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

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19 **Abstract**

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

41

42 **Keywords :** Digestive tract, Human microbiota, Methanogens.

43

44 **1. Introduction**

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

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

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

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

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

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

## 73 **2. Methods**

### 74 **2. 1. Research strategy and article selection**

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

### 82 **2. 2. Setting**

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

## 87 **3. Methods for studying human archaea in clinical microbiology**

### 88 **3.1. Direct microscopic examination and fluorescent *in situ* hybridization (FISH)**

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

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

### 99 3.2. Molecular approach

100 There are many routinely used PCR-based detection systems for archaea, one is targeting the  
101 archaeal 16S rRNA gene (forward primer: 5'- TCCAGGCCCTACGGG-3'; reverse primer:  
102 5'- YCCGGCGTTGAMTCCAATT-3'; probe: FAM 5'-  
103 CCGTCAGAATCGTTCCAGTCAG-3') [16], another one is targeting the *mcrA* gene  
104 encoding a methyl-coenzyme-M reductase subunit (an enzyme involved in methanogenesis)  
105 (forward primer: 5'- GCTCTACGACCAGATMTGGCTTGG -3'; reverse primer: 5'-  
106 CCGTAGTACGTGAAGTCATCCAGCA -3'; probe: FAM 5'-ARGCACCKAACAMCAT  
107 GGACACWGT -3') [17] and haloarchaea-specific primers Halo 1F and Halo 1R [20]. Also, a  
108 real-time PCR protocol targeting the *rpoB* gene encoding for the beta subunit of the RNA  
109 polymerase, could be used for the detection of *Methanobrevibacter smithii* (forward primer:  
110 Ms\_rpoBF, 5'-AAGGGATTTGCACCCAACAC-3'reverse primer: Ms\_rpoBR, 5'-  
111 GACCACAGTTAGGACCCTCTGG-3'; probe: Ms\_rpoBVIC, 5'-  
112 ATTTGGTAAGATTTGTCCGAATG-3') and *Methanosphaera stadtmanae* forward primer:

113 Stadt\_16SF, 5'-AGGAGCGACAGCAGAATGAT-3' Reverse primer: Stadt\_16SR, 5'-  
114 CAGGACGCTTCACAGTACGA-3'; probe: Stadt\_16SFAM, 5'-  
115 TGAGAGGAGGTGCATGGCCG-3' [21].

### 116 3.3. Culture approach

117 There is different culture approach for archaea isolation from human clinical sample. For  
118 methanogens culture we use a technique which is consisting of the use of two compartments  
119 for the aerobic culture of methanogens in the presence of *Bacteroides thetaiotaomicron* (*B.*  
120 *thetaitaomicron*) producing hydrogen, the aerobic culture of methanogens being made  
121 possible by using antioxidants. In brief, the sample is seeded in the Hungate tube containing  
122 SAB broth supplemented with ascorbic acid, uric acid and glutathione as anti-oxydants and  
123 inoculated with *B. thetaiotaomicron* to produce hydrogen. Subculture seeded on a Petri dish  
124 containing SAB medium supplemented with agar and deposited in the upper compartment and  
125 the lower compartment contains a culture of *B. thetaiotaomicron* [22]. Recently, we  
126 developed a new technique for methanogens isolation in clinical microbiology. This new  
127 technique uses a chemical method form hydrogen production that allow a constant hydrogen  
128 production and increase a number of methanogens colonies compared to biological method  
129 [23]. In addition, we used new culture conditions to culture halophilic archaea. The culture  
130 enrichment and isolation procedures for the culture of halophilic prokaryotes were performed  
131 in a Columbia broth medium (Sigma-Aldrich), modified by adding (per Litre):  $MgCl_2 \cdot 6H_2O$ ,  
132 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  
133 glucose. The pH was adjusted to 7.5 with 10 M NaOH before autoclaving. All additives were  
134 purchased from Sigma-Aldrich. Four concentrations of NaCl were used (150 g L<sup>-1</sup>, 200 g L<sup>-1</sup>  
135 and 250 g L<sup>-1</sup>) [24].

