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L'isolement par culture des méthanogènes d'intérêt clinique

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Je soussigné, Virginie Pilliol, déclare par la présente que le travail présenté dans ce manuscrit est mon propre travail, réalisé sous la direction scientifique de Elodie Terrer et co-encadré par Ghiles Grine, dans le respect des principes d'honnêteté, d'intégrité et de responsabilité inhérents à la mission de recherche. Les travaux de recherche et la rédaction de ce manuscrit ont été réalisés dans le respect à la fois de la charte nationale de déontologie des métiers de la recherche et de la charte d'Aix-Marseille Université relative à la lutte contre le plagiat.

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Liste de publications et participation aux conférences

1) Liste des publications[1] réalisées dans le cadre du projet de thèse :

   1. **Pilliol V.**, Guindo C.O., Terrer E., Aboudharam G., Drancourt M., Grine G. Culturing clinical *Methanobrevibacter smithii* using GG medium in a minimal anaerobe atmosphere. J Microbiol Methods. 2023;207:106704. doi: 10.1016/j.mimet.2023.106704. Epub 2023. PMID: 36907565.

   2. Khelaifia S., **Pilliol V.**, Belkacemi S., Tassery H., Terrer E., Aboudharam G. Culturing the Human Oral Microbiota, Updating Methodologies and Cultivation Techniques. Microorganisms. 2023;11(4):836. doi: 10.3390/microorganisms11040836. PMID: 37110259; PMCID: PMC10143722.

   3. **Pilliol V.**, Morsli M., Terlier L., Hassani Y., Malat I., Guindo C.O., Davoust B., Lamglait B., Drancourt M., Aboudharam G., Grine G., Terrer E. *Candidatus* Methanosphaera massiliense sp. nov., a methanogenic archaeal species found in a human fecal sample and prevalent in pigs and red kangaroos. Microbiol Spectr. 2024:e0514122. doi: 10.1128/spectrum.05141-22. Epub ahead of print. PMID: 38189277.

   4. **Pilliol V.**, Beye M., Terlier L., Balmelle J., Kacel I., Lan R., Aboudharam G., Grine G., Terrer E. *Methanobrevibacter massiliense* and *Pyramidobacter piscolens* Co-Culture Illustrates Transkingdom Symbiosis. Microorganisms. 2024; 12(1):215. doi.org/10.3390/microorganisms12010215.

   5. **Pilliol V.**, Boualam M., Hameich A., Tellissi L., Drancourt M., Aboudharam G., Tassery H., Grine G., Terrer E. *Methanobrevibacter oralis*: from oral cavity commensalism to opportunistic pathologies. (Soumis dans Journal of Oral Microbiology)

[1] Cette liste comprend les articles publiés, les articles soumis à publication et les articles en préparation ainsi que les livres, chapitres de livre et/ou toutes formes de valorisation des résultats des travaux propres à la discipline du projet de thèse. La référence aux publications doit suivre les règles standards de bibliographie et doit être conforme à la charte des publications d'AMU.

6. **Pilliol V.,** Boualam M., Terlier L., Drancourt M., Aboudharam G., Grine G., Terrer E. Dental calculus *Methanobrevibacter massiliense-Pyramidobacter piscolens*, a millennium-old association. Research Square. 2023. PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-3423779/v1]

2) Participation aux conférences[2] et écoles d'été au cours de la période de thèse :

1. Communication Orale. Deuxième prix de la meilleure communication orale de la Journée de la Recherche de l'Ecole de Médecine Dentaire, Marseille.
   Pilliol V., Terrer E. Optimisation de la culture des *Archaea* méthanogènes. (23/09/2021)

2. Communication Orale. « Work In Progess » à l'IHU Méditerranée Infection, Marseille.
   Pilliol V., Grine G., Terrer E. Présentation générale du projet de thèse. (13/09/2022)

3. Communication Orale. « Work In Progess » à l'IHU Méditerranée Infection, Marseille.
   Pilliol V., Grine G., Terrer E. *Candidatus* Methanosphaera massiliense sp. nov. questions transfer between human and animal microbiota. (18/01/2022)

4. Communication Orale. Premier prix de la communication orale de la Journée de la Recherche de l'Ecole de Médecine Dentaire, Marseille.
   Pilliol V., Terrer E. *Archaea* méthanogènes et pulpe dentaire. (24/05/2022)

5. Communication Orale (en anglais). Journées Annuelles du CNEOC (Collège National des Enseignants en Odontologie Conservatrice), Rennes.
   Pilliol V., Terrer E. Methanogenic *Archaea* and dental pulp. (01 et 02/07/2022)

6. Communication Affichée Internationale. Congrès de la SFBBM (Société française de biochimie et de biologie moléculaire), Paris.
   Pilliol V., Terrer E. Methanogenic *Archaea* and dental pulp. (04 et 05/07/2022)

[2] Le terme « conférence » est générique. Il désigne à la fois « conférence », « congrès », « workshop », « colloques », « rencontres nationales et/ou internationales » … etc.
Indiquer si vous avez fait une présentation orale ou sous forme de poster.

7. Communication Affichée Internationale. Congrès de l'IADR (International Association for Dental Research), Marseille.
   Pilliol V., Terrer E. Methanogenic *Archaea* and dental pulp. (du 15 au 17/09/2022)

8. Communication Orale (distanciel, en anglais). Congrès APH Europa.
   Pilliol V., Terrer E. *Candidatus* Methanosphaera massiliense sp. nov. questions transfer between human and animal microbiota. (22/02/2023)

9. Communication Orale. Premier prix de la communication orale de la Journée de la Recherche de l'Ecole de Médecine Dentaire, Marseille.
   Pilliol V., Terrer E. *Candidatus* Methanosphaera massiliense sp. nov. questions transfer between human and animal microbiota. (23/05/2023)

10. Communication Orale (distanciel, en anglais). Journée des jeunes chercheurs de la SFBBM.
    Pilliol V., Terrer E. *Candidatus* Methanosphaera massiliense sp. nov. questions transfer between human and animal microbiota. (15/06/2023)

11. Communication Orale. « Work In Progess » à l'IHU Méditerranée Infection, Marseille.
    Pilliol V., Grine G., Terrer E., *Methanobrevibacter massiliense* and *Pyramidobacter piscolens* Co-Culture Illustrates Transkingdom Symbiosis. (05/09/2023)

12. Communication Orale. Séminaire à l'IHU Méditerranée Infection, Marseille.
    Pilliol V., Terrer E. *Methanobrevibacter massiliense* and *Pyramidobacter piscolens* Co-Culture Illustrates Transkingdom Symbiosis. (22/12/2023)

Résumé

Les *Archaea* méthanogènes (méthanogènes) sont des micro-organismes appartenant au domaine des *Archaea*, l'un des trois domaines du vivant à côté des bactéries et des eucaryotes. Les méthanogènes produisent du méthane à partir de différents substrats issus du métabolisme bactérien et sont des acteurs importants des microbiotes humains, présents notamment dans l'intestin et la cavité orale. Elles sont de plus en plus intéressantes d'un point de vue clinique. Elles sont associées à plusieurs pathologies humaines comme les pathologies abcédées ou encore l'endocardite infectieuse. Plus généralement elles sont également impliquées dans les dysbioses. Néanmoins, la culture des méthanogènes demeure complexe en raison de leur sensibilité à l'oxygène et de leurs exigences spécifiques. Les protocoles de culture sont dits fastidieux, et constitue un défi majeur en microbiologie clinique. Ainsi, cette thèse a pour objectif d'améliorer les connaissances sur les méthanogènes d'intérêt clinique par l'optimisation des conditions d'isolement par culture. Une première revue de littérature sur les méthodes de culture microbiennes applicables au microbiote oral a permis de faire le point sur les méthodes de culture des méthanogènes. Une deuxième revue a permis de synthétiser les connaissances sur *Methanobrevibacter oralis* (*M. oralis*), la méthanogène la plus fréquemment retrouvée dans la cavité orale. Cette analyse exhaustive a également répertorié les multiples aspects de l'étude des méthanogènes, allant de leur caractérisation phénotypique à leur éventuel rôle pathologique, en passant par leur détection dans les microbiotes passés et présents, ainsi que les associations probables avec d'autres bactéries. Au niveau expérimental, cette thèse présente le développement d'un nouveau milieu de culture, le milieu GG®, permettant de s'affranchir de l'utilisation de l'hydrogène, gaz dangereux nécessitant des normes de sécurité particulières. Le milieu GG® a ainsi permis la culture et l'isolement d'une nouvelle espèce de méthanogènes, *Candidatus* Methanosphaera massiliense, ainsi que la redécouverte de *Methanobrevibacter massiliense* (*M. massiliense*) en coculture avec la bactérie *Pyramidobacter piscolens* (*P. piscolens*) dans une nouvelle situation clinique, la pulpe dentaire infectée. Cette forte association en culture entre *M. massiliense* et *P. piscolens* nous a conduit à nous intéresser à l'antiquité de cette relation. L'analyse des données métagénomiques provenant de tartres anciens issues d'études antérieures a révélé l'ancienneté et la persistance de cette association. De plus, elle a apporté des informations supplémentaires pour comprendre le déclin de *M. massiliense* au profit de *M. oralis* au moment de la révolution industrielle au 18ème siècle. Ainsi, grâce à l'optimisation de la culture, ce travail de thèse a contribué à élargir le répertoire des méthanogènes cultivées chez l'Homme pouvant avoir un intérêt clinique et à enrichir notre compréhension des dynamiques d'évolution des microbiotes humains à la fois à l'échelle de l'individu mais également à l'échelle de l'histoire humaine.

Mots clés: *Archaea*, méthanogène, milieu GG, *Methanobrevibacter oralis*, *Candidatus* Methanosphaera massiliense, *Methanobrevibacter massiliense*, *Pyramidobacter piscolens*.

Abstract

Methanogenic *Archaea* (methanogens), belonging to the *Archaea* domain, one of the three domains of life alongside bacteria and eukaryotes, are microorganisms known for their production of methane from various substrates derived from bacterial metabolism. They play crucial roles in human microbiomes, notably in the gut and oral cavity. Increasingly, they are of clinical interest due to their association with various human pathologies such as dysbioses, abscess-related diseases, and infectious endocarditis. However, culturing methanogens remains challenging due to their sensitivity to oxygen and specific requirements, posing a significant hurdle in clinical microbiology protocols. The objective of this thesis is to enhance understanding of clinically relevant methanogens by optimizing isolation conditions through culture techniques. A literature review on microbial culture methods applicable to the oral microbiota provided insights into methanogen culture techniques. Another review synthesized knowledge on *Methanobrevibacter oralis* (*M. oralis*), the most frequently encountered methanogen in the oral cavity. This comprehensive analysis also cataloged various aspects of methanogen study, from phenotypic characterization to potential pathological roles, including their detection in past and present microbiomes and likely associations with other bacteria. Experimentally, this thesis introduced the development of a new culture medium, GG®, eliminating the need for hydrogen, a hazardous gas requiring specific safety standards. GG® medium allowed the culture and isolation of a new methanogen species, *Candidatus* Methanosphaera massiliense, possibly acquired through a carnivorous diet in one individual, as well as the rediscovery of *Methanobrevibacter massiliense* (*M. massiliense*) in co-culture with the bacterium *Pyramidobacter piscolens* (*P. piscolens*) in a new clinical condition, endodontic infection. The strong culture association between *M. massiliense* and *P. piscolens* prompted an investigation into the antiquity of this relationship. Analysis of metagenomic data from ancient tartar, sourced from previous studies, revealed the ancient and persistent nature of this association. Moreover, it provided additional insights into the decline of *M. massiliense* in favor of *M. oralis* during the 18th-century industrial revolution. Thus, through culture optimization, this thesis contributed to expanding the repertoire of methanogens cultivated in humans with potential clinical relevance and enriched our understanding of human microbiome evolution dynamics at both individual and historical scales.

Keywords: Archaea, methanogen, GG medium, *Methanobrevibacter oralis*, *Candidatus* Methanosphaera massiliense, *Methanobrevibacter massiliense*, *Pyramidobacter piscolens*.

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Je dédie cette thèse à ma très chère maman.

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Table des matières

Table des matières **12**
Affidavit **3**
Affidavit **4**
Liste de publications et participation aux conférences **5**
Résumé **8**
Abstract **10**
Remerciements **11**
Introduction **12**
Chapitre I: Revues de littérature **16**
Préambule du chapitre I 16
Article 1: Culturing the Human Oral Microbiota, Updating Methodologies and Cultivation Techniques 18
Article 2: *Methanobrevibacter oralis:* from oral cavity commensalism to opportunistic pathologies 36
Chapitre II: Optimisation des conditions de l'isolement par culture des méthanogènes **105**
Préambule du chapitre II 105
Article 3: Culturing clinical *Methanobrevibacter smithii* using GG medium in a minimal anaerobe atmosphere 107
Chapitre III: *Candidatus* Methanosphaera massiliense sp. nov. **114**
Préambule du chapitre III 114
Article 4: *Candidatus* Methanosphaera massiliense sp. nov., a methanogenic archaeal species found in a human fecal sample and prevalent in pigs and red kangaroos 115
Chapitre IV: *Methanobrevibacter massiliense* **132**
Préambule du chapitre IV 132
Article 5: *Methanobrevibacter massiliense and Pyramidobacter piscolens Co-Culture Illustrates Transkingdom Symbiosis* 134
Article 6: Dental calculus *Methanobrevibacter massiliense-Pyramidobacter piscolens, a millennium-old association* 151
Conclusion et perspectives **161**
Références bibliographiques **164**
Annexes **167**

Introduction

Les *Archaea* sont des êtres procaryotes, unicellulaires et dépourvus d'organite à l'instar des bactéries mais phénotypiquement indistinguables de celles-ci, ayant par exemple des formes de coque ou bacille (1). Elles sont connues depuis plus de 150 ans par les microbiologistes et, déjà en 1868, A. Béchamp décrivait le rôle des microbes dans la méthanogenèse, un processus aujourd'hui presque exclusivement associé aux *Archaea* (1). Elles constituent désormais un domaine distinct de la vie à côté des bactéries (*Bacteria*) et des eucaryotes (*Eukaryota*) depuis leur reclassification en 1977 par C.R. Woese grâce aux analyses phylogénétiques portant sur le gène de l'ARNr 16S. Bien que leurs mécanismes moléculaires (réplication, transcription et traduction) ressemblent à ceux des eucaryotes, les *Archaea* possèdent des lipides membranaires uniques et des parois cellulaires sans peptidoglycane (1). Leur ADN est organisé avec des histones, similairement aux eucaryotes. Elles peuvent posséder des voies métaboliques qui leurs sont propres comme les *Archaea* méthanogènes (appelées également méthanogènes) qui produisent du méthane. Elles ont souvent été qualifiées d'extrémophiles car retrouvées dans des environnements extrêmes comme les sources chaudes (thermophiles) ou les lacs salés (halophiles) (2). Or, nous savons maintenant que la plupart sont mésophiles et largement répandues dans l'environnement et chez les êtres-vivants dont l'Homme (2). Elles pourraient représenter une fraction importante de la biomasse terrestre (2).

Chez l'Homme, la première Archaea identifiée a partir de selles en 1982 était *M.smithii* (3). On sait désormais que les *Archaea* sont également présentes au niveau de différents microbiotes humains, notamment l'intestin (4) et la cavité orale (5), représentées essentiellement par les méthanogènes appartenant au phylum *Euryarchaeota*. Elles utilisent les composés métaboliques produits par les autres micro-organismes tels que l'hydrogène gazeux ou le formate pour réduire le CO_2 (hydrogénotrophes), les composés méthylés ou le méthanol (méthylotrophes) ou encore l'acétate (acétotrophes) pour produire du méthane (CH_4) (6). Les méthanogènes, les *Archaea* halophiles et les *Nanoarchaea* plus récemment sont les trois seuls groupes d'*Archaea* à avoir été cultivés dans le microbiote humain, tandis que plusieurs autres groupes, comprenant les *Crenarchaeota* et les *Thaumarchaeota*, ont été seulement détectés par des méthodes de biologie moléculaire et le séquençage (7).

Les méthanogènes sont dites d'intérêt clinique car on a pu les retrouver dans certaines situations pathologiques : la gingivite, la parodontite, la péri-implantite, l'infection endodontique (8), les pathologies abcédées (tels que les abcès cérébraux, musculaires et péri-appendiculaires) (9), la sinusite réfractaire (10), l'endocardite infectieuse (« Archaémie », présence des *Archaea* dans la circulation sanguine) ou encore

les dysbioses (telles que la vaginose (11) et les infections urinaires (12)) (13). Cependant, il demeure complexe de déterminer avec précision leur impact positif ou négatif sur la santé humaine, notamment si elles peuvent agir directement comme agents pathogènes, favoriser la croissance d'autres micro-organismes en soutenant leur métabolisme, ou si leur absence pourrait être préjudiciable (14).

