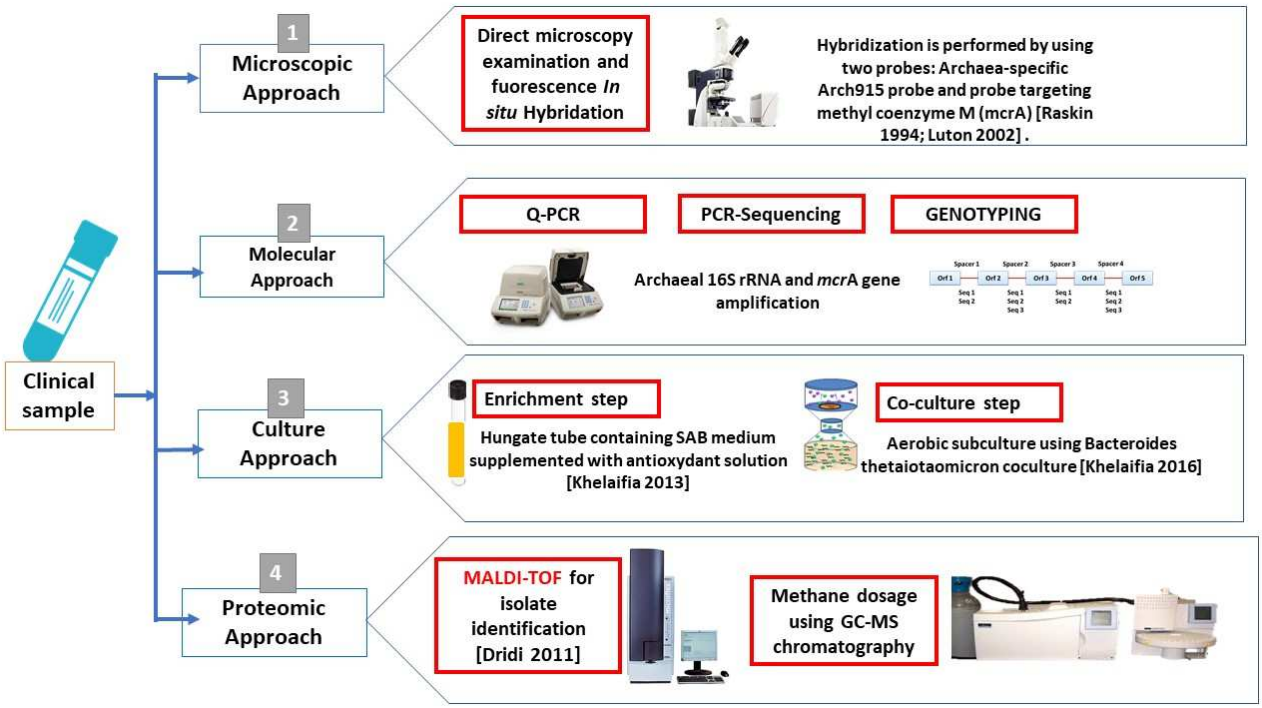


Figure 1. Repertoire of methanogens in human digestive microbiota.



633 Figure 2. Flow chart of selection of included articles in the review. Each box indicates the
634 number of selected articles.



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636 Figure 3. Methods for studying human archaea in clinical microbiology.

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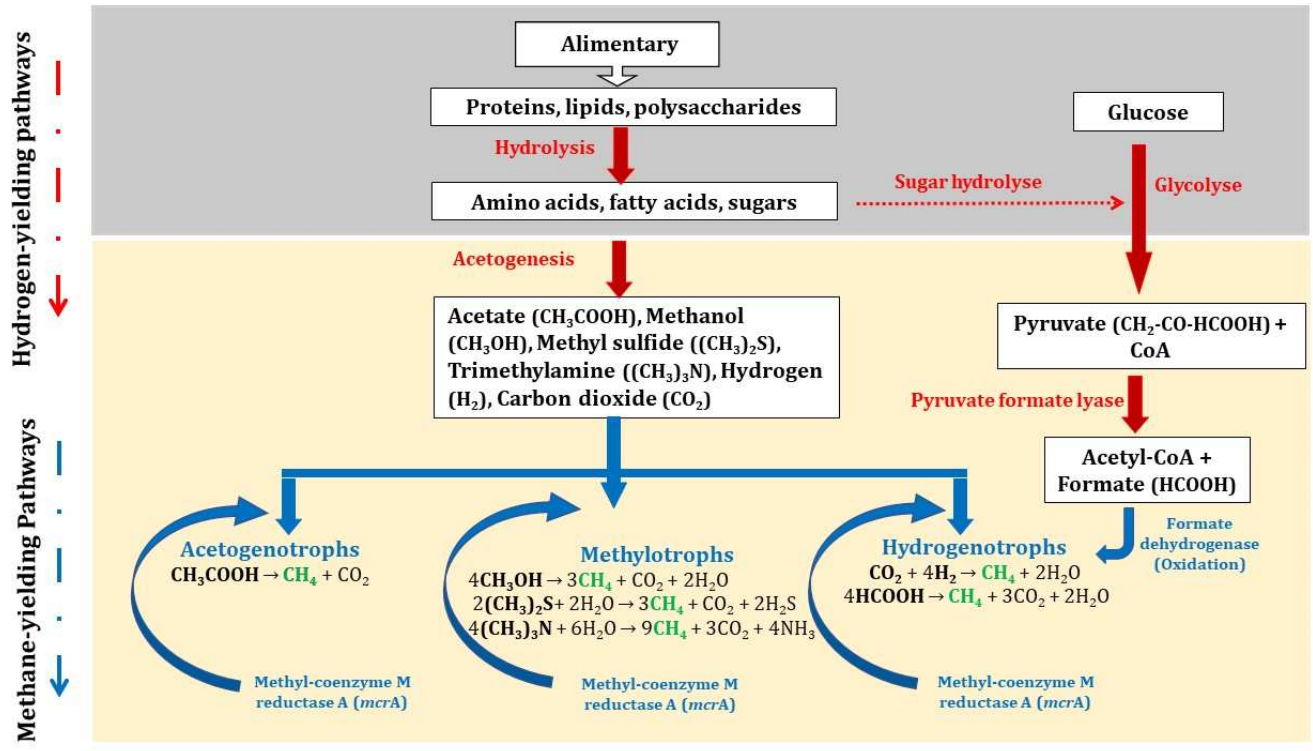

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639 Figure 4. Metabolisms supporting archaeal methanogenesis in aerobic (gray background) and
640 anaerobic (yellow background): Bacterial enzymatic pathways in red characters and
641 methanogens enzymatic pathways in blue characters.

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