### 136 **3.4. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry** 137 **(MALDI-TOF MS)**

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

### 147 **3.5. *In vitro* susceptibility testing**

148 Most antibiotics used to fight bacteria are *in vitro* inactive against methanogens [35]. Indeed,  
149 intestinal *Methanobrevibacter smithii* (*M. smithii*) isolates are highly resistant to beta-lactams,  
150 aminoglycosides, glycopeptides, lincosamides and fluoroquinolones and susceptible to  
151 metronidazole, fusidic acid, rifampicin, bacitracine and squalamine [35–38]. Lovastatine is a  
152 pro-drug, which needs to be metabolized by anaerobes before being active against *M. smithii*  
153 [39]. The *in vitro* susceptibility of methanogens to chloramphenicol is variable: *M. smithii*,  
154 *Methanobrevibacter oralis* (*M. oralis*) and *Methanomasillicoccus luminyensis* (*M.*  
155 *luminyensis*) encodes a chloramphenicol O-acetyltransferase and exhibit minimum inhibitory  
156 concentration (MIC) up to 25 mg/L, in contrast to *Methanosphaera stadtmanae* (*M.*  
157 *stadtmanae*) which exhibits MIC of 4 mg/L [36].

## 158 **4. Role of Human-associated methanogens**

### 159 **4.1. Direct physical contact might support symbiotic metabolism**

160 Methanogens in different human microbiota are in close contact with bacteria. This  
161 association between methanogens and bacteria has been used to develop a method of  
162 cultivating methanogens in co-culture with *B. thetaiotamicron* which produces hydrogen and  
163 therefore allows the production of methane by methanogens by consuming hydrogen [22].  
164 *Methanobrevibacter massilensis* was co-cultivated for the first time in the oral cavity with the  
165 bacterium *Pyramidobacter piscolens* [40] and a recent article has shown Syntrophy via  
166 Interspecies H<sub>2</sub> Transfer between *Christensenella* and *Methanobrevibacter* genus [41].

#### 167 **4.2. Methanogens and carbohydrates degradation**

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

#### 181 **4.3. Methane release and H<sub>2</sub> consumption**

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

190 In an anaerobic respiration, methanogens oxidize carbon such as CO<sub>2</sub> as a terminal electron  
191 acceptor. Thus, methanogens are common in habitats that are poor in other electron acceptors,  
192 (O<sub>2</sub>, NO<sub>3</sub>, Fe<sup>3+</sup> and SO<sub>4</sub><sup>2-</sup>). Methanogens as strict anaerobes have long been classified to be  
193 limited to anoxic habitats. However, recent studies have shown that some methanogens are  
194 able to produce methane in oxygenated soils [49] and even in human microbiota [18].  
195 Methanogens were classified into three biochemical groups based on the substrates using for  
196 hydrogen production: hydrogenotrophic, acetoclastic and methylotrophic [6,41,49] (Fig. 4).  
197 The most group described in human microbiota is hydrogenotrophic methanogens who  
198 oxidize H<sub>2</sub>, formate or a few simple alcohols and reduce CO<sub>2</sub> to CH<sub>4</sub> [1,6,41].

#### 199 **4.4. Heavy metal transformation**

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

#### 205 **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

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

## 233 **5. Human associated methanogens and human diseases**

### 234 **5.1. Methanogens associated with dysbiosis**

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

242 There are three methanogens involved in dysbiosis of the oral microbiota, namely *M. oralis*  
243 (most involved), *Methanobrevibacter massiliense* (*M. massiliens*) and *M. smithii* [77]. They  
244 have been found in periodontal dysbiosis such as periodontitis, peri-implantitis and gingivitis  
245 [77–83]. In a recent study conducted in our laboratory, Sogodogo *et al* found *M. oralis*, *M.*  
246 *smithii* and *M. massiliense* in sinus abscesses, more precisely in refractory maxillary sinusitis  
247 [84].

248 *M. smithii* is so far the only archaea implicated in vaginal dysbiosis including vaginosis  
249 [85,86]. The role of archaea in dysbiosis of the urinary microbiota was studied in a recent  
250 study by Grine *et al* [85]. In this study, the authors used a polyphasic approach (PCR-  
251 sequencing and culture) to highlight *M. smithii* as the only archaea involved in urinary tract  
252 infection [85].

### 253 **5.2. Methanogens associated with abscesses**

254 There are only three studies carried out on the role of archaea in human abscesses and all  
255 these studies were done in our laboratory [87–89]. In a study conducted by Drancourt *et al*,  
256 *M. oralis* was isolated and cultured from a brain abscess [87]. In this study, the authors  
257 carried out a polyphasic approach (PCR-sequencing, metagenomics and culture) on brain  
258 abscess samples. They were able to detect 8/18 positive samples by qPCR, 28/32 positive  
259 samples by metagenomics and succeeded in isolating by culture the *M. oralis* in a brain  
260 abscess sample taken from a 51-year-old woman with a history of dental avulsion of one  
261 month [87]. *M. smithii* was detected by PCR specific for 16S RNA and *mcrA* genes in a  
262 paravertebral muscle abscess in a 41-year-old man suffering from lumbar swelling with night  
263 sweats and chronic fever [88]. In a study by Nkamga *et al*, *M. oralis* was detected by PCR-  
264 specific sequencing of the 16S RNA and *mcrA* genes in a brain abscess sample from a 30-  
265 year-old woman suffering from persistent headaches for four days and a left temporal abscess  
266 [89].