Avant ce travail de thèse, seules neuf espèces de méthanogènes ont pu être cultivées voire isolées chez l'Homme. Il s'agit de *Methanobrevibacter smithii* (*M. smithii*) (3), *Methanobrevibacter oralis* (*M. oralis*) (15), *Methanobrevibacter arboriphilicus* (16), *Methanobrevibacter massiliense* (*M. massiliense*) (17), *Methanomassiliicoccus luminyensis* (*M. luminyensis*) (18) and *Methanosphaera stadtmanae* (*M. stadtmanae*) (19), *Candidatus* Methanomethylophilus alvus (20), *Candidatus* Methanomassiliicoccus intestinalis (21) et *Candidatus* Methanobrevibacter intestini (22–24). *M. smithii* et *M. stadtmanae* sont les deux principales méthanogènes retrouvées dans le tractus digestif (25) tandis que *M. oralis* est l'espèce prédominante de la cavité orale (5). La culture des méthanogènes est particulièrement fastidieuse. Tout d'abord, elles sont particulièrement sensibles à l'oxygène qui est toxique pour elles, leur culture doit donc se faire dans des conditions strictement anaérobies. De plus, elles ont des besoins spécifiques notamment une source d'hydrogène gazeux, inhibiteur pour de nombreux autres micro-organismes, et différentes sources de carbone dont le CO_2, les composés méthylés ou encore l'acétate ainsi que différents cofacteurs et vitamines (26). Néanmoins, seule la culture et l'isolement permet de confirmer la présence effective de micro-organismes viables dans un microbiote donné, ceci permettant de les étudier plus en détails afin de mieux comprendre leur métabolisme, leurs conditions optimales de croissance et leur potentiel rôle en santé humaine.

Ainsi, ce travail de thèse porte sur l'isolement par culture des méthanogènes d'intérêt clinique. Son objectif principal était de perfectionner les méthodes de culture et d'isolement de ces micro-organismes à partir d'échantillons issus des microbiotes humains, permettant ainsi leur étude à travers divers paramètres. En cultivant et en isolant ces méthanogènes, nous pouvons les observer, les caractériser, examiner leur métabolisme, analyser leurs interactions avec d'autres micro-organismes du microbiote, ainsi que comprendre leurs sources potentielles d'acquisition, leur évolution et leur éventuel rôle dans le développement ou la progression de certaines maladies.

Une première partie de cette thèse s'est articulée autour de deux revues de littérature. La première a été consacrée à la mise à jour des connaissances sur les méthodes de culture des trois domaines de la vie dans le microbiote oral, incluant les *Archaea* et les méthanogènes. La seconde revue s'est focalisée sur *M. oralis*. Nous constaterons que depuis son isolement en 1994 (15), les connaissances à son sujet n'ont cessé de croître, mais demeurent encore largement insuffisantes pour une compréhension globale de son rôle dans la santé humaine.

La deuxième partie de ce travail a porté sur l'optimisation de la culture des méthanogènes en développant un nouveau milieu de culture de référence dans notre laboratoire, le milieu GG®. Ce milieu présente l'avantage de ne pas nécessiter l'utilisation d'hydrogène gazeux, un gaz explosif soumis à des normes d'utilisation strictes.

La troisième partie décrit la découverte d'une nouvelle espèce de méthanogènes isolée à partir d'un échantillon de selle humaine, *Candidatus* Methanosphaera massiliense (*Cand.* M. massiliense). Étant également détectée chez le porc destiné à la consommation, nous avons émis l'hypothèse que cette espèce pourrait être acquise par le biais de l'alimentation.

Enfin, la quatrième partie propose une étude plus approfondie de *M. massiliense*, une méthanogène précédemment co-cultivée avec la bactérie *Pyramidobacter piscolens* (*P. piscolens*) à partir d'un échantillon de plaque dentaire prélevé sur un individu souffrant de parodontite sévère (17). Dans ce travail de thèse, *M. massiliense* a été à nouveau co-cultivée avec *P. piscolens* à partir d'un nouvel échantillon clinique, la pulpe dentaire infectée, offrant ainsi l'opportunité d'approfondir la caractérisation de cette espèce particulièrement difficile à cultiver. La forte dépendance à la bactérie *P. piscolens* pour la culture nous a également conduit à rechercher cette association dans le tartre ancien par des analyses statistiques basées sur des données métagénomiques de plusieurs études.

Une dernière partie présentera les principales conclusions et les perspectives de ce travail de thèse.

Chapitre I: Revues de littérature

Préambule du chapitre I

Le présent chapitre présente deux revues de littérature distinctes, chacune nous ayant permis d'acquérir les connaissances nécessaires pour la suite de ce travail de thèse.

La première revue s'est concentrée sur le microbiote oral, illustrant l'importance cruciale de la culture dans l'étude des micro-organismes. La culture est la méthode la plus ancienne utilisée par les microbiologistes et elle a grandement contribué à améliorer les connaissances scientifiques et médicales sur les microbes. Toutefois, elle présente plusieurs inconvénients et peut être en effet fastidieuse, couteuse et chronophage. C'est pourquoi elle a été progressivement abandonnée au profit approches moléculaires telles que les techniques de PCR et la métagénomique. Ces dernières méthodes ont largement contribué à l'extension des connaissances sur les microbiotes et ont mis en évidence l'existence de nombreux microbes non encore cultivés. Ceci a conduit à un regain d'intérêt pour les méthodes de culture, développant des méthodes et milieux toujours plus adaptés aux micro-organismes fastidieux et notamment ceux de la cavité orale. Les méthodes de culture adaptées à l'isolement des méthanogènes sont ce qui nous intéresse particulièrement dans ce travail de thèse. Ces méthodes ont évolué depuis Hungate et le roll-tube vers des méthodes de coculture avec des bactéries et de culture aérobie de ces micro-organismes anaérobies en utilisant des antioxydants. Toutefois, il semble que de nouvelles espèces restent encore à découvrir et à isoler afin d'étendre le répertoire des méthanogènes et des *Archaea* en général vivant dans le microbiote oral et pouvant avoir un réel intérêt clinique.

La deuxième revue de littérature est une revue systématique qui explore toutes les facettes connues de *M. oralis*, la méthanogène la plus fréquemment retrouvée dans la cavité orale. Son isolement en 1994 par Ferrari et al. a permis sa description phénotypique, jetant ainsi les bases de futurs travaux de recherche (15). Malgré le fait que les méthanogènes soient toujours moins étudiées que les bactéries, les connaissances sur *M. oralis* ont progressé au fil du temps. Cette exploration approfondie de la littérature a également été l'occasion de faire le point sur les méthodes de caractérisation et d'étude des méthanogènes au travers l'exemple de *M. oralis*, incluant l'analyse du métabolisme, des génomes disponibles, ainsi que la détection et l'identification par diverses techniques de biologie moléculaire et de spectrométrie de masse. Nous avons également fait le point sur sa sensibilité aux antimicrobiens, sa présence dans différents microbiotes humains et animaux, les pathologies auxquelles elle peut être associée, ainsi que les potentielles sources et dynamiques d'acquisition et ses interactions avec les autres micro-organismes au sein d'un microbiote donné. Ces différents aspects constituent autant de domaines

passionnants pour l'étude de nouvelles espèces de méthanogènes qui nous ont été utile pour la suite de ce travail de thèse.

Article 1: Culturing the Human Oral Microbiota, Updating Methodologies and Cultivation Techniques

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Review

Culturing the Human Oral Microbiota, Updating Methodologies and Cultivation Techniques

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Abstract: Recent years have been marked by a paradigm shift in the study of the human microbiota, with a re-emergence of culture-dependent approaches. Numerous studies have been devoted to the human microbiota, while studies on the oral microbiota still remain limited. Indeed, various techniques described in the literature may enable an exhaustive study of the microbial composition of a complex ecosystem. In this article, we report different methodologies and culture media described in the literature that can be applied to study the oral microbiota by culture. We report on specific methodologies for targeted culture and specific culture techniques and selection methodologies for cultivating members of the three kingdoms of life commonly found in the human oral cavity, namely, eukaryota, bacteria and archaea. This bibliographic review aims to bring together the various techniques described in the literature, enabling a comprehensive study of the oral microbiota in order to demonstrate its involvement in oral health and diseases.

Keywords: culture; human oral microbiota; bacteria; archaea; fungi; culturomics

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1. Introduction

The human oral cavity is a complex ecosystem favourable to the development and establishment of numerous bacterial, archaeal, viral, fungal and unicellular eukaryota species [1]. These different kingdoms of life cohabit and evolve within this complex ecosystem, composing what is commonly known as "microbiota" [2]. This is represented by a complex community that persists and develops on the surfaces of the oral cavity, generally in the form of a multi-species biofilm which transits through the saliva [3]. This biofilm, when growing on the teeth, is referred to by the generic term "dental plaque" [3]. The unique properties of the oral cavity and its constituent organs make the composition of the oral microbiota characteristic of the site it colonises and distinguishes it from that of neighbouring ecosystems, such as the skin, respiratory and digestive tracts [4]. These properties play a major role in determining the oral microbiota and its composition [4]. Several parameters are involved in determining which species can colonise, grow and settle to become a major or minor component of the microbiota, including the quality and quantity of saliva, the biotype and phenotype of the periodontium, the depth of the periodontal pockets around the teeth and the quality of the dental tissue [5].

The oral microbiota maintains a symbiotic relationship with its host [6]. The resident microorganisms of the oral cavity help fight pathogens, down-regulate undesirable and potentially pro-inflammatory responses, and promote oral health [7,8]. However, this symbiotic relationship remains fragile and susceptible to change depending on social, economic, dietary and nutritional factors; sedentary lifestyle, autonomy, hygiene compliance, smoking and alcohol consumption as well as genetic and physiological factors [5,9–12]. These factors directly influence the composition of the microbiota and also its behaviour

and its evolution from a commensal microbiota to pathogenicity [13]. Since the notion of infectious disease first appeared, humans have tried to classify microorganisms as pathogens or commensals as part of the local and resident microbiota, evolving in perfect symbiosis with their host [14]. It is now known that some bacterial species or even communities may have properties that escape our classification systems [15]. The distinction between pathogenic and commensal bacteria is becoming an increasingly complex and ambiguous task, especially when the microorganisms usually found in healthy states are involved in complex interactions with their host leading to a state of disease or infection [15]. Recent years have been marked by a paradigm shift in the study of the human microbiota, with a re-emergence of culture-dependent approaches [16–21]. Recent studies have been mainly devoted to cultivating the gut microbiota and studies on the oral microbiota remain very limited. The oral microbiome is highly diverse. Approximately 774 indigenous species are present in the oral cavity, including 54% cultivated and named species, 14% cultivated but not yet named species, and 32% remaining uncultivated phylotypes [6] (the Human Oral Microbiome Database, https://www.homd.org/; accessed on 23 January 2023). Recently, molecular-based studies have demonstrated that the oral microbiota is more diverse and complex and plays a vital role in the pathogenesis and development of many oral and systemic diseases [22,23].

In this literature review we review new strategies that have been developed to improve the culture and isolation of microbes from the human microbiota that can be applied to cultivate the oral microbiota, as well as their field of application.

2. Current Strategies to Study the Human Oral Microbiota

2.1. Next-Generation Sequencing and Metagenomics

Next-generation sequencing (NGS) is a new molecular approach applied to study the microbial diversity of diverse ecological niches [24,25]. NGS has enabled a more in-depth and non-specific exploration of the human microbiota by generating a huge amount of data leading to a better understanding its composition, mainly through amplicon sequencing and whole-genome metagenomics [26,27]. NGS has enabled a rapid and comprehensive analysis of microbial diversity in various ecological niches, which has propelled it to the forefront to gradually replace the tedious and time-consuming standard culture techniques [24,28].

However, the limitations of NGS are quickly identified. Indeed, the absence of a standardised protocol and the variability of the used DNA extraction techniques, as well as the variability of the primers used for amplification, create biases that make the results dependent on the technique used [25]. In addition, the depth bias associated with all molecular methods, due to limited sensitivity, results in the neglect of minority species [29]. One of the major shortcomings of molecular techniques remains the lack of information related to the viability, adaptation, and availability of the strains for further in vivo studies [18,21].

The limitations and lack of information generated by molecular techniques encourage us to return to the sources of the fundamental research in microbiology, namely microbial culture [18]. Indeed, microbial culture represents the basic component of microbiology, and has long been side-lined at the expense of molecular methods [18,24]. Much recent work underlines the need to combine molecular techniques such as NGS with recent and innovative approaches such as culturomics in order to overcome the lack of these techniques and to gain a global and exhaustive view of the composition of the microbiota of a given ecological niche [21].

2.2. Culturomics: A New Concept Applicable to Oral Microbiota

Culturomics is a high-throughput culture-based concept created in 2012 with the goal of isolating all species that make up the human digestive microbiota [30]. Since then, many studies have combined high-throughput culture and metagenomics with the goal of reducing the dark matter of the microbiota, consisting of unassigned and unmatched sequences, through the isolation and sequencing of previously uncultured species [20].

Culturomics has proven itself over the last ten years, and its application has been made possible by rapid colony identification using Matrix Assisted Laser Desorption Ionization/Time Of Flight Mass Spectrometry (MALDI-TOF MS) [30]. Culturomics has demonstrated its effectiveness and contribution to the exploration of the gut microbiota over the last decade by significantly expanding the field of knowledge regarding the repertoire of the human microbiota by isolating new or previously uncultured species [18–21,30]. Indeed, to date, more than 2770 different species, including 800 new species, have been isolated by culturomics from human specimens from different sites including: human gut, skin, urinary tract, vaginal and respiratory microbiota [31].

Various studies throughout the world have shown the interest of diversifying culture media and conditions to broaden the scope of culture, in addition to the use of specific media and targeted approaches (Table 1). Indeed, the use of selective and non-selective media has allowed a continuous progression of knowledge concerning the diversity of the human microbiota [32]. The oral microbiota is undoubtedly very diverse, as shown by the Human Oral Microbiome Data Base (https://www.homd.org/; accessed on 23 January 2023), which currently contains more than 774 different species (https://www.homd.org/; accessed on 23 January 2023) (Figure 1) [33,34]. Currently, metagenomics-based estimates suggest a global number of around 1200 species grouped into 19 different phyla, namely *Absconditabacteria* (SR1), *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Gracillibacteria* _(NG02), *Ignavibacteriae*, *Lentisphaerae*, *Proteobacteria*, *Saccharibacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes* WPS-2, and *Euryarchaeota* (https://www.homd.org/taxa/tax_hierarchy; accessed on 23 January 2023) [22]. The majority of cultured species are grouped in only six phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, and *Spirochaetes* (Figure 2) [22].

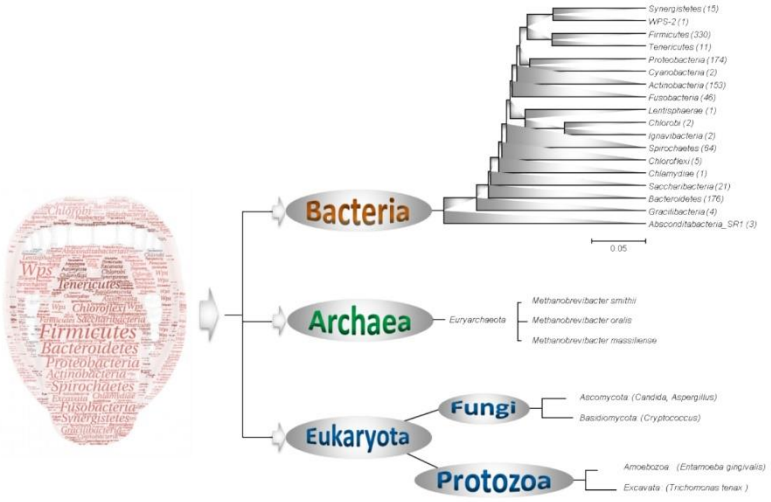

Figure 1. Phylogenetic diversity of the 19 phyla isolated or detected at least once in the human oral cavity. The number of species per phylum is presented between parents in the phylogenetic tree. The illustration on the left of the figure was made using the online tool wordle (www.wordle.net; accessed on 23 January 2023); the size of the name of each phylum is proportional to the number of species it contains. The phylogenetic tree was created using MEGA software. Figure 1 was built according to data collected from the Human Oral Microbiome Database; https://www.homd.org/; accessed on 23 January 2023.

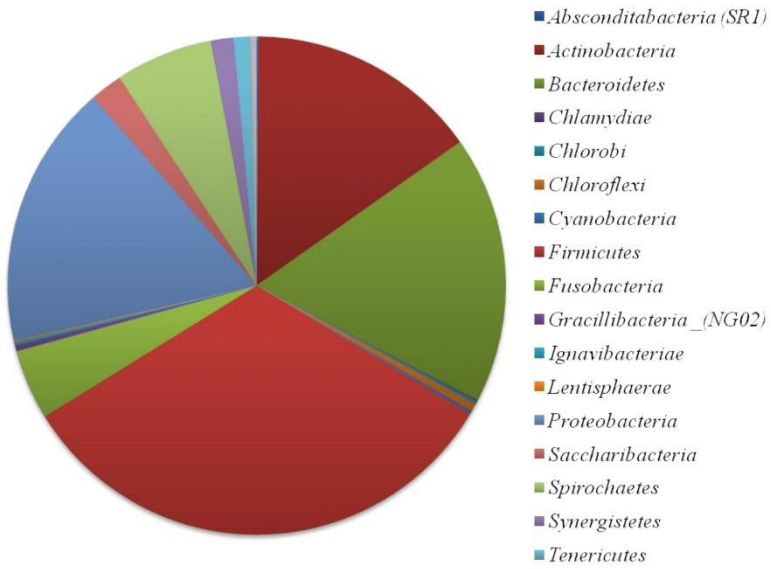

Figure 2. Quantitative distribution of cultured and uncultured bacterial diversity composing the oral microbiota represented by number of species composing each phylum. Each phylum is represented by a different colour, as shown on the right. (Figure 2 was built according to data collected from the Human Oral Microbiome Database; https://www.homd.org/; accessed on 23 January 2023).

The oral cavity is the main entrance and an obligatory passage for colonising microbes ending their journey in the digestive tract. The colonisation of these microbes to the oral ecosystem is also possible thanks to the favourable living conditions for the development and establishment of various microorganisms including bacteria, archaea, fungi, viruses, giant viruses, Candidate Phyla radiation group (CPR) and unicellular eukaryotes. Microbial culturomics may be a helpful tool for studying the oral microbiota by culture in order to broaden the spectrum of cultivated species of this ecosystem.

2.3. Rapid Culture of Anaerobic Bacteria Using YCFA Medium

Yeast extract-casein hydrolysate fatty acids (YCFA) culture medium is a rich medium widely used in the literature to culture strict anaerobes [35,36]. It is composed of growth factors, antioxidants, volatile fatty acids and vitamins essential for their growth (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1611.pdf; accessed on 23 January 2023).

One very interesting approach to cultivating anaerobic bacteria in record time was proposed by Naud et al., in 2020, using YCFA medium as well as the medium of blood culture bottles (BioMérieux, Marcy l'Etoile, France) supplemented with rumen [36]. The culture investigations, which were performed on only two stool samples, resulted in the isolation of 121 different bacterial species in only three weeks, of which 104 species were cultured within 24 h of incubation (Table 1). This is an unprecedented technical achievement, making it possible to use bacterial culture as a rapid and accurate diagnostic tool [36].

According to another study conducted by Browne et al., the YCFA culture medium allowed the cultivation of 137 species, 68 of which were completely new species, and 63 of which belonged to the phylum *Firmicutes*, four to *Bacteroidetes* and one to *Actinobacteria* [35]. This medium also gave exceptional results during a study by Forster et al. in 2019 on the diversity of the human digestive macrobiota, by succeeding in cultivating 273 different

bacterial species, of which 105 were new bacterial species distributed over three phyla: 91 species belonged to the phylum *Firmicutes*, 13 to the phylum *Bacteroidetes*, and one to the phylum *Proteobacteria* [37].

The oral microbiota consists of a large majority of anaerobic microorganisms [38,39]. YCFA medium is suitable for the culture of these oxygen-intolerant microorganisms and the studies described above demonstrate the high yield of culture using this medium to cultivate anaerobes [35,36]. Moreover, this medium is able to cultivate the most represented phyla in the human oral cavity, namely *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* [35,36]. The YCFA medium may constitute a good culture base for future investigations to explore the diversity of the oral microbiota. So far, and according to the sources we have consulted, no study exploiting the potential of the YCFA medium to study the diversity of the oral microbiota has been conducted.

2.4. Specific Conditions and Media for a Targeted Culture

2.4.1. Spore-Forming Bacteria in the Oral Cavity

Recent studies have described the isolation and characterisation of spore-forming bacteria from the oral cavity in the context of pathogenicity [40] or as an integral part of the oral microbiota [41]. Despite these limited studies, the number of spore-forming bacteria colonising the oral cavity is limited to a few cultivated species, mainly from the genera Bacillus or *Peanibacillus* [41]. Numerous techniques have been optimised for the specific culture of spore-forming bacteria in various domains, including the intestinal microbiota (Table 1).

2.4.2. Alcohol Decontamination

In 2016, Browne et al. described the use of improved pre-culture conditions that allowed for the targeted isolation of spore-forming bacteria [35]. This technique consisted of pre-incubating the sample in an equal volume of 70% alcohol for four hours at room temperature under aerobic conditions (Table 1). A wash was then performed prior to plating on a rich culture medium under anaerobic conditions [35].

The alcohol selection or disinfection technique was then adapted and modified by the culturomics team in 2020 to specifically isolate spore-forming bacteria that could be used in bacteriotherapy trials for the treatment of *Clostridium difficile* infections from human stool samples [42]. A total of 254 bacterial species were identified, of which nine represented new species. Of the species isolated in this study, 242 have never been included in clinical trials and represent potentially interesting new candidates for bacterial therapy trials [42].

2.4.3. Thermal Shock

Thermal shock is a selective technique based on the use of high temperature for the isolation of spore-forming bacteria. One of the culture protocols validated by the culturomics approach is to expose the sample to a constant temperature of 80 °C in a dry bath for 20 min to select spore-forming or spore-producing bacteria. The exposed sample is then subcultured on Columbia agar plates in anaerobic conditions [18].

2.4.4. The Use of Antioxidants to Improve Anaerobic Conditions

Antioxidants have long been used to establish an anaerobic atmosphere or to contribute to its improvement [43,44]. Indeed, antioxidants such as sodium sulphide (Na_2S), or amino acids such as cysteine, are found in old culture media optimised for the culture of anaerobic bacteria but also for the culture of methanogenic archaea for which sensitivity to oxygen is widely demonstrated [17,43,44]. Since then, many studies have focused on the beneficial effect of antioxidants for the culture of fastidious anaerobes. A more recent study conducted by La Scola et al., in 2014, succeeded in cultivating six strictly anaerobic bacteria and seven aerobic bacteria aerobically on Schaedler agar culture medium supplemented with 0.1 g glutathione and 0.5 g ascorbic acid. Among the species cultivated during this study, the authors mention the *Fusobacterium necrophorum* and *Ruminococcus gravus* species, known

for their extreme sensitivity to oxygen [45]. Their results suggest that using this unique medium it would be possible to cultivate anaerobic and aerobic bacteria aerobically in a standard incubator, enabling the renewal of the anaerobic culture [45].

In 2015, Dione et al. were able to expand the spectrum of bacterial species grown with antioxidants by culturing aerobic, microaerophilic, and anaerobic bacteria on a single medium by a simple addition of antioxidant consisting of 0.1 g glutathione +1 g uric acid +1 g ascorbic acid to Shedler agar medium [46]. This technical feat enabled the culture of 251 different bacterial species and showed that it was possible to perform antibiograms of anaerobic bacteria in aerobic conditions without affecting the sensitivity of the results [46]. This technique was then applied to methanogenic archaea culture to successfully isolate 13 strains of *Methanobrevibacter smithii* and nine strains of *Methanobrevibacter oralis* from 100 stools and 45 oral samples [16].

The work of Million et al., in 2020 confirmed the beneficial effect of antioxidants on the culture of anaerobes [47]. In this case, antioxidants were able to decrease the oxidative stress experienced by anaerobic bacteria of the genus *Clostridium* and maintain an increased production of volatile fatty acids such as butyrate, isobutyrate and isovalerate when grown in the presence of three antioxidant molecules: glutathione, ascorbic acid and uric acid. Volatile fatty acids are known to contribute to the maintenance and active resilience of host–bacterial mutualism against mucosal oxygen and uncontrolled oxidative stress in vivo [47].

Recently, new molecules with antioxidant activities have been described in the literature, such as phenolics, flavonoids and carotenoids [48]. These molecules with antioxidant potential should be tested for their antioxidant effect in order to improve or even establish the anaerobic atmosphere necessary for the culture of anaerobic bacteria in the oral cavity.

2.4.5. Bacterial Co-Culture

Co-culture has been widely used in recent years to make possible the culture of certain microorganisms that so far had remained uncultured. Co-culture is based on the capacity of some bacteria to produce or secrete active substances, cofactors or growth factors of interest to other species lacking them [16]. Numerous approaches have been used either to accelerate the bacterial growth, by the addition of missing unknown nutrients in the culture medium, or of known nutrients not commercially available [16,49–51]. To illustrate the contribution of co-culture to the field of microbial culture, and in particular oral cavity microbes, we took the example of methanogenic archaea.

The culture of methanogenic archaea is fastidious and requires an external supply of H_2 and CO_2 required for their growth [17]. In 2016, Khelaifia et al. succeeded in substituting the gaseous supply of H_2 and CO_2 by a co-culture with *Bacteroides thetaiotaomicron* used as the sole source of hydrogen and CO_2 in a dual chamber culture device. In this device, *B. thetaiotaomicron* were grown in the bottom chamber in Schaedler liquid medium supplemented by antioxidants producing enough hydrogen and CO_2 to allow growth of the methanogenic archaea *Mathanobrevibacter smithii* on solid medium and isolate viable colonies [16]. Previous studies have used cultured species to boost the growth of other fastidious bacteria using diffusion chambers or a double layer of agar separated by porous membranes [49,50]. Co-culture is therefore becoming a good alternative for laboratories lacking the technical means to culture fastidious bacteria and methanogenic archaea from all sources, whether biological or environmental [16].

2.4.6. Selective Media Based on Antimicrobial Agents, Antibiotics and Phages

The ability of some microorganisms, including bacteria and archaea, to resist antimicrobial agents is one of the most important public health problems of recent years, but also poses an interesting possibility for microbiologists to decontaminate samples for the purpose of the specific isolation of these resistant microorganisms [52,53]. Methanogenic archaea are known to be resistant to the majority of antibiotics used in antimicrobial therapeutics and the treatment of infectious diseases, except for metronidazole derivatives, chloramphenicol and fusidic acid [54,55]. Many studies have focused on this ability found

in some microorganisms to facilitate their isolation from a complex environment. The culture medium optimised by Khelaifia et al., in 2013, is now one of the most widely used media for the selective culture of methanogenic archaea [16] (Table 1). One of the peculiarities of this medium, in addition to its complex nutrient composition (thus considered a rich medium), is the addition of a cocktail of antibiotics aimed at eliminating Gram-positive and Gram-negative bacteria, fungi and yeasts [16,54].

The disadvantage of the use of antibiotics is the obligatory selection of resistant strains [52,53]. Bacterial growth can also be inhibited by the use of phages, which reduces the potential development of resistance associated with the use of antibiotics [56]. Indeed, bacteriophages are viruses that specifically infect certain bacteria. In the case of lytic phages, host bacteria are destroyed by their phage through a process of cell lysis [18]. This specific lysis capacity can therefore be used to specifically eliminate majority bacteria in a polymicrobial culture of any origin, including the human oral cavity, in order to cultivate minority and slow growing bacteria.

2.4.7. The Genomic-Reverse Culture Technique

Reverse genomics is one of the most promising new perspectives in the cultivation of uncultured microorganisms in all areas of life [57]. This technique exploits the ability of antibodies to bind specifically to a given organism, to isolate it by culture, and then sequence its genome from a simple culture or complex ecosystem [57]. Using this method, Cross et al. successfully isolated and cultured three species of *Saccharibacteria*, including the TM7 strain known to be an integral part of the human oral macrobiota [57]. They also isolated and cultured species from the human oral SR1 bacteria group, which are members of a previously uncultured bacterial lineage [57].

A more recent study has also demonstrated the potential of reverse genomics by identifying a common system capable of culturing all members of the *Saccharibacteria* group from any human sample [58]. This method is also applicable to any other sample or specimen of diverse origin [58]. In this study, the authors describe their success in obtaining in-silico specific epitopes for *Saccharibacteria* spp., distributed in four different transmembrane proteins. Subsequently, they suggest the use of all these epitopes to synthesise antibodies targeting the Saccharibacteria species in order to cultivate them. According to the authors, this strategy can also be applied to archaea, or to other phyla/taxa, such as the Parcubacteria phylum and the DPANN group (*Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota*, and *Nanohaloarchaeota*) [58]. Thus, the use of this methodology can help cultivate new microbes and enrich our knowledge of the composition of the human microbiota in general and the oral microbiota in particular. Most microorganisms in the human oral cavity from all taxonomic levels remain uncultured to date [59]. Reverse genomics can be a significant asset for the cultivation of uncultured microorganisms and can be applied to all species from all known ecosystems [57]. These species, once isolated and cultured, can be characterised and studied to better understand both the role they play in the oral microbiota but also their actual involvement in oral disease and health [60].

2.5. Treponemes of the Oral Cavity

Treponemes are spiral-shaped motile prokaryotes belonging to the genus *Treponema* and the order Spirochaetales, in the family *Spirochaetaceae* [61,62]. These microorganisms colonise the human intestinal [63], oral [64] and genital [65] microbiota, as well as those of animals. Most of these microorganisms are part of the commensal microbiota, but some species are opportunistic pathogens, such as *Treponema pallidum*, the causative agent of syphilis in humans [66]. Other species have been directly associated with a high prevalence in patients with periodontitis and gingivitis, such as *Treponema denticola*. Indeed, numerous studies have revealed the direct involvement of certain *Treponema* species in the severity and extent of periodontal lesions [67,68]. *T. denticola* is the most widely studied of these, and its virulence factors have been identified. This bacterium is a member of the Sokransky

red complex [69] and has the ability to bind to fibroblasts and epithelial cells via dentilisin protease, thus becoming cytotoxic and causing cell death [70].

Treponemes are a typical example of fastidious microorganisms with complex nutritional requirements. They cannot grow on minimal media, and their culture requires special and complex nutrients. Most *Treponema* species grow at temperatures ranging from 37 °C to 40 °C, anaerobically and/or micro-aerobically [71]. Several culture media have been reported in the literature, specifically designed to support the growth of these bacteria, and several modifications have been made to one or more of these media, including the addition of vitamins, carbohydrates, volatile fatty acids and serum of animal or human origin (Table 1) [71]. Initially, media and techniques previously designed for the culture of *T. pallidum* led to the isolation of the first treponemes associated with the human genital mucosa (*Treponema calligyrum*, *Treponema refringens*) [72] and the oral cavity (*T. denticola*) [73]. Thus, the nutritional requirements deduced from attempts to grow *T. pallidum* led to the formulation of specific culture media, adapted to the culture of *Treponema* spp., such as Growth Medium (GM-1) [74], Enriched Medium 10 (M10) [75], Oral Treponemes Isolation medium (OTI) [76], New oral spirochete medium (NOS) [77], Oral Treponemes Enrichment Broth (OTEB) [78], Oral Microbiology and Immunology, Zürich medium (OMIZ) [79], and more recently T-Raoult [71]. Teponemes culture media are summarized in Table 1.

The genus *Treponema* is the predominant genus of *Spirochaetaceae* colonising the oral cavity. Although these organisms are part of the normal microbiota, they have, to date, been neglected by microbiologists, and the number of cultured species is thus far limited to only ten species [71]. However, studies based on molecular methods by direct amplification of 16S DNA from oral cavity samples have revealed the existence of more than 80 different phylotypes belonging to the genus *Treponema* that remain uncultured so far [80]. Hence, there is a pressing need to develop new identification and culture methods specifically targeting *Treponema* from clinical samples, to allow the extension of the *Treponema* repertoire associated with human microbiota and, more particularly, the oral microbiota. This can be achieved by isolating new species to phenotypically characterise them and sequence their genomes in order to study their roles in human oral health and pathology to determine their role within this complex microbiota.

2.6. Archaea of the Oral Cavity

Archaea are unicellular prokaryotes forming a separate domain of life from bacteria, eukaryotes and giant viruses [81]. They were first isolated from extreme environments, such as hot springs [82] and salty lakes, but it is now known that they are ubiquitous and can be found in all known ecosystems, including living organisms [83,84]. In humans, methanogenic and some halophilic archaea species are principally found [85,86]. Archaea have colonised all human mucosa, particularly intestinal [87] and oral mucosa [88], and have been associated with dysbiosis [89] as well as abscessed pathologies [90].

Culturing methanogenic archaea is a long and fastidious process, due to their oxygen intolerance and specific nutritional needs [17]. Most methanogenic archaea require an external source of hydrogen and carbon dioxide for their growth [17]. In 1969, Hungate pioneered methanogenic archaea culture. He first did so in a strict anaerobic liquid medium, and then in a solid medium in roll tubes, which allowed a control of the culture atmosphere in general, as well as the addition of hydrogen and carbon dioxide at various concentrations in particular [44]. Since then, different media appropriate to the requirements of each known species have been optimised (Table 1) [17]. Recently, the SAB® medium has been developed and optimised in order to be versatile and enable the culture and isolation of most methanogenic archaea species [17]. The addition of antioxidants to agar SAB® medium also enabled the aerobic culture of methanogenic archaea, by placing the agar plates in the upper part of a double chamber, with a lower part containing liquid culture medium inoculated with *B. thetaiotaomicron* as the sole continuous source of hydrogen and carbon dioxide [16]. Subsequently, Guindo et al. improved the double chamber culture

technique by proposing a new concept, based on the production of hydrogen through a chemical reaction between acetic acid and iron filings [91].