267

## 268 **6. Conclusions and perspectives**

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

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

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

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

289

#### 290 **Conflicts of interest**

291 There is no conflict of interest

292

#### 293 **Highlights**

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

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

299

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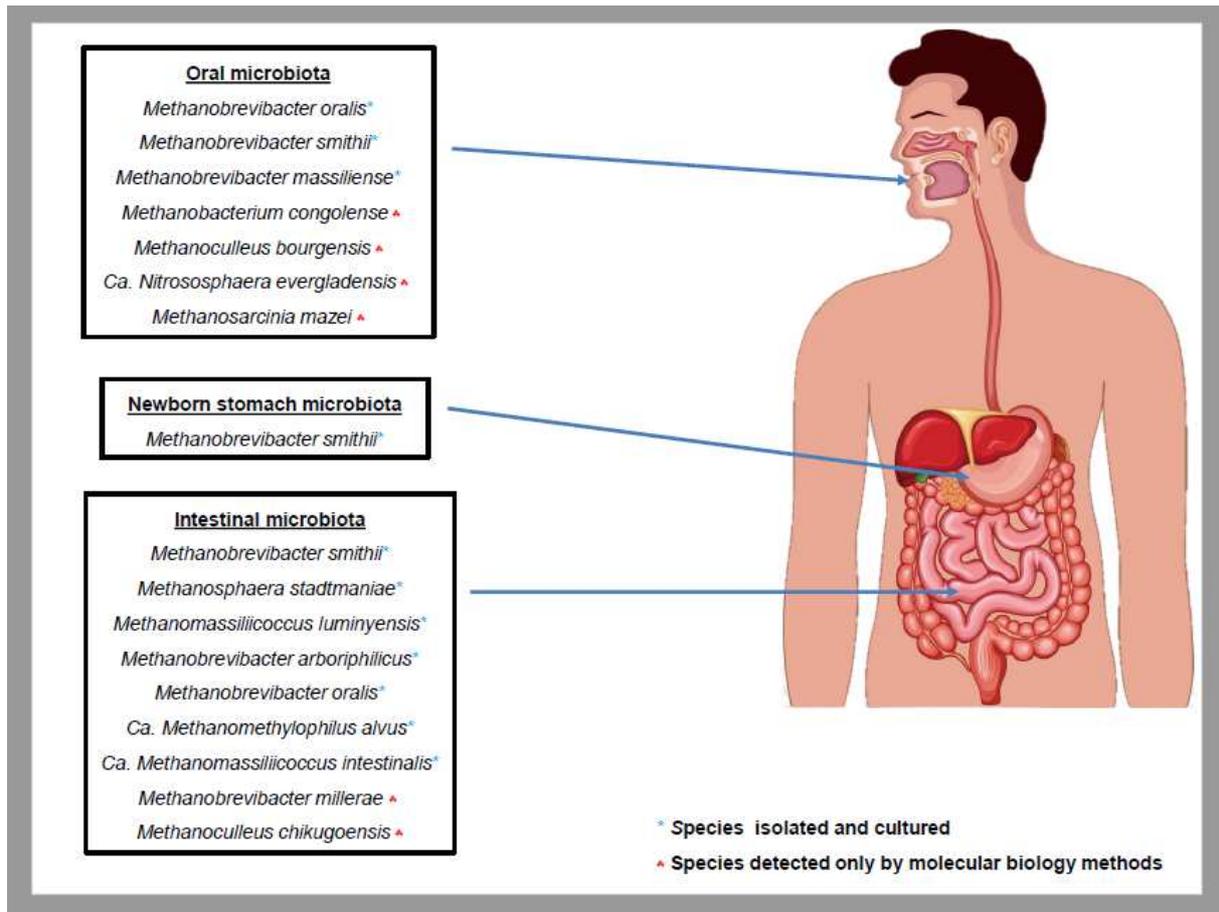
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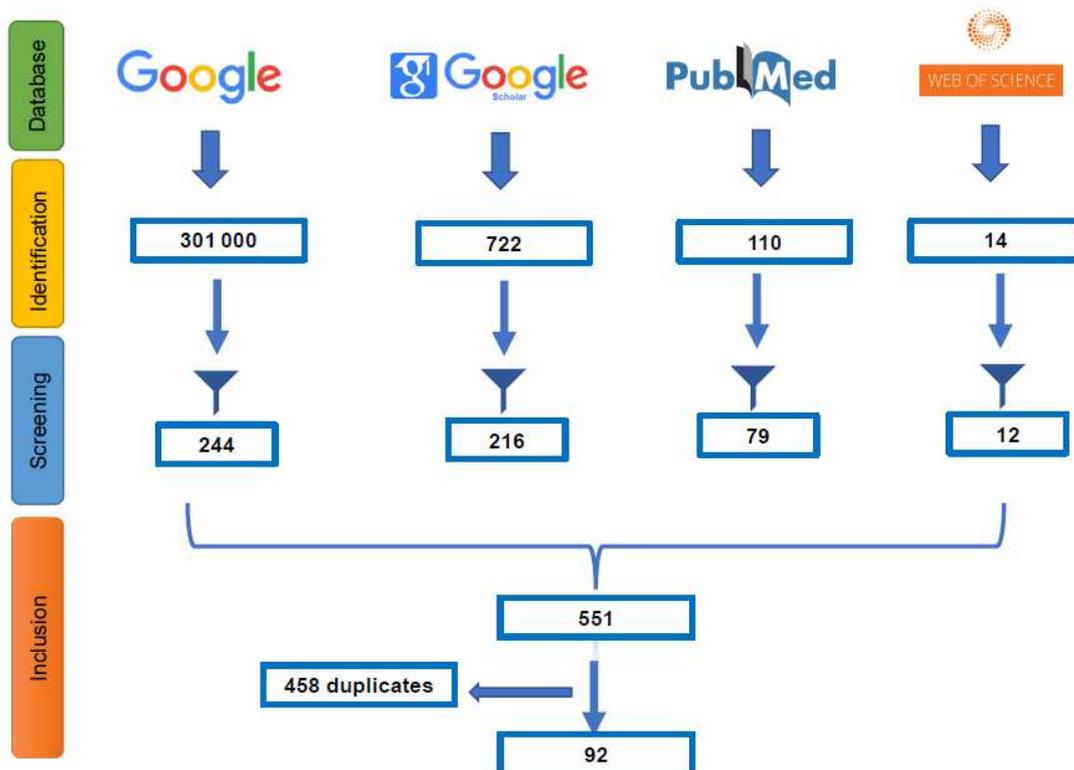
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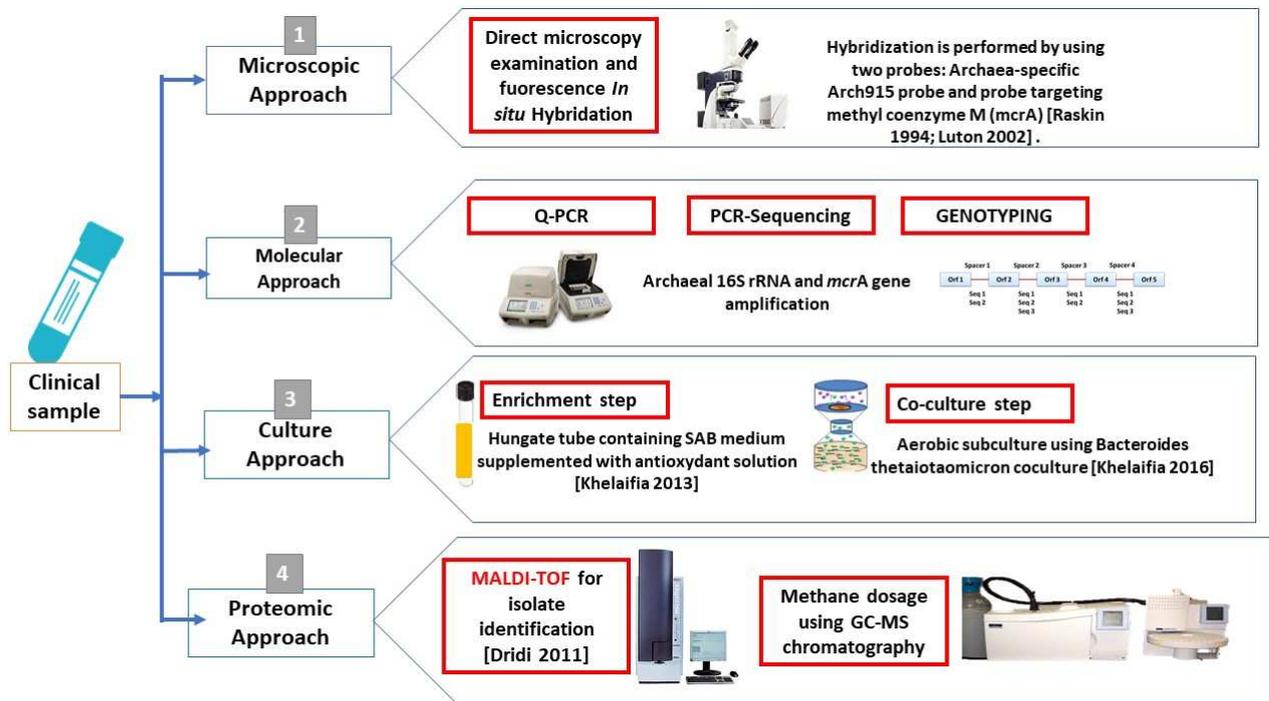
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Figure 1. Repertoire of methanogens in human digestive microbiota.