In the oral cavity, methanogenic archaea are part of the commensal microbiota, especially in individuals who smoke [92]. They are also associated with periodontal pathologies and especially with periodontitis [93] and peri-implantitis [94]. However, they have also been detected in inflammatory and infected dental pulp [95]. *Methanobrevibacter oralis*, *M. smithii* and *Methanobrevibacter massiliense* are the sole methanogenic archaea cultured from oral cavity specimens, mainly saliva and dental plaque (Figure 1) [93]. Furthermore, the discovery of *Nanopusillus massiliensis* in dental plaque samples and its co-culture with *M. oralis* revealed the *Nanoarchaeota* phylum in the human oral cavity [96]. Finally, other archaea species have only been detected by molecular tools, including *Methanobacterium congolense*, *Methanocellus bourgensis*, Candidatus *Nitrosphaera everglandensis*, *Methanosarcinia mazei* [88,97] as well as non-methanogenic species belonging to the phylum *Thermoplasmata* [98,99].

Despite recent investigations regarding the culture of archaea, as well as technological advances and new culture methods, there remains a significant discord between the diversity of archaea in the gut microbiota and that of the oral cavity. To date, only three archaea species have been isolated from the oral cavity compared to seven from the digestive tract. This difference may be explained by the interest accorded to the gut microbiota recent years, particularly in the context of the Human Microbiome Project, which has focused on characterisation of the human gut microbiota [100]. In contrast, the oral microbiota remains neglected by microbiologists, and a lack of investigation and research work is notable, especially in the field of the culture of methanogenic archaea [83,88]. Additional efforts are needed to extend the repertoire of these microorganisms by first using molecular detection and diagnostic tools and then culture-based methods available in the literature to isolate them in order to better understand their metabolism and their involvement in human health and pathologies.

2.7. Fungi and Yeasts of the Oral Cavity through the Example of Candida

Fungi are a minor component of the oral microbiota. The diversity of this component is mainly represented by three genera, namely *Candida*, *Aspergillus*, and *Cryptococcus* (Figure 1) [101]. These commensal microorganisms are regularly present in low concentrations without causing infection, but they can become opportunistic pathogens when their host is immunodeficient [102]. The majority of oral fungal infections are forms of candidiasis [103]. These infections may be superficial and affect the mucosa, or they may penetrate the epithelium and be disseminated through the haematogenous pathway with serious consequences. Mucosal infections are seen in neonates and the elderly, two groups with suboptimal immune function [101]. They also affect people with suppressed immune systems [103].

Since the discovery of the first microbes [104], many techniques have been developed to study and cultivate fungi and yeasts from the oral cavity. Indeed, multiple references are proposed by manufacturers to enable an easy and selective culture of these microorganisms, including Sabouraud dextrose agar, malt agar, potato dextrose agar, CZAPEK, Colombia agar, Dixon agar, modified Leeming Notman agar, YPD medium and glycine-vancomycin-polymyxin B agar [32].

Recently, a new culture medium called FastFung® was developed by the Culturomics, Fungi and Yeasts Team at the IHU Méditerranée Infection, Marseille, with the objective of cultivating fastidious fungi [105]. The FastFung® medium was compared to the standard Sabouraud agar medium for the culture of 98 fungal and 20 bacterial strains. The positive culture rate of fungal strains was 100% compared to 95% for Sabouraud, and the inhibition of bacterial strains was 100% compared to 20% for Sabouraud medium [105].

The FastFung® culture medium was subsequently tested for the specific culture of *Malassezia* yeasts [106]. The aim of this study was to compare the performance of three skin sampling methods and two culture media for the detection of *Malassezia* yeasts by

culture from human skin samples. The FastFung® medium showed promising results for the specific culture of *Malassezia* yeast and was proposed for direct application in routine laboratory clinical diagnosis [106].

The FastFung® medium is a promising culture medium due to the results provided for the culture of fungi and yeasts from clinical samples. Therefore, this medium can be adapted for future studies for the cultivation of fungi and yeasts from the oral microbiota.

2.8. Protozoa of the Oral Cavity: Through Amoebae and Trichomonas

Protozoa are microscopic, unicellular eukaryotes. Each protozoan typically exists as an independent cell, living freely as a phagotrophic microorganism [107]. In some species, the cells join together to form colonies. Many protozoa are pathogenic and can cause diseases such as malaria, while others are commensals in the digestive tract of ruminants and wood-eating insects [107].

Protozoa are also part of the oral microbiota, of which *Entamoeba gingivalis* is the most frequent representative, followed by *Trichomonas tenax* (Figure 1) [108]. Oral protozoan infections have been shown to be mainly associated with periodontal disease [8]. Indeed, their prevalence is much higher in patients with periodontal disease than in those with healthy periodontium [108]. Currently, the detection of oral cavity protozoa is mainly performed by direct microscopy or by PCR targeting the 18S gene [8,109]. Culture-based approaches applied to these organisms are very rare in the recent literature. The few studies which have been published use a minimal culture medium with an agar base comprised between 1.5% and 2% [110]. The ATCC 1171 TYGM-9 culture medium (https://www.atcc.org/products/30956; accessed on 23 January 2023) adopted from the work of Clark et al. in 1992 and 1997, remains the reference medium for the culture of protozoa from the human oral cavity [111–113]. New investigations are, therefore, necessary to improve the culture of these fastidious organisms and new culture media must be optimised to broaden the research spectrum and isolate new species that have remained uncultured.

Table 1. Update and description of culture techniques and their research perspectives.

Techniques	Usual Denomination	Applications and Perspectives	References
Next-generation sequencing	NGS	Molecular-based study of the microbial diversity of diverse ecological niches.	[24–27]
Culturomics	Culturomics	Culture-based study of the microbial diversity of the human microbiota.	[18–20,30]
Rapid culture of anaerobic bacteria using YCFA medium	Rapid culture	Culture-based study of stool samples.	[36]
Blood culture bottles supplemented with Rhumen	Rapid culture	Culture-based study of stool samples.	[36]
Alcohol decontamination	NO	Spore-forming bacteria.	[35,42]
Thermal shock	NO	Spore-forming bacteria.	[18]
Antioxidants	NO	Improvement of anaerobic conditions for aero-intolerant bacteria culture.	[16,45,46]
Co-culture	NO	Improvement of fastidious bacteria culture.	[16,49–51]
Selective media	Antibiotics and phages	Selective culture of antibiotics and phase-resistant organisms.	[20,54,56]
The genomic-reverse culture technique	The genomic-reverse	Saccharibacteria TM7 group.	[57,58]
Oral Treponema enrichment	T-Raoult medium	*Treponema* spp.	[71]
Specific media for Treponema culture	OMIZ-WP = OMIZ-Pat	*Treponema* spp.	[79]
	OTEB	*Treponema* spp.	[78]

Table 1. *Cont.*

Techniques	Usual Denomination	Applications and Perspectives	References
	NOS	*T. pectinovorum* *T. denticola* *T. socranskii* subsp. *Socranskii* *T. vincentii*	[77]
	OTI	*T. pectinovorum*	[76]
	Oral spirochete medium	*T. denticola*	[114]
	PDDTp	*T. phagedenis* *T. denticola*	[115]
	Spirolate Medium	*T. vincentii*	[116]
	Thioglycolate medium	*Treponema reiter* *T. phagedenis* *T. refringens* *T. vincentii*	[117]
	Pre-reduced *T. denticola* broth	*T. denticola*	[118]
	Growth Medium (GM-1)	*T. denticola*	[74]
	Medium 10 (M10)	*T. socranskii*	[75]
	Human oral medium (HO)	*T. phagedenis* *T. refringens* *T. denticola* *T. vincentii*	[119]
Hungate roll tubes	Roll tubes	Methanogenic archaea culture. and strictly anaerobic bacteria.	[44]
Double chamber technique	SAB-medium	Methanogenic archaea culture.	[16]
Modified double chamber technique		Methanogenic archaea culture.	[97]
FastFung®	FastFung®	Fungi and yeasts.	[105]
The ATCC 1171 TYGM-9 culture medium	ATCC 1171	Protozoa of the oral cavity.	[110]

NO: unspecified.

3. Discussion

Since the discovery of the first microbes by Anthonie van Leeuwenhoek in the 17th century (1683) [104], microbial culture was one of the first methods used to study the human microbiota [18]. Since then, microbial culture has greatly contributed to improving the scientific and medical knowledge of the microbes studied as well as of the diseases they provoke [19]. Microbial culture has long been considered tedious, costly and time consuming [20,21]. As a result, microbiologists have gradually abandoned it in favour of new molecular approaches such as PCR techniques and metagenomics [20,21]. Indeed, metagenomics has been considered as one of the most promising techniques for studying microorganisms without culturing them. It was first applied in 2005 to study the human intestinal microbiota with the prospect of revealing all remaining uncultured microbes [18]. Subsequently, culture-based studies were gradually abandoned and metagenomics became the reference method used by microbiologists to study complex ecosystems [24]. However, this technique presented many biases related to the extraction methods and the type of primers used for the amplification of genomic DNA, which made the results obtained non-reproducible [120]. In addition, a depth bias makes all species with a population below 1×10^5 or even 1×10^6 per gram of sample undetectable by this technique [19,24,120]. Nevertheless, metagenomics has contributed to the expansion of knowledge about the uncultured part of the human microbiota, which does not correspond to any known microbe, commonly referred to as "dark matter" [19,20]. It is a question of an uncountable number of sequences not assigned to any known microorganism, which metagenomics is unable to identify [19,20].

In recent years, microbial culture has experienced a re-emergence thanks to environmental microbiologists who have developed culture conditions close to the original environment of bacteria in order to facilitate their cultivation [18]. Numerous approaches

have subsequently proven their value in microbial culture, such as microbial culturomics, which has made considerable progress in the field of culture and especially in the repertoire of human microbiota, enabling the culture of more than 2770 spaces of bacteria, archaea, fungi and yeasts, as well as unicellular eukaryotes [31]. In contrast, few studies have focused on the oral microbiota by culture, and studies performed in this field remain limited. Indeed, the oral cavity hosts a complex microbiota composed of more than 700 different bacterial species colonising the surface of the teeth and the oral mucosa [22]. After the gut microbiota, it is the second microbiota in humans in terms of number and diversity [22].

The study of the human microbiota has become a major public health issue given its involvement in human health and disease, which goes beyond our understanding. Several studies in the literature have clearly demonstrated its involvement not only in disease but also in human health. Its involvement in the regulation of the response to anti-cancer treatments has been demonstrated [121–123]; as well as the participation in the metabolic homeostasis of certain species like *Akkermansia muciniphila*, which could have a protective effect against metabolic disorders [124]. Indeed, the involvement of *Faecalibacterium prausnitzii* and *A. muciniphila*, two of the most abundant bacteria in the human gastrointestinal tract, in the marked attenuation of colitis, weight loss, and decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines in patients with chronic inflammatory bowel disease has been demonstrated [125,126]. In the oral cavity, numerous studies have described a protective role attributed to the oral microbiota. According to Sekirov et al., 2010, the microbiota may provide its host with a physical protective barrier against incoming pathogens by competitive exclusion [7]. Indeed, many actions of the microbiota can have a beneficial effect on its host, such as the occupation of attachment sites, the consumption of nutrient sources and the production of antimicrobial substances [7]. The microbiota also acts on the immune system of its host by stimulating the production of various antimicrobial compounds such as defensins, cathelicidins and C-type lectins, known to be produced generally in the digestive tract of mammals [7,127].

The oral microbiota has also been incriminated in certain infectious and tumoral pathologies of the oral cavity as well as in numerous systemic pathologies such as diabetes, cardiovascular diseases and certain distant tumour illnesses [23].

It now seems clear that the oral microbiota plays an essential role in the maintenance of oral health in particular and of human health in general. The alteration of this microbiota has often been associated with oral and systemic pathologies. However, the role of the oral microbiota in the onset and development of these diseases remains unresolved, and further investigations are needed to characterise the oral microbiota and elucidate its involvement in human and oral pathologies. Culture-based studies offer good potential in this regard, and microbial culture still has much to contribute to the study of microbiota in order to obtain clinical isolates, to study their metabolism and their behaviour towards antimicrobial agents used in clinical practice, as well as to sequence their genome to better understand their implication in the process of oral infectious diseases.

4. Patents

S.K. is co-inventor of a patent on the culture of anaerobic bacteria (CAS 28-FR1757574); S.K. is co-inventor of a patent for the preservation of bacteria (1H53 316 CAS 25). S.K. is co-inventor of patent: "Milieu et procede de culture et extraction des archaea methanogenes" (FR1254779A). S.K. is co-inventor of a patent on the use of antioxidants to aerobically cultivate anaerobic bacteria and methanogenic archaea (1H52437 cas 32FR). S.K. and S.B. are co-inventors of a patent on the use of vinblastine or vincristine to improve the culture of treponemes (FR 19 13945).

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Article 2: *Methanobrevibacter oralis:* from oral cavity commensalism to opportunistic pathologies

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Methanobrevibacter oralis: from oral cavity commensalism to opportunistic pathologies

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Submitted manuscript

Abstract. 3
Key points. 4
Bibliography strategy. 7
Antiquity of *M. oralis*. 8
General microbiology. 12
First insights into oral methanogens and the isolation of *M. oralis*. 12
Phenotypic characterisation. 12
Molecular detection and identification. 13
Genomes and diversity. 15
Culture methods. 16
Antimicrobial susceptibility testing. 18
M. oralis in the human microbiota. 19
M. oralis in pathological situations. 20
Oral dysbiosis. 20
Abscesses. 26
Other pathologies. 27
M. oralis in animal microbiota. 29
M. oralis and its host. 29
Pathogenicity and host response. 29
Acquisition and dynamics. 31
M. oralis and bacteria. 32
M. oralis and other archaea. 35
Perspectives and conclusion. 37
Acknowledgements. 42
Conflict of interest statement. 42
Author Contributions statement. 42
Data Availability Statement. 42
References. 43

submitted manuscript

Abstract.

Methanobrevibacter oralis (*M. oralis*) has predominated human oral microbiota methanogenic archaea as far back as the Palaeolithic era in Neanderthal populations and gained dominance from the 18th century onwards. Initially discovered in dental plaque samples collected from two apparently healthy individuals, *M oralis* was then detected in various oral cavity sites in healthy individuals and those diagnosed with oral pathologies, notably periodontal diseases, and endodontic infections. *M oralis* was further detected in brain abscesses and respiratory tract samples, bringing its clinical significance into question. Transmission pathways, possibly involving maternal milk and breastfeeding, remain to be clarified. The culture and molecular methods used for its identification are challenging due to their fastidiousness. Next-generation sequencing contributed five draft genomes, each approximately 2.08 Mb (+/- 0.052 Mb) with a 27.82 (+/- 0.104) average GC%, and two ancient metagenomic assembled genomes. This review summarises the current knowledge about *M. oralis*, highlighting its prevalence, associations, symbiotic relationships, and potential pathogenicity. Understanding its role in oral and systemic health may inform targeted therapeutic interventions and encourage further exploration of the dynamics of the human microbiota.

Key words: *Methanobrevibacter oralis*, methanogen, oral microbiota, dysbiosis, periodontitis, endodontic infection, abscess, ancient dental calculus.

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Key points.

1. *Methanobrevibacter oralis*, the most predominant methanogen in human oral microbiota, traces back to the Palaeolithic era, showing dynamic relationships with human evolution.
2. The intricate interactions of *Methanobrevibacter oralis* within the oral microbiota and its potential role in oral and systemic pathologies need further investigation as a diagnostic biomarker and therapeutic target.
3. Despite its long-standing association with humans and oral cavity prevalence, *Methanobrevibacter* oralis' genomic, phenotypic, and metabolic characteristics remain largely unexplored, urging comprehensive studies to uncover its unique ecological niche and health implications.

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Introduction.

Methanobrevibacter oralis (*M. oralis*) is one of ten methanogenic archaea (methanogens) identified in the oral microbiota (1), alongside *Methanobrevibacter smithii* (*M. smithii*) and *Methanobrevibacter massiliense* (*M. massiliense*), all three of which were isolated through culture methods (2). *M. oralis* was originally isolated in 1994 from two dental plaque samples collected from two apparently healthy individuals (3). Since then it has then consistently emerged as the most prevalent methanogen associated with the oral microbiota (4), exhibiting a dynamic relationship with human evolution, dating back to the Palaeolithic era in Neanderthal populations (5–8).

Exploration of *M. oralis* surpasses mere confirmation of its presence. The quantification of *M. oralis* has emerged as a potential diagnostic biomarker and a therapeutic target for some oral diseases (9), given its distinctive antibiotic resistance profile (10). Its intricate interactions within the oral microbiota, involving various micro-organisms such as bacteria (11) and nanoarchaea (12), reveal a complex network of relationships. Unravelling these complex interactions and dependencies within microbial ecosystems could provide valuable insights into the role of *M. oralis* in maintaining microbial balance and influencing health outcomes. Beyond its oral domain, *M. oralis* extends to an extra-oral realm, with notable occurrences in brain abscesses (13,14). This extra-oral presence suggests a potential for systemic implications and prompts a reassessment of its medical significance. The intricate dynamics of *M. oralis* in the broader context of human health remain largely uncharted territory.

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The objective of this literature review is to provide an in-depth exploration of *M. oralis* in oral and extra-oral sites and networking connections with other micro-organisms, emphasising the need for a holistic approach. By summarising existing knowledge and delving into unexplored facets, this review aims to pave the way for future investigations, unlocking the full spectrum of the impact of *M. oralis* on oral and systemic health.

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Bibliography strategy.

This review is based on scientific literature published up to December 2023, obtained from the PubMed, Google Scholar, and Web of Science databases. A specific query was formulated to identify articles related to *M. oralis*, using the search terms "*Methanobrevibacter oralis*" or "*M. oralis*". Articles were screened based on their titles and abstracts, and were required to be original research articles published in English, specifically addressing the presence of *M. oralis* in humans and animals, and other relevant studies. Out ofthe initially identified 1099 articles (PubMed: n=56; Web of Science: n=56; Google Scholar: n=987), 73 were reviewed after removing duplicates and applying a meticulous screening process. Additionally, a manual search aiming to elucidate ecological niches and hosts where *M. oralis* was absent, yielded 15 additional relevant studies, contributing to a broader understanding of the microbial ecology of this specific methanogen. Furthermore, references cited in the selected papers were examined to identify and incorporate any additional relevant articles that could enhance the overall comprehension of the topic. For comparison, the same search was conducted for *M. smithii* using the same three databases and the search terms "*Methanobrevibacter smithii*" or "*M. smithii*", resulting in a total of 9702 publications (PubMed: n= 295; Web of Science: n= 517; Google Scholar: n=8890). This initial bibliometric comparison suggests that *M. oralis* has been studied to a much lesser extent than *M. smithii*.

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Antiquity of *M. oralis*.

DNA Illumina sequencing detected *M. oralis* in two middle Palaeolithic (about 50 000 BP) sediment samples from the El Salt site in Spain, containing millimetric coprolites and faecal biomarkers in sufficient proportions to suggest a *Homo* origin (7). The oldest evidence for *M. oralis* in humans was, however, obtained by metagenomic analysis of a dental calculus sample collected from an approximately 48 000-year-old Neanderthal individual suffering from dental abscess, in the El Sidrón cave (El Sidrón 1) in Spain (5). This sample yielded an almost complete *M. oralis* genome sequence named *M. oralis* subsp. *neanderthalensis*, thought to have diverged from the modern *M. oralis* strain JMR01 about 12 600 years BP, far later than the genomic divergence of Neanderthals from *Homo sapiens* which took place between 45-000 to 75 000 years BP (5). Comparing metagenomic taxonomy classifications with four different databases and using a nucleotide-to-nucleotide alignment-based taxonomic classification, a MALTn-RefSeqGCS taxonomic analysis performed on previously published data (including those from (5)) also revealed the presence of *M. oralis* in an additional Neanderthal dental calculus sample from the Spy Cave (Spy II) in Belgium dated 36 000 years BP (6).

Later in the prehistoric timeline, *M. oralis* was also detected in ancient Japanese *Homo sapiens*, spanning both the "Jomon" hunter-gatherer period (3000 years BP) and the "Edo"' agriculturalist period (400–150 years BP) by dental calculus aDNA metagenomics (15). Specifically, this study highlighted a higher abundance of *M. oralis* in women with periodontal disease (32%), evidenced by higher levels of bone loss during the agriculturalist "Edo" period than in men (5%). Although not statistically significant, this shed light on potential gender-specific dynamics in oral

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158 health during that historical era. Likewise, *M. oralis* was reported in dental calculus
159 samples collected from nine individuals (dating from 1479 to 495 years BP) in
160 California in the United States (16).

161 In a broad metagenomic investigation spanning various historical sites, aDNA
162 extracted from both dentin and dental calculus in 48 individuals revealed that dental
163 calculus proved to be less contaminated and more DNA-rich in oral microbes, making
164 it a better choice for ancient oral microbiota studies (17). *M. oralis* was identified
165 exclusively in dental calculus in seven individuals: 4/36 from Ireland (600–1300
166 Common Era (CE), 2/2 from Guadeloupe (975–1395 CE), and 1/2 from the
167 Netherlands (1611–1866 CE) (17). Metaproteomics analysis of dental calculus
168 sampled in 21 individuals buried in a 1100-1450 CE mediaeval cemetery in Tjærby,
169 Denmark, detected several proteins from *M. oralis* which were found to be
170 significantly more abundant in the group with an abundance of periopathogenic
171 species compared to the other group associated with oral health (18). *M. oralis* was
172 also found in higher abundance in 76 individuals from Middenbeemster in the
173 Netherlands dated from 1611–1866 CE than in the 31 individuals from various other
174 sites (19). This was confirmed by another metagenomics study including 65
175 individuals from the same site of Middenbeemster, and eight from Convento de los
176 Mercedarios de Burtzeña (CMB), Spain, both dated from the 19th century industrial
177 era (20).

178 PCR-sequencing of hundreds of dental calculus samples collected from six
179 archaeological sites in France, dated from the 14th to the 19th century detected *M.*
180 *oralis* in 11/56 (19.6%) of samples free of PCR inhibition (56/100), with significantly
181 lower prevalence in past populations compared to modern ones (21). Additionally, the
182 metagenomic reconstruction of oral microbiomes from 44 ancient foragers and

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183 farmers in the Balkans and the Italian peninsula spanning a large period from the
184 Palaeolithic era to the Early Middle Ages, and comparison with historical samples
185 confirmed the increased abundance of *M. oralis* in historical samples from the 18th
186 and 19th centuries (22). Furthermore, a comprehensive analysis spanning the
187 Neolithic period to the early Middle Ages, focusing on methanogen diversity and
188 evolution in the oral microbiome, revealed two previously unidentified archaeal
189 species which were predominant before the 18th century (8). Intriguingly, *M. oralis*
190 emerged exclusively in samples from the Middle Ages and became dominant from
191 the 18th century. However, another study reported that *M. oralis* was more abundant
192 in ancient samples (23).

193 In a non-human study exploring the oral microbiome of ancient Egyptian
194 baboons from the end of the pharaonic era (9th–6th centuries BP) and historical
195 baboons from the 19th century through metagenomic analysis, *M. oralis* was identified
196 in one historical baboon and one Egyptian mummy. This presence is likely to be
197 attributable to horizontal foodborne transmission, a consequence of captive breeding
198 practices (24).

199 In essence, *M. oralis* appears not to be historically confined to *H. sapiens*, as it
200 has been found in Neanderthals. Indeed, the earliest evidence of *M. oralis* was
201 identified in coprolitic sediment (7) and dental calculus (5) from these hominids.
202 Neanderthal or *Homo neanderthalensis* represented a species closely related to
203 *Homo sapiens*. Inhabiting Europe and Western Asia between approximately 400 000
204 and 40 000 years ago, both species displayed distinctive anatomical features,
205 including robust bodies and elongated skulls (25). Neanderthals exhibited
206 adaptations to diverse environments. Proficient hunters and gatherers, they adhered
207 to an omnivorous diet, using a range of resources from large animal meats to plant

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foraging (5,25). While the draft genome of *M. oralis neanderthalensis* was recovered from the dental calculus of a Neanderthal individual with a non-meat-centric diet at the El Sidrón site in Spain (5), subsequent detection in a site associated with meat consumption, the Spy site in Belgium, contradicted the hypothesis that meat consumption influenced the presence of *M. oralis* in Neanderthals (6). *M. oralis* was further detected in prehistoric *H sapiens* and possible transmission between the two *Homo* species is still open (5). The advent of agriculture at the beginning of the Neolithic area appears to have led to an increased abundance of *M. oralis* (15). Nonetheless, the precise impact of agriculture on oral microbiota remains uncertain, as the modifications appeared to have unfolded gradually (22). Interestingly, the introduction of tobacco in Europe during the 16th century did not seem to have a discernible impact on *M. oralis* (20). Later, *M. oralis* was sporadically detected in numerous studies and identified in populations across several continents, including pre-contact populations in America (16). This underscores the widespread presence of *M. oralis* in *H. sapiens* long before the onset of the European colonisation of America (16). A notable shift in its abundance and prevalence occurred in Europe (8,21,22). Collectively, these three studies suggest a notable rise in the prevalence and abundance of *M. oralis*, particularly from the 18th century onwards, possibly influenced by societal changes and potentially linked to the generalisation of sugar consumption during the industrial era. Further investigations into the evolutionary timeline of *M. oralis* across different human populations in different parts of the world and its potential interactions with dietary and lifestyle changes could provide deeper insights into its prevalence and significance in oral microbiomes over time **(Figure 1)**.

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232 **General microbiology.**

233

234 **First insights into oral methanogens and the isolation of *M. oralis*.**

235

236 The search for methanogenic archaea in the human oral cavity began in 1987
237 by culturing samples of dental plaque from patients who had not brushed their teeth
238 for 24 hours (26). The authors aimed to find methanogens in the subgingival plaque
239 from the gingival crevice, an ecological niche favourable to the growth of anaerobic
240 micro-organisms (26). Methanogens belonging to the *Methanobrevibacter* genus
241 were isolated from three of ten samples, confirming their presence in the oral cavity
242 (26). Along with this first study, another survey used the culture approach to reveal
243 the presence of methanogens in the dental plaque of patients with periodontal
244 disease (27). The predominant cultivated genus was *Methanobrevibacter*. Finally, in
245 1994, *M. oralis* was isolated and characterised from the subgingival plaque of two
246 apparently healthy patients, and the results were published by Ferrari *et al.* (strain
247 DSM 7256) (3). This occurred twelve years after the isolation of *M. smithii* (28) and
248 nine years after *Methanosphaera stadtmanae* (*M. stadtmanae*) (29), both from
249 human faeces.

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251 **Phenotypic characterisation.**

252

253 *M. oralis* has been described as a non-motile, non-spore-forming
254 coccobacillus with tapered ends or short oval rods of 0.4–0.5 µm in width and 0.7–1.2
255 µm in length, observed by two or short chains. *M. oralis* is gram-positive to gram-

variable after four days of culture, and is autofluorescent at 420 nm, as are other methanogens **(Figure 2)**. Less is known about its cell wall and membrane, contrary to other methanogens, but in transmission electron microscopy, *M. oralis* harbours a tri-stratified wall with deep invaginations, as observed in *Methanobrevibacter ruminantium* but not in *M. smithii* or *M. arboriphilicus* (3).

Molecular detection and identification.

Molecular detection is typically carried out through PCR and RT-PCR, and is sometimes supplemented by sequencing identification. These systems target either the 16S rRNA or the *mcrA* gene encoding methyl coenzyme reductase A involved in methanogenesis **(Table 1)**.

Five distinct systems targeting the 16S rRNA gene have been developed. Pairs of primers (forward primer 300fEyAr and reverse primer 954rEyAr (30) and forward primer SDArch0333aS15 (31) and reverse primer SDArch0958aA19 (32)), initially designed to detect and identify archaea including *M. oralis*, have been used in several studies, although a first attempt failed to detect archaea in endodontic infections using the universal primers for archaea Arch21F and SDArch0958aA19 (33) **(Table 1)**. The primers pair SDArch0333aS15/SDArch0958aA19 was also combined with a TAMRA probe in a 5′ nuclease assay and ABI Prism 7900HT Sequence Detection System (Applied Biosystems) for fast RT-PCR (4). Later, Vianna *et al.* (34–36) used a previously published Sybr Green RTQ-PCR system (A109F (37) and A934R (38) to identify and quantify archaea in endodontic infections and other oral conditions **(Table 1)**. Another 16S rRNA-gene based Sybr Green RT-PCR

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system (probes Mbb279F and Mbb709R) was designed to detect *M. oralis* and closely-related methanogens (39). Finally, a RT-PCR system targeting the methanogen 16S rRNA gene was used to detect, without specification, all methanogens, including *M. oralis* (40–42).

Three systems targeting the *mcrA* gene detected *M. oralis* **(Table 1)**: the ME1 and ME2 primers, previously published (43), and used to study methanogen diversity in faecal samples from patients diagnosed with intestinal diseases (44); the LuF and LuR primers (45), also used in various studies **(Table 1)**; and the mcrAFor and mcrARev primers that successfully detected *M. oralis* in sinusitis and oral samples using standard PCR and sequencing (46,47).

Finally, one *M. oralis*-specific RT-PCR system targeting a chaperonin gene (*M. oralis*-cnp602F and *M. oralis*-cnp602R, the forward and reverse primers and the probe *M. oralis*-cnp602P) made it possible to quantify the *M. oralis* load (9). This specific system was further used to detect *M. oralis* in plaque samples (2,12,48,49), maternal milk (50), and brain abscesses (13), and also to quantify *M. oralis* in peri-implantitis cases (51) **(Table 1)**.

Furthermore, several Fluorescent In Situ Hybridization (FISH) probes have been designed to detect archaea including *M. oralis*. The Arch 915 probe targets archaea 16S rRNA and, coupled with different fluorochromes, has visualised *M. oralis* in various samples (12,21,47,48,52). The SBGA-1 probe, designed on the 16S rRNA gene, ahs detected oral methanogens in dental plaque samples (4). A third FISH probe, based on the probe LuR, targeted the methanogen *mcrA* gene, including *M. oralis* (47).

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303 Finally, metagenomics and Next-Generation Sequencing (NGS) detected *M.*
304 *oralis* in various samples (53,54), including ancient specimens (5–
305 8,15,17,19,22,24,55), while a comprehensive study of over 1000 gut samples failed
306 to detect *M. oralis* (56).

307 **Genomes and diversity.**

308

309 Only five draft *M. oralis* genomes were available in the NCBI database at the
310 time of this review (December 2023) **(Table 2)**. The first draft genome, published in
311 2014, was obtained from the *M. oralis* strain JMR01 isolated in our laboratory from
312 the human gut (57). A second draft genome of *M. oralis* DSM 7256, isolated from
313 human subgingival plaque in 1994 by Ferrari *et al.*, was published in 2016 (57, 58).
314 Two other draft genomes were deposited by our laboratory, *M. oralis* CSUR P5920
315 (name M2), isolated from human breast milk (50) and the reference genome of *M.*
316 *oralis* YH, isolated from dental plaque, along with its nanoarchaeal symbiont,
317 *Nanopusillus massiliensis* (12). Following these reports, the *M. oralis* genome is
318 about 2.08 Mb (+/- 0.052) with an average 27.82 GC% (+/- 0.104) and an average of
319 1896 protein coding genes (+/- 41) (**Table 2**). It harbours two or three CRISPR loci
320 and associated proteins (Cas). Based on the *M. oralis* strain JMR01 draft genome,
321 multispacer sequence typing (MST) using four spacer primer systems revealed at
322 least nine genotypes in *M. oralis*, several variants of which could be carried by a
323 single individual (49).

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326 **Culture methods.**

327

328 Methanogen culture is fastidious, as methanogens are strictly anaerobic
329 and thus require specific conditions of culture and isolation. The first ever *M. oralis*
330 isolate was cultivated in a modified Balch *et al.* medium 1, called anaerobic growth
331 medium (MB), a liquid medium preserved in a serum bottle incubated under 80%
332 H_2/20% CO_2 atmosphere (202.6 kPa) for eight days (3). The headspace was
333 repressurised every three to four days (3). Serial dilutions were then subcultured on
334 an MB agar plate for 15-20 days and multiple further transfers with antibiotics made
335 pure isolation of the *M. oralis* strain DSM 7256 possible. Ferrari *et al.* detailed the
336 optimal growth conditions according to neutral pH value (6.9 to 7.4), NaCl
337 concentration (0.01 to 0.1 M), temperature (36 °C to 38 °C), and emphasised the
338 need for a 80% H_2 /20% CO_2 atmosphere and the presence of a mixture of volatile
339 fatty acids and faecal extracts. Meanwhile, formate, acetate and methanol were
340 reported as being dispensable (3). Other studies used the medium 119a
341 (https://www.dsmz.de) under 80%H_2 /20% CO_2 atmosphere (1 bar) at 37 °C and pH
342 7, as recommended by the Leibniz Institute DSMZ (59–63). This medium was
343 compared to a new adopted culture medium called SAB medium to improve the
344 culture and isolation of mesophilic methanogens associated with the human
345 microbiota. *M. oralis* DSMZ 7256 grew faster in the SAB medium (three-day
346 incubation with a 18-hour doubling time) than in modified DSMZ 119 medium (seven-
347 day incubation and a 21-hour doubling time) (61). SAB medium was used to establish
348 the repertoire of methanogens cultivated from severe periodontitis (2) and to study *M.*
349 *oralis* genetic variants (49). Subsequently, SAB medium was optimised for bedside
350 sampling, enabling the aerobic culture of methanogens including *M. oralis* (64). Oral

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samples were collected in an Ae-Ana transport medium (Culture-Top, Marseille, France) initially designed for aerobic conservation during the sampling and transport of anaerobic bacteria. After this, 1 mL was aerobically transferred into a Hungate tube containing 5 mL of a modified liquid SAB medium with a growing culture of H_2-CO_2, producing *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*). The composition of the medium was supplemented with three antioxidants (uric acid, ascorbic acid and glutathione), while glucose was required for *B. thetaiotaomicron* growth. After nine days, methane-positive cultures were inoculated onto SAB modified-solid plates with antibiotics to remove bacteria and fungi, and placed into the upper part of the two-chamber flask and incubated at 37 °C for seven days. Ultimately, nine *M. oralis* strains were isolated. This technique was also used to isolate *M. oralis* from oral fluid (52,65) and human milk (50). Furthermore, in order to routinise methanogen culture in the laboratory, hydrogen-producing *B. thetaiotaomicron* was successfully replaced by 1.5 g of iron filings, 200 mL of distilled water and 150 µL of acetic acid. The action of this weak acid on the iron produced sufficient hydrogen to enable the isolation of four additional strains of *M. oralis* (66).

Growing colonies could be identified by peptide profiling using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as a rapid and low cost technique to identify cultured micro-organisms including archaea, using one of two reported protocols (59,65). Both protocols involved a specific protein extraction from broth medium. *M. oralis* reference spectra were absent from the Brüker-Daltonics database, but five *M. oralis*-strain spectra were added to our laboratory database, and successfully identified *M. oralis* from 14 clinical isolates (59,65).

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376 **Antimicrobial susceptibility testing.**

377

378 *M. oralis* was initially isolated with MB medium containing cefalotin,
379 clindamycin, kanamycin, and vancomycin, and several dilutions were necessary to
380 obtain a pure culture (3). Later, the antimicrobial resistance pattern of methanogens
381 was determined using the macrodilution technique. *M. oralis* was found to be
382 resistant to amphotericin B, ampicillin, streptomycin, gentamycin, rifampicin,
383 ofloxacin, tetracycline (MIC > 100 mg/L), and vancomycin (MIC > 50 mg/L);
384 moderately susceptible to chloramphenicol and bacitracin (MIC < 25 mg/L); and
385 susceptible to metronidazole and ornidazole (MIC < 1 mg/L) (60). The *M. oralis*
386 susceptibility profile was, therefore, similar to *M. smithii* except for bacitracin, to which
387 *M. smithii* was susceptible (MIC < 1 mg/L). Also, in contrast to *M. smithii*, the *M.*
388 *oralis* genome lacks the chloramphenicol-O-acetyltransferase gene, despite its
389 moderate susceptibility to chloramphenicol. A further study pointed towards
390 resistance to chloramphenicol (MIC = 50 mg/L) and ceftriaxone (MIC = 100 mg/L),
391 two antibiotics commonly used to treat brain abscesses (13). The correlation of the
392 anti-archaeal activity of imidazole derivatives was confirmed with their hydrophobicity,
393 in particular with the aim of improving periodontal treatment (62). Later, *M. oralis*
394 susceptibility was extended to biocides. *M. oralis* was found to be susceptible to
395 squalamine (MIC = 0.5 mg/L) and derivatives (from 0.5 to 5 mg/L), paracetic acid
396 used to disinfect medical devices (MIC = 1.5 g/L), and chlorhexidine (MIC = 0.2 mg/L)
397 (63). The anti-cholesterol pro-drug lovastatin was also found to be effective on
398 human methanogens including *M. oralis* (MIC = 4 mg/L) by interfering with isoprenyl
399 synthesis and disrupting cell wall synthesis, but had no known effect on intestinal
400 bacteria and was already known to inhibit methanogenesis in livestock (67). As for

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clinical relevance, a first study found no significant influence of metronidazole combined with amoxicillin and mechanical treatment compared to mechanical treatment alone for periodontal therapy (68). Another study showed that adding metronidazole with or without amoxicillin was more effective at reducing archaeal load in periodontal disease (69). More anecdotally, the possible inhibitory effect of *Neolamarckia cadamba* leaf extract on *M. oralis* was reported by studying its relative abundance in an in vitro fermentative digester by metagenomic analysis (70). Finally, antibiotics targeting infection-causing bacteria may prove ineffective against *M. oralis* and methanogens, given their enzymes, metabolic pathways, cell walls, and membranes, which are distinct from bacteria, leading to potentially significant clinical consequences.

M. oralis in the human microbiota.

M. oralis was first isolated in the oral cavity, more specially from dental plaque samples of two healthy individuals, suggesting that it could be a member of the normal oral microbiota (3). It also has been detected from the subgingival biofilm of healthy teeth and implants (71), and detected and cultured from the saliva of patients with no periodontal disease. However, a positive correlation was found between the occurrence of *M. oralis* and tobacco-smoking (52,66). Moreover, *M. oralis* may occupy additional oral niches in pathological situations, including subgingival and pocket dental plaque from patients with periodontitis or peri-implantitis, pulp inflammation and infection (1). Finally, *M. oralis* appears to be the most prevalent methanogen associated with oral mucosa in both healthy and pathological situations,

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and also as a planktonic micro-organism in the saliva. However, *M. oralis* does not seem to be restricted to the oral cavity, as it also has been detected in the human gut microbiota (44), the respiratory tract (48), and the vagina (54), and has been isolated from human faeces (57) and milk (50). In particular, its adaptation to the human gut has not been clearly defined, as other studies did not report the presence of *M. oralis* (56,72), or reported it in a low prevalence (44,73). A lack of correlation between the presence of methanogens in the gut and the oral cavity was mentioned by Brusa *et al.* in 1993 (74). Given that *M. oralis* is unable to grow at a pH below 6, it may be destroyed by the acidity of the stomach (3). Therefore, its colonisation within the human gut could occur in the presence of gastric pathologies or anti-acid treatments. **(Figure 3)**

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M. oralis in pathological situations.

Oral dysbiosis.

Periodontal diseases.

Gingivitis and periodontitis.

Biofilm-induced gingivitis is defined as a reversible inflammation limited to the gingiva which does not extend to the periodontal attachment, while irreversible attachment loss has been described for periodontitis, possibly leading to tooth loss (75). Periodontal diseases have been linked to other pathologies such as

449 endocarditis, stroke, and diabetes for example, and many conditions for gingival and
450 periodontal pathologies have been described by the American Academy of
451 Periodontology but are not detailed here (76). Gingivitis and periodontitis microbiota
452 have been studied, particularly by Socransky, who defined five bacterial complexes
453 related to periodontal disease (77,78). However, methanogens and *M. oralis* were not
454 sufficiently considered in such studies.

455 Methanogens were firstly enriched from subgingival plaque in both healthy and
456 periodontally diseased patients, before the isolation of *M. oralis* from dental plaque
457 samples collected from healthy individuals (3,26,27,74). Only one study reported the
458 detection of *M. oralis* in 2/3 Malian patients with gingivitis (47). A first study detected
459 methanogens by PCR-sequencing in 37/48 (77%) of patients with periodontitis, with
460 no negative control group, and *M. oralis* was majoritarily identified (31/37; 84%) (30).
461 Later, a quantifying case control study with patients diagnosed with different stages
462 of periodontitis and controls from healthy sites and healthy individuals was conducted
463 (4). The prevalence of archaea was 36% for 50 periodontitis patients and *M. oralis*
464 was identified in 81% of clones from six patients. Archaea appeared to be restricted
465 to periodontitis sites, and a correlation between archaeal load and disease severity
466 and bacterial load was established (4). Importantly, no archaea were detected from
467 healthy controls (sites or individuals), as confirmed in further studies (35,36,79,80).
468 The presence of methanogens was then associated with periodontitis-positive
469 patients (11/49 (22%), 0/30 healthy patients) and pocket depth (> 6 mm), and were
470 detected in both chronic (6/32, 19%) and aggressive periodontitis (5/17, 29%). The
471 *M. oralis* and *M. oralis*-phylotype-like were predominant in four tested patients (80).

472 Additional studies have revealed the presence of *M. oralis* in healthy sites, yet
473 consistently observed a higher prevalence and/or abundance in diseased sites, some

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474 with specific attention to chronic, aggressive, or mixed types of periodontitis. *M. oralis*
475 was detected in 20 healthy individuals and 20 patients diagnosed with aggressive
476 periodontitis but the abundance of archaea was significantly higher in diseased sites
477 (81). Moreover, the abundance of the archaeal species *M. oralis, M. smithii,*
478 *Methanomassiliicoccus luminyensis,* and *M. stadtmanae* were significantly higher in
479 periodontal disease when compared to healthy sites by metagenomics analysis.
480 Functional analysis revealed that fermentation and methanogenesis were
481 predominant energy transfer metabolisms in disease (82). The prevalence of *M.*
482 *oralis* was significantly higher in chronic periodontitis patients (6/15, 40%) than in
483 orally healthy individuals (1/15, 6.7%) (83). Moreover, *M. oralis* was successfully
484 identified in all seven sequenced samples, and a higher prevalence of methanogens
485 was found in periodontitis sites (53% of mild periodontitis sites and 64% of
486 moderate/advanced periodontitis sites) compared to peri-implantitis sites (10%) (84).

487 Accordingly, an *M. oralis*-specific RT-PCR system disclosed that *M. oralis* load
488 significatively correlated with the periodontitis severity score, despite an absence of
489 significant prevalence between periodontitis (12/22, 55%) and healthy patients (3/10,
490 30%) (9). This correlation of *M. oralis* load with pocket depth (and age) was later
491 confirmed, while the correlation with gender was not (39). Finally, Huynh *et al.* were
492 the only team to reintroduce methanogen culture to confirm the presence of living
493 micro-organisms (2). Their results showed that *M. oralis* was present in a living state
494 in 31/65 patients (47.7%%) with periodontitis, compared to only 1/15 healthy controls
495 (6.7%), suggesting that studies based solely on PCR may overestimate the presence
496 of viable *M. oralis* in healthy individuals' samples, providing further evidence that *M.*
497 *oralis* wis implicated in periodontitis (2).

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498 To conclude, there is substantial evidence highlighting the significant role of *M.*
499 *oralis* in periodontitis. From its consistent presence in periodontal pockets to its
500 association with disease severity, these findings emphasise the clinical relevance of
501 this micro-organism in the context of periodontitis.

502

503 **Peri-implantitis.**

504

505 Peri-implantitis is an inflammatory disease similar to periodontitis in terms of
506 implicated bacterial species and as it leads to soft tissue and bone loss with the
507 appearance of a pocket around the implant.

508 *M. oralis* is the majoritarily detected methanogen in peri-implantitis sites (90%
509 of clones), and a significantly higher abundance of archaea (12/25 (48%) of peri-
510 implantitis sites) than in healthy implant sites in individuals with peri-implantitis (4/25,
511 8%) and in healthy individuals (2/25, 4%) has been reported (71). However, the
512 methanogens association with peri-implantitis remain controversial, as they have
513 been detected in both peri-implantitis sites (15/30, 50%) and healthy sites (16/28,
514 57%) without any significant difference (51). However, one recent study detected no
515 methanogen in healthy sites versus in 10% of peri-implantitis sites, 53% in mild
516 periodontitis sites, and 64% in moderate/advanced periodontitis sites (84). In
517 contrast, another metagenomics study which did not identify methanogens at the
518 level species, revealed that the *Methanobrevibacter* genus was more abundant in
519 peri-implantitis than in periodontitis (85), and suggested that the core microbiota of
520 individuals with peri-implantitis and periodontitis are different. This contradiction
521 deserves further investigation.

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522 **Pericoronitis.**

523

524 Pericoronitis is an infectious disease of the soft tissues around partially
525 erupted teeth, especially third mandibular molars (86). *M. oralis* was detected by the
526 PCR-sequencing method in 3/11 samples (27%) of subgingival plaques of third
527 molars with symptomatic pericoronitis and was not detected in asymptomatic
528 pericoronitis (87).

529

530 **Endodontic pathologies.**

531

532 Micro-organisms can colonise the dental pulp through coronal or root surfaces
533 in the presence of defective restorations, dental caries, fractures, or any situation
534 involving communication between the oral cavity and the endodont. The infectious
535 process can be either symptomatic or asymptomatic, and includes pulp inflammation,
536 necrosis, apical periodontitis, apical abscess, and bone and soft tissue infection (88).
537 The primary infection is caused by micro-organisms that initially invade the root
538 canal, while the secondary infection is caused by micro-organisms colonising the root
539 canal after a professional treatment (88). The endodontic microbial community is
540 mainly composed of anaerobes, is less diverse in the secondary infection (88), and
541 archaea are not so often included in studies.

542 A first study erroneously concluded that there was no archaea implication in
543 the endodontic infection after failing to detect archaeal DNA. This was probably due
544 to use of the wrong primers (89). An *M. oralis*-like species was finally detected by RT-
545 PCR in 25% (5/20) in asymptomatic primary infected dental pulp samples,

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546 representing 0.28% to 2.53% of the total microbial community (34). *M. oralis* was
547 formally identified shortly after in the same samples (35). The presence of *M. oralis*
548 was again reported as the main archaea in the primary infection (59.4% (19/32) (96),
549 (97) as well as in the secondary infection (37.5% (12/32), (90), (91)), and in inflamed
550 pulp (85% (17/20), (92)). Furthermore, Vickerman *et al.* (93) did not manage to
551 establish a link between the symptomatic status and the presence of *M. oralis* in the
552 root canal, as an *M. oralis*-like species was detected in 1/20 (5%) asymptomatic
553 patients and 1/14 (7%) symptomatic patients. However, another study reported that
554 symptomatic cases which were positive for bacteria and archaea were significantly
555 higher (16/22, 73%) than symptomatic cases positive for bacteria alone (21/47, 45%),
556 without identifying archaea at the species level (94). Two studies based on bacterial
557 16 rRNA PCR analysis reported the presence of *M. oralis*, one in the root ends and
558 periradicular soft tissue in 1/16 samples (95), and one before endodontic treatment in
559 1/6 teeth but not after, suggesting the efficacy of the endodontic procedure at
560 removing *M. oralis* from the infected canal (96). This data suggests that *M. oralis* may
561 be a neglected member of the pathological microbial community in the endodontic
562 infection process. The association between infection progression and symptomology
563 remains unclear and warrants further investigation.

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564

565 **Dental caries.**

566

567 Dental caries was defined by the World Health Organization (WHO) as a
568 localised pathological process of external origin, occurring after eruption,
569 accompanied by a softening of hard tissues and progressing towards the formation of
570 a cavity (97). Pitts *et al.* give another definition, stating that "dental caries is a biofilm-

mediated, sugar-driven, multifactorial, dynamic disease that results in the phasic demineralization and remineralization of dental hard tissues" (98). Surprisingly, *Methanobrevibacter* methanogens were not detected, in contrast to the *Thaumarchaeota* phylum and *Methanocellales* order, in one carie sample investigated by archaea-specific 16S RNA gene cloning and sequencing (99). Further analysis and other primers should be tested for a better understanding the potential role of archaea in dental caries.

Halitosis.

Halitosis is a bad odour from the oral cavity caused by intra- or extra-oral situations and is related to the production of malodorous compounds by bacteria. The presence of archaea in the supra gingival plaque, subgingival plaque, and tongue have been highly correlated to halitosis compared to healthy controls, particularly in subgingival sites (100). However, archaea have not been defined at the species level, and the relationship between archaea including *M. oralis* and other micro-organisms in the production of malodorous compounds should be investigated.

Abscesses.

M. oralis has been associated with several cases of infection and abscesses, revealing its potential pathogenicity for other sites than periodontal or endodontic tissue. Indeed, *M. oralis* was detected in 1/11 (9%) brain abscesses, confirmed by metagenomic analysis (14). *M. oralis* was then cultured from one index brain abscess

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pus specimen, and RTQ-PCR reported that *M. oralis* had a significantly higher prevalence in brain abscesses than in other brain tissue controls, being detected in 7/8 (87.5%) of methanogen-positive pus specimens (7/18, 39.8% of total brain abscesses pus specimens), and in 1/27 (3.7%) controls without brain abscess (13). *M. oralis* was also reported in 1/4 (25%) cases of peri-appendicular abscesses (41), always with other bacteria, and in 2/100 (2%) cases of orthopaedic prosthesis infection (42).

Other pathologies.

Respiratory diseases.

M. oralis could also be part of the microbiota in patients with refractory sinusitis who have not received nitroimidazole derivatives, as it was detected in 4/9 (44%) pus specimens which were positive for methanogens and cultured from one of them (25%) (46). However, a case control study revealed no association between archaea and chronic rhinosinusitis (2/20 (1%) in the healthy control group and 5/40 (12.5%) in the disease groups) (101). Moreover, another study detected *M. oralis* in the respiratory tract from 19/527 (3.6%) sputum samples and bronchoalveolar lavages, indicating the possible involvement of methanogens in other respiratory diseases (48).

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Inflammatory diseases.

M. oralis was identified in pooled samples of patients with Crohn's Disease (29/48, 50% of sequenced clones) and Irritable Bowel Syndrome (3/48, 6.3% of sequenced clones), but no correlation was found between *M. oralis* and digestive tract diseases. Methanogens were detected in equivalent numbers of individuals (range from 45% to 50%, mainly corresponding to *M. smithii*) in colorectal cancer, polypectomised, irritable bowel syndrome, and control groups. Their incidence was notably reduced in the inflammatory bowel disease groups, with 24% for ulcerative colitis and 30% for Crohn's disease (44).

Cancer.

Less is known about methanogens and cancer, however one study (102) showed a depletion of methanogens in patients with colorectal cancer, while another (103) revealed that archaeal metabolites, especially in the oral cavity and gut, could have an influence on the tumour microenvironment and carcinogenesis. A further study reported a positive correlation between colorectal cancer and *M. smithii,* but no data was provided concerning *M. oralis* (104). However, a LEfSe analysis indicated a substantial contribution of *M. oralis* to the differentiation between HPV16-positive and HPV16-negative groups of women, suggesting a possible association between *M. oralis* and HPV16 infection (54). Given the carcinogenic nature of HPV16, this association assumes heightened importance, emphasising the need for further exploration of the role of *M. oralis* in the composition of the vaginal microbiome and its potential implication in cancer predisposition among women infected with HPV16.

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643 ***M. oralis* in animal microbiota.**

644

645 Few recent studies have reported the presence of *M. oralis* in animals'
646 microbiota. It was notably detected in 7/10 (70%) dogs with severe periodontitis but
647 not in healthy dogs or those with mild or moderate periodontal disease, hinting at a
648 potential connection to canine gum health (53). Additionally, dental calculus analysis
649 identified *M. oralis* in a 19th-century baboon and a mummified domestic baboon from
650 the Pharaonic era (9th–6th centuries BP). This discovery not only provides insights
651 into the ancient oral microbiota of animals but also raises questions about potential
652 human influences on a potential interspecies transmission of *M. oralis*. Interestingly,
653 a contemporary wild chimpanzee, in contrast, showed no evidence of *M. oralis*,
654 underscoring the distinctive variations in oral microbial communities among closely-
655 related primate species (24). Likewise, while some studies reported the presence of
656 *M. oralis* in the rumen of non-dairy and dairy cows (105,106), a comprehensive study
657 by Guindo *et al.* did not confirm these observations when it investigated dogs, cats,
658 cows, sheep, horses, and pigs, in which *M. oralis* was not detected (107).

659

660 ***M. oralis* and its host.**

661

662 **Pathogenicity and host response.**

663

664 While aggressive periodontitis does not necessarily require antibiotics to
665 reduce the prevalence of archaea (68), periodontal treatment consisting in root
666 scaling with adjunctive antibiotics for patients with chronic periodontitis is more

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667 effective at reducing archaea and the prevalence and load of *M. oralis* than
668 mechanical treatment alone. Both treatments, however, are associated with an
669 improvement in periodontal health (69). In particular, this reduction was not
670 influenced by changes in prokaryotic biomass. The same findings emerged
671 concerning root canal treatment, where *M. oralis* was not detected after irrigation with
672 3% H_2O_2 and intracanal medication, and there was no significant difference in total
673 DNA extracted before and after treatment (96). However, these findings provide
674 indirect evidence of the pathogenicity of *M. oralis*, as its absence post-treatment
675 remains effective, despite the stability in the total microbial load.

676 The first direct evidence of pathogenicity was provided by the inoculation of an
677 *M. oralis* monoculture or *M. oralis* with *S. intermedius* in a mouse model leading to
678 animal death (17/22 (77.3%) and 75/104 (72.1%) mice died, respectively). This was
679 significantly higher than deaths caused by inoculation of *S. intermedius* alone (32/95
680 (33.7%), showing for the first time the direct pathogenicity of this methanogen (13).
681 Studies were then conducted focusing on the immunogenicity of *M. oralis,* revealing
682 that sera from patients with periodontitis contained IgG against *M. oralis* (108). These
683 antibodies are directed against *M. oralis* group II chaperonin (Cpn-1 and Cpn-2
684 subunits) and may cross react with human group II chaperonin CCT, highlighting their
685 potential role in periodontal diseases and providing avenues for future research into
686 their involvement in autoimmune responses (108,109).

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687 **Acquisition and dynamics.**

688

689 The source of acquisition and the dynamics of transmission of methanogens,
690 particularly *M. oralis,* remain unsolved. Researchers have successfully cultured
691 viable *M. oralis* from 1/20 (0.5%) maternal milk samples, suggesting a possible route
692 of transmission through breastfeeding (50). However, *M. oralis* was not found in the
693 colostrum (50), meconium (110), or gastric juices of one-day-old newborns (111), in
694 contrast to *M. smithii* (50,110,111). Surprisingly, another study identified *M. oralis* as
695 the predominant methanogen in meconium, placenta, and amniotic fluid samples
696 from newborns, as well as in oral, rectal, and vaginal samples from mothers,
697 indicating that its presence was not influenced by the delivery mode (112). *M. smithii*
698 was not detected in this study, raising questions about a potential mix-up between *M.*
699 *oralis* and *M. smithii* (112). Finally, researchers have not yet explored the presence of
700 *M. oralis* in the oral cavity of newborns and older infants, leaving the key time when
701 *M. oralis* is acquired unknown. Moreover, the easily exchangeable nature of oral
702 fluids presents potential opportunities for the person-to-person transmission of *M.*
703 *oralis*, occurring during interactions between mothers and children, including shared
704 food consumption, fomite contact, and kissing (52). This hypothesis gained support
705 with the discovery of *M. oralis* in domestic baboons, which are in close contact with
706 humans and share similar lifestyles and diets (24). Moreover, the identification of *M.*
707 *oralis* in the human-built environment hints at the possibility of environmental
708 mediation in interhuman transmission (113). In particular, multispacer sequence
709 typing (MST) studying genetic variants of *M. oralis* may be useful in investigating the
710 dynamics of *M. oralis* populations and inter-individual transmission (49). Intriguingly,
711 common foods such as confectionery products, fresh fruit, cheese, vegetables, meat,

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712 and fish do not appear to be a source of *M. oralis*, suggesting that food might not
713 play a significant role in its transmission pathways (114). However, diet and antibiotic
714 use can influence the microbiota throughout an individual's lifetime, given that *M.*
715 *oralis* thrives in symbiosis within specific bacterial niches. External factors including
716 oral treatments such as mouthwashes and the intake of antibiotics, notably
717 metronidazole, may influence the presence of *M. oralis* in complex microbiota (10,62)
718 either directly or indirectly, through associated bacteria. Furthermore, *M. oralis* seems
719 to exhibit a global distribution across multiple continents, including Europe, Africa,
720 Asia, North, and South America **(Figure 4)**. However, it is evident that not all
721 countries and specific regions have been thoroughly investigated. Exploring diverse
722 areas could prove valuable, particularly in studying the dynamics of transmission and
723 its potential correlation with dietary patterns.

724

725 ***M. oralis* and bacteria.**

726

727 *M. oralis* has been identified alongside various previously selected and
728 targeted bacteria in different oral pathologies, suggesting potential symbiotic
729 partnerships. In cases of pericoronitis, *M. oralis* was consistently present in all sites
730 which were positive for *Fusobacterium nucleatum* (*F. nucleatum*), while
731 *Campylobacter gracilis*, *Prevotella melaninogenica*, *Veillonella dispar, Filifactor*
732 *alocis*, and *Tannerella forsythia* (*T. forsythia*) were found in both positive and
733 negative archaeal sites (87). Furthermore, in one symptomatic endodontic case,
734 *M. oralis* was co-detected with *Prevotella intermedia* (*P. intermedia*), *Porphyromonas*
735 *endodontalis* (*P. endodontalis*), *Porphyromonas gingivalis* (*P. gingivalis*),
736 *Peptostreptococcus micros* (*P. micros*), *Streptococcus* sp., *F. nucleatum,* and

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737 *T. forsythiae,* but not with *Treponema denticola* (*T. denticola*) or *Enterococcus*
738 species, while in an asymptomatic case, it was detected alongside *P. gingivalis,*
739 *P. micros, Streptococcus sp., F. nucleatum*, and *T. forsythia* (93). Interestingly, no
740 specific associations were reported with selected bacteria (*T. denticola, T. forsythiae,*
741 *P. gingivalis, F. nucleatum, P. intermedia*) in cases of periodontitis and peri-
742 implantitis (84). Furthermore, despite the formation of a unique core microbiome in
743 severe periodontal disease cases in dogs, including *M. oralis*, *Christensenellaceae*
744 *sp, Bacteroidales sp*, Family XIII sp, *Peptostreptococcus canis*, and *Tannerella* sp,
745 the correlation of *M. oralis* with bacterial species has not been studied (53). Other
746 studies have specifically reported both positive and negative associations with *M.*
747 *oralis*, contributing to a better understanding of complex microbial relationships. In
748 periodontitis, a negative association was found with *Treponema* spp. (4), despite the
749 co-detection of *T. denticola* with *M. oralis* in an asymptomatic endodontic sample and
750 its absence in another *M. oralis*-positive symptomatic sample (93). The negative
751 association of archaea, including *M. oralis*, with *T. denticola* was confirmed in another
752 study, possibly explained by their shared role as hydrogen consumers (90). Similarly,
753 mutual exclusion with other hydrogen consumers such as sulfate-reducing bacteria
754 (SRB) and acetogens suggests their potential as alternative syntrophic partners for
755 secondary fermenting periodontal pathogens (36). The positive association with
756 *P. intermedia* in endodontic infection was supported by Horz *et al.,* who suggested
757 the broader substrate range for fermentation, including carbohydrates and proteins,
758 exhibited by *Prevotella* species, including *P. intermedia*, compared to other genera
759 within the *Bacteroidetes* domain, such as *Porphyromonas* and *Tannerella*, which are
760 asaccharolytic and may be more suitable to *M. oralis* (39). Interestingly, in Malian
761 patients with different oral conditions such as gingivitis and periodontitis, *M. oralis*

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762 was detected with bacteria that were not reported in other studies including *Delftia*
763 *acidovorans, Microbacterium oxydans, Pseudomonas putida*, *Citrobacter freundii,*
764 *Brevundimonas aurantiaca, Rhizobium radiobacter, Microbacterium kitamiense,*
765 *Peptoniphilus harei* and *Klebsiella pneumoniae*. This finding suggests that the
766 microbial partnerships of *M. oralis* may vary depending on the geographic location,
767 shedding light on potential regional differences in oral microbiota composition (47).

768 Moreover, *M. oralis* has been found alongside other bacteria in diverse extra-
769 oral conditions. These bacteria were detected and cultured impartially, without prior
770 biases, to explore the microbial diversity of the sample. *M. oralis* has been identified
771 in cases of refractory sinusitis, coexisting with various bacterial species, including
772 *Corynebacterium accolens*, *Staphylococcus aureus* (*S. aureus*), *Raoultella*
773 *ornithinolytica*, *Streptococcus pseudintermedius*, *Pseudomonas aeruginosa*
774 (*P. aeruginosa*), *Staphylococcus epidermidis* (*S. epidermidis*), *Corynebacterium*
775 *propinquum*, *Corynebacterium avidum*, and *Propionibacterium acnes* (46). In
776 particular, *S. epidermidis* was found in all four cases positive for *M. oralis*,
777 *P. aeruginosa* in two out of four cases, and *Corynebacteria* species in three out of
778 four cases. Furthermore, *M. oralis* was identified in a peri-appendicular abscess with
779 *Escherichia coli* (41), in a brain abscess with *Aggregatibacter*
780 *actinomycetemcomitans* (14), and in another case of a brain abscess with
781 *Porphyromonas endodontalis* and with *S. intermedius* (13). Co-infection experiments
782 in mice revealed significantly higher mortality rates when *M. oralis* was present with
783 *S. intermedius*, indicating the severity of combined infections (13). Additional co-
784 detection with *S. aureus* and *S. epidermidis* in orthopaedic prosthesis infection cases
785 suggests a potential syntrophic association with *Streptococcus* sp. and
786 *Staphylococcus* sp. (42). Given the facultative anaerobic nature of these two species,

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enabling adaptation to both aerobic and anaerobic conditions, coupled with their inherent defence mechanisms against oxygen and reactive oxygen species (ROS), the establishment of a biofilm may provide a favourable niche for methanogens to thrive (42).

While methanogens, including *M. oralis*, have traditionally been viewed as secondary colonisers, relying on syntrophic interactions with bacterial partners, particularly in hydrogen transfer, their mere co-detection with bacteria does not necessarily imply dependency, and most of the studies presented here focused only on known oral pathogens. Investigating positive associations may reveal potential partners for *M. oralis* such as *P. intermedia*. Such insights hold the potential to enhance cultivation techniques. Conversely, exploring negative associations, such as competition, particularly in terms of hydrogen utilisation and varying degrees of mutual exclusion, notably with *Treponema* sp., may be useful for a comprehensive understanding of microbial community dynamics. Further research, incorporating correlation analysis and co-culturing, is pivotal to produce nuanced insights into both dependencies and competition among micro-organisms. **(Figure 5)**

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***M. oralis* and other archaea.**

M. oralis was the predominant methanogen in oral samples and was mainly detected alone, suggesting competition within the *Methanobrevibacter* genus. However, some studies revealed that this co-exclusion was not strict and *M. oralis* seemed to be able to coexist with other *Methanobrevibacter*. Indeed, *M. oralis* was co-detected in 3/34 subgingival dental plaque samples, 31 of which contained only one phylotype (30). Moreover, *M. oralis* was co-detected with *Methanobrevibacter*

812 phylotype SBGA-1 in four plaque samples from four patients with periodontitis (80)
813 and was also co-detected with *Methanobacterium congolense/curvatum*. This is a
814 hydrogenotrophic methanogen initially isolated from an anaerobic digester in Congo,
815 in a peri-implantitis group and in a healthy control group, although the other
816 methanogen had a lower prevalence (71). *M. oralis* was co-detected in endodontic
817 samples with a *Methanobrevibacter* species associated with *Synergistes* sp. (35).
818 However, the ability of *M. oralis* to share its ecological niche was confirmed by Grine
819 *et al.* who co-cultured *M. oralis* and *M. smithii* from saliva (66).

820 *M. oralis* has also been detected alongside other members of the archaea
821 domain. Firstly, *M. oralis* and a *Thermoplasmatales* species were co-detected in the
822 oral cavity (115). Then, *Nanopussillus massiliense* (*N. massiliense*) was co-detected
823 with *M. oralis* in 4/102 (3.92%) dental plaque specimens and co-isolated with *M.*
824 *oralis* from one dental plaque specimen (12). *N. massiliense* was the first
825 nanoarchaea isolated in the human microbiota, followed by *Nanopusillus phocaeans*
826 with *M. smithii* from the human gut, with other nanoarchaea living in the environment
827 with their archaeal host (Hassani *et al.*, in press). Nanoarchaea revealed a small
828 genome with reduced metabolic function which appeared to be strongly dependent
829 on their host. This finding added a new layer to our understanding of symbiotic
830 relationships in microbial ecosystems. While methanogens were historically regarded
831 as organisms which were heavily reliant on bacteria and their metabolic byproducts,
832 this discovery provided a more nuanced perspective. It revealed that methanogens
833 such as *M. oralis*, could also play a vital role as hosts, supporting the existence of
834 smaller micro-organisms. This symbiotic interdependence highlights the intricate
835 dynamics within microbial communities, underscoring the diverse ways in which
836 organisms interact and rely on one another in the intricate tapestry of life **(Figure 5).**

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837 **Perspectives and conclusion.**

838

839 This comprehensive review explores the intricate realm of *M. oralis*. It is
840 noteworthy that this methanogen has received considerably less attention than the
841 gut-predominant *M. smithii*, despite being the predominant methanogen in the human
842 oral cavity. Comparative microbiology would illuminate the specificities of *M. oralis*
843 reviewed in this study compared with *M. smithii*, another human mucosae-associated
844 microbiota methanogen (Malat *et al*., submitted for publication).

845 This review suggests that *M. oralis* is primarily associated with humans, with
846 little or no clear evidence of its detection in animals, even those in close contact with
847 humans. In contrast, *M. smithii* has been detected in several animal species (107).
848 Specifically, *M. oralis* has been found in only three animal species (baboons, dogs,
849 and cows), indicating a possible unique association with humans (24,53,105,106).
850 However, this difference may arise from the focus on gut or faecal samples in animal
851 studies, while samples from the oral cavity are not routinely examined.

852 More particularly, *M. oralis* predominates in the human oral cavity, with a
853 notable presence in the respiratory tract (46,48) and an association with cerebral
854 abscesses (13,14), indicating an affinity for these anatomically linked regions. In
855 contrast, *M. smithii* exhibits a higher propensity to colonise both the oral (2) and
856 digestive microbiota (116), and extends its presence to extra-intestinal sites such as
857 the heart valves (40), the urinary tract (117), and the vagina (118), underscoring its
858 ability to adapt to various anatomical niches.

859 Accordingly, *M. oralis* is a long-standing companion of humans and has been
860 documented since the Palaeolithic era and Neanderthal times (5,7). In contrast, the

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861 oldest evidence of *M. smithii* in humans dates to about 14 000 years ago (23,119).
862 Such a long-standing association suggests some degree of indifference to huge
863 variations in alimentation through the millennia. However, temporal changes in its
864 prevalence within dental calculus since the 18th century present a compelling
865 historical narrative (8,21,22). This shift could potentially be influenced by societal
866 changes such as the widespread introduction and consumption of sugar, while it is
867 not associated with sugar-promoted dental caries, raising questions about changes in
868 bacterial composition that may favour micro-environments which are conducive to
869 *M. oralis* and the decline of *M. massiliense* associated with *Pyramidobacter*
870 *piscolens,* an asaccharolytic bacteria (21,120). Exploring the concept of a "ghost"
871 bacterium associated with *M. oralis*, which can degrade sugars and proteins, could
872 provide valuable insights for future investigations, and may explain why *M. oralis*
873 became the predominant oral methanogen. Overall, this review suggests that much
874 more paleomicrobiology study must be undertaken to pain a more representative
875 picture of the dynamics of the oral cavity microbiota, which has included
876 methanogens for millennia, in correlation with dietary shifts.

877 The determinants of this unique situation of *M. oralis* in the oral cavity of
878 humans remain to be elucidated. At the genomic level, the *M. oralis* and *M. smithii*
879 reference genomes are closely related, as both microbes contain DNA in one circular
880 chromosome without a plasmid, their chromosomes are a similar size of 1.95 Mb
881 (*M. oralis* strain YH, GCA_912073625.1) versus 1.85 Mb (*M. smithii* strain ATCC
882 35061; PS; DSMZ 861, GCA_000016525.1). This suggests that more subtle
883 differences have yet to be found and, indeed, fine comparative genomics work has to
884 be done, along with phenotypic comparisons highlighting the adaptative mechanisms
885 of *M. oralis* to a more limited ecological niche than *M. smithii*. However, these

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886 considerations are restricted by the limited, basic microbiological knowledge of
887 *M. oralis*. Indeed, only five genomes (12,50,57,58) and two MAGs (5,8) are available,
888 leaving the genetic diversity of *M. oralis* unknown **(Table 2)**. Also, for example,
889 MALDI-TOF-MS unambiguously differentiates between both species by their unique
890 yet diverse peptide footprints in the 2-20 KDa range, indicating that both species do
891 not express the same protein interactions with the environment (59,65). However,
892 only one cultured *M. oralis* representative has been successfully preserved in the
893 long term, confirming the challenges associated with its conservation and culture (3).
894 Consequently, few studies have focused on its phenotypic characteristics, and its cell
895 wall and membrane composition in particular remain unknown. Exploring metabolic
896 pathways and conducting substrate affinity tests, especially for substrate
897 combinations, could facilitate its routine cultivation and a better understanding of its
898 interplay with the *M. oralis* microenvironment.

899 This environment must contribute towards the unique situation of *M. oralis* in
900 the oral cavity, comprising at least the mucosae-associated microbiota and the host
901 mucosae. In particular, re-examining the concept of "ghost" bacteria in this context,
902 especially considering its positive association with the saccharolytic and proteolytic
903 bacteria, *P. intermedia,* which could be one "ghost" bacterium accompanying *M.*
904 *oralis* (39), could provide valuable insights into the predominance of *M. oralis* in the
905 oral cavity, while symbiotic *N. massiliense*, which is thought to profoundly change the
906 behaviour of *M. oralis* in the microbiota and the host, was discovered by our research
907 group in 2021 (12). Likewise, the interplay between *M. oralis* and the host mucosae,
908 and related factors such as immunoglobulins and innate immune system cells, has
909 been almost completely ignored and will have to be investigated, including from the
910 perspective of *M. oralis* as a neglected local, opportunistic pathogen. A host

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response to *M. oralis* local pathologies has been ascertained by the detection of *M. oralis*-specific antibodies in patients' sera (108,109).

Increasingly compelling pieces of evidence support the role of *M. oralis* in local, regional, and systemic pathologies. Whether detected or not in healthy individuals or in sites free of gingivitis or periodontitis, *M. oralis* is consistently more prevalent and/or abundant in samples collected from individuals diagnosed with periodontitis than from apparently healthy individuals (4,35,36,79–82,84,83,2,9). This gains significance given the distinctive antibiotic resistance profile *M. oralis* to a large spectrum of antibiotics, with the exception of imidazole derivatives, necessitating careful consideration of treatment strategies (60,62). The implication of *M. oralis* in the pathogenicity of peri-implantitis, however, is less supported, probably due to a limited number of investigated patients and controls, and further research is needed to understand this aspect (51,84,85). Nonetheless, the quantification of *M. oralis* is a promising diagnostic biomarker for oral pathologies and a potential therapeutic target for oral diseases (9). Beyond the oral cavity, *M. oralis* has been found in extra-oral locations, particularly in brain abscesses (13,14). This observation prompts a re-evaluation of its potential dissemination through the bloodstream, despite the fact that it has not been detected in archaemia (40), likely originating from the oral cavity, suggesting broader medical implications beyond oral health. Moreover, the potential link with *Streptococcus* species leads us to reconsider the potential involvement of *M. oralis* in dental caries (13,46), despite one unsuccessful study, as *Streptococcus mutans* is the species most commonly associated with caries (98).

In summary, this comprehensive review explores *M. oralis* within oral and extra-oral microbiology, emphasising its multifaceted nature and calling for further nuanced exploration. Despite uncertainties about its role in oral pathologies, its

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936 quantification as a diagnostic marker and potential therapeutic target holds promise.
937 The complex interactions with micro-organisms, temporal shifts in prevalence, and
938 extra-oral manifestations, especially in brain abscesses, highlight its broader medical
939 relevance. This review advocates for a holistic understanding of *M. oralis*, unlocking
940 therapeutic avenues and revealing its intricate role in human health.

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Conflict of Interest statement.

MD is a previous cofounder and GG a previous employee of Culture-Top, one product of which is cited in this review paper. The other authors declare no conflict of interest.

Author Contributions statement.

Conceptualisation, ET; methodology, ET, GG, and VP; validation, ET and, GG; data curation, VP, MB, AH and, LT; writing—original draft preparation, VP; writing—review and editing, MD, ET and GG; supervision, ET, HT and GA; project administration, ET; funding acquisition, ET. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement.

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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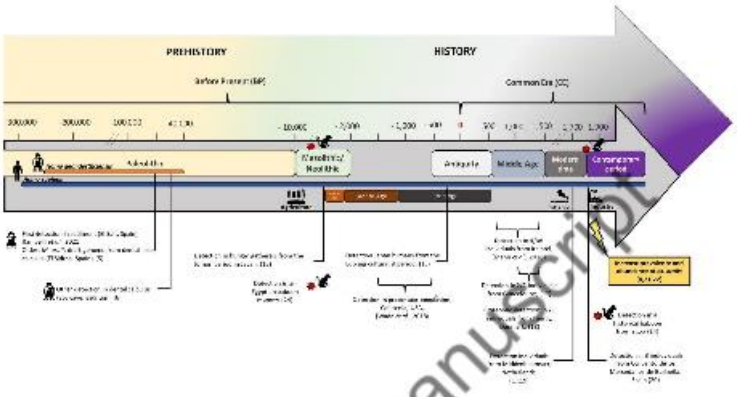
1325 **Figures.**

1326 **Figure 1: Timeline of *M. oralis* antiquity.**



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1329 **Figure 2: Microscopy features of *M. oralis* A.** Gram coloration, *M. oralis* appears

1330 as Gram-variable coccobacilli, by pairs or short chains. **B.** Electron microscopy

1331 (SU5000 HITACHI, 10 KV, X 40.000), zoom on *M oralis* diplococcobacilli. **C.**

1332 Confocal microscopy, autofluorescence at 420 nm (LSM 900 (Carl Zeiss Microscopy

1333 GmbH).

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1338 **Figure 3: Localisation of *M. oralis* in microbiota.**



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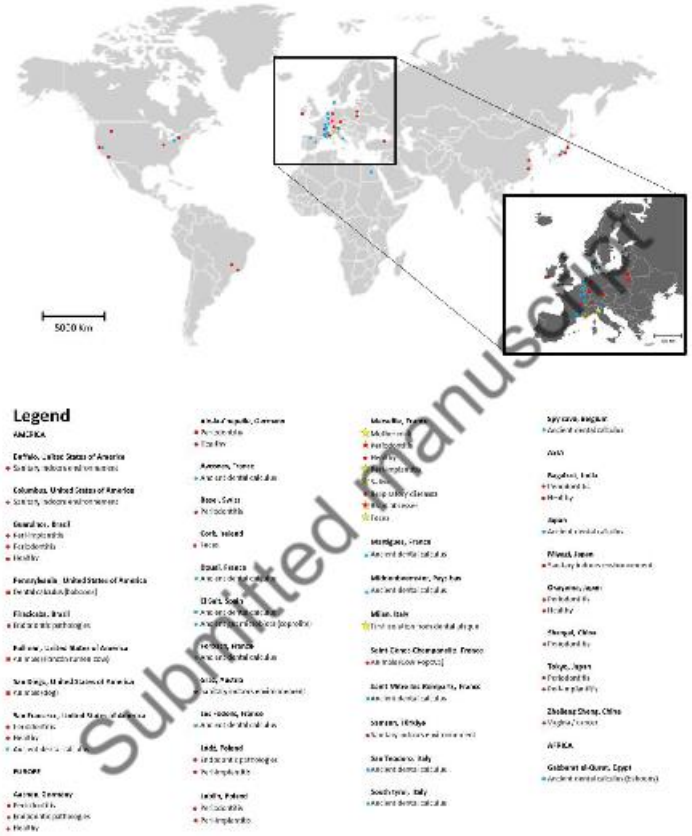
1341 **Figure 4: Past and present geographic distribution of *M. oralis*.**



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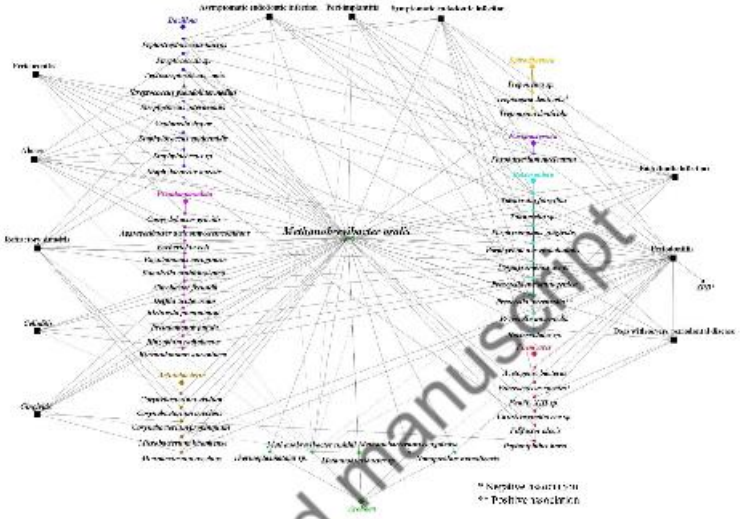
1345 **Figure 5: *M. oralis* interactions with bacteria and other *Archaea*.**



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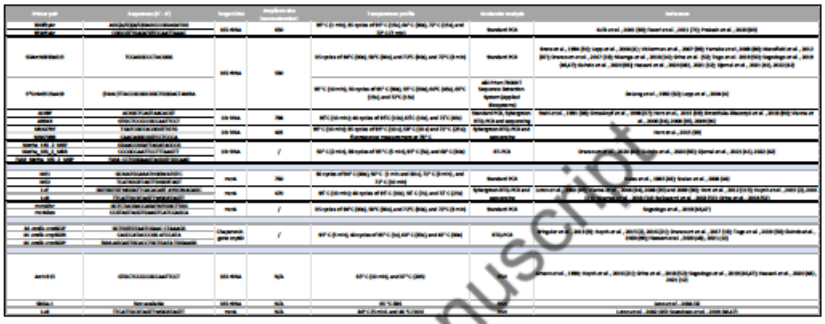
1348 **Tables.**

1349 **Table 1: The different PCR systems able to detect and amplify *M. oralis***

1350 **sequences.**



1351

1352 **Table 2: *M. oralis* genomes features.**

Organism Name	Methanobrevibacter oralis	Methanobrevibacter oralis	Methanobrevibacter oralis	Methanobrevibacter oralis	Methanobrevibacter oralis	Methanobrevibacter oralis	Methanobrevibacter oralis
Organism Groups	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota
Strain	YH	DSM 7326	MGYG-HGUT-02163	MS CSUR P6020	JMW-1	N/A	N/A
BioSample	SAMEA4568756	SAMN14867345	SAMEA3865866	SAMEA104579764	SAMEA3138857	N/A	N/A
BioProject	PRJEB40774	PRJNA315790	PRJEB33866	PRJEB34873	PRJEB4880	PRJEB43389	N/A
Sample	Human dental plaque	Human dental plaque	Human feces	Human milk	Human feces	Ancient dental calculus from an Early Middle Age Homo sapiens	Ancient dental calculus from a Neanderthal
Assembly	GCA_912073625.1	GCA_905340715.1	GCA_902364065.1	GCA_904259635.1	GCA_940539525.1	N/A	N/A
Total Length (bp)	1,903,936	2,147,823	2,080,511	2,134,480	2,107,831	1,372,986	2,076,642
No. of Sequences	14	136	60	106	14	265	14
GC Content (%)	27.9	27.8	27.8	27.7	27.8	27.6	29.9
N50	306,900	46,270	66,369	45,268	56,2296	8,712	360,780
Gap Ratio (%)	0	0	0.00030396	0.00038713	0.011572668	0	66.456,596
No. of CDSs	1,920	2,011	2,279	2,300	2,260	1,153	890
No. of tRNA	2	2	2	2	2	0	2
No. of rRNA	29	30	31	30	31	12	30
No. of CRISPRS	2	2	2	3	2	1	6
Coding Ratio (%)	84.9	80.7	82.1	81.8	81.2	66.8	4.7
Completeness (%)	100.00	100	96.33	100	96.33	82.41	34.3
Contamination (%)	0	0	0	0	0	0.926	0

1353

Submitted manuscript

Les figures et tableaux sont repris ci-après.

Figure 1: Timeline of *M. oralis* antiquity.

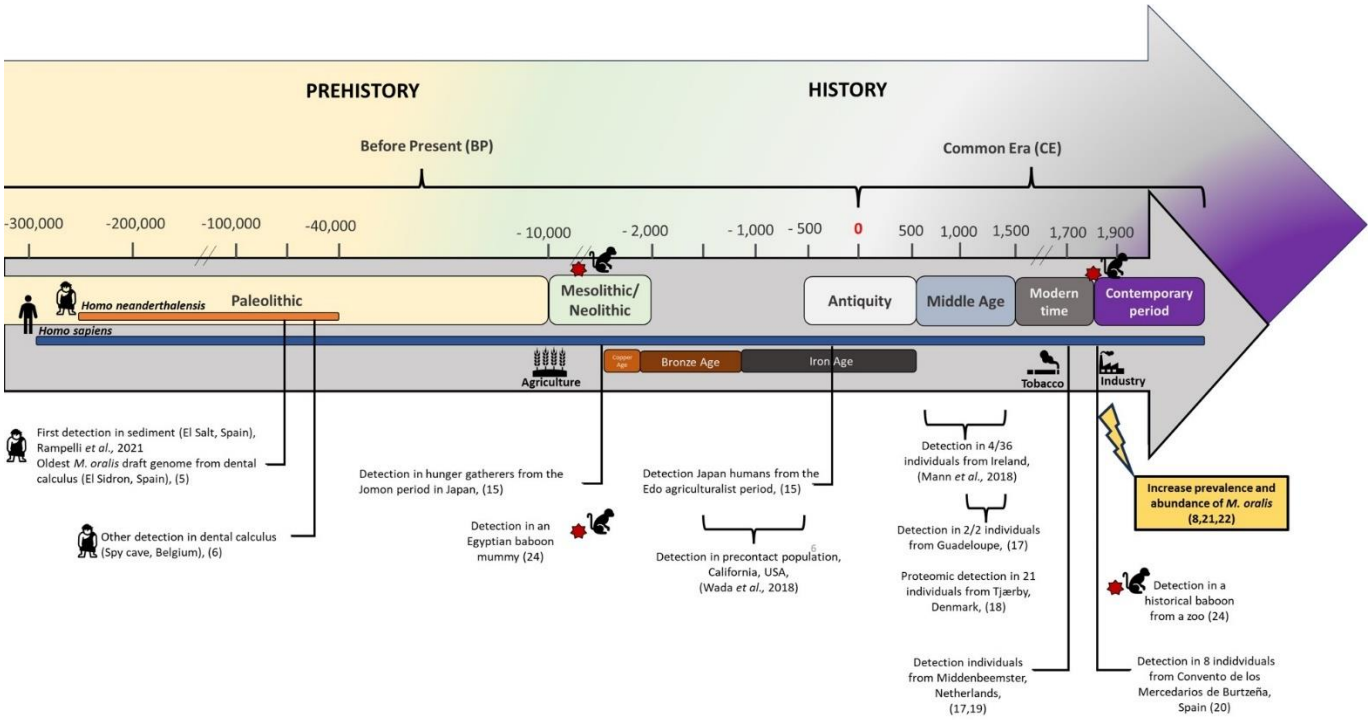