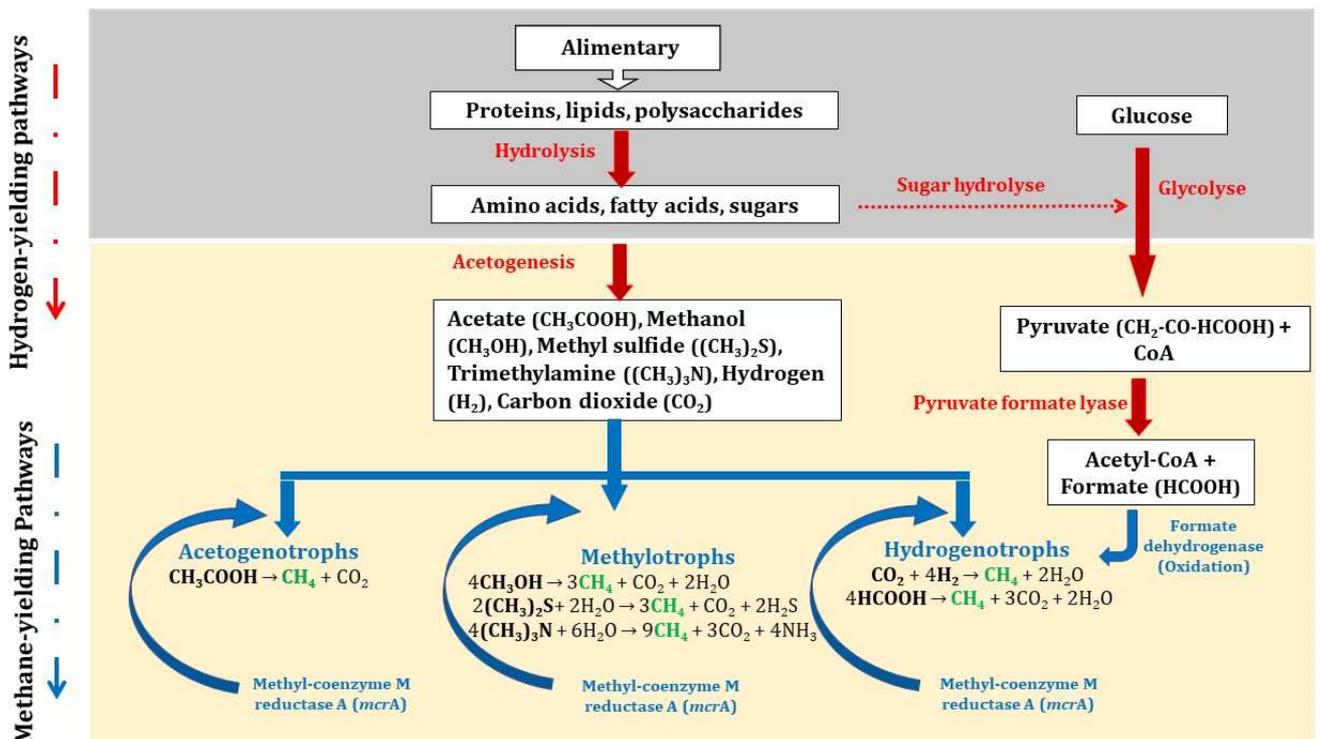
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633 Figure 2. Flow chart of selection of included articles in the review. Each box indicates the  
 634 number of selected articles.



635  
 636 Figure 3. Methods for studying human archaea in clinical microbiology.

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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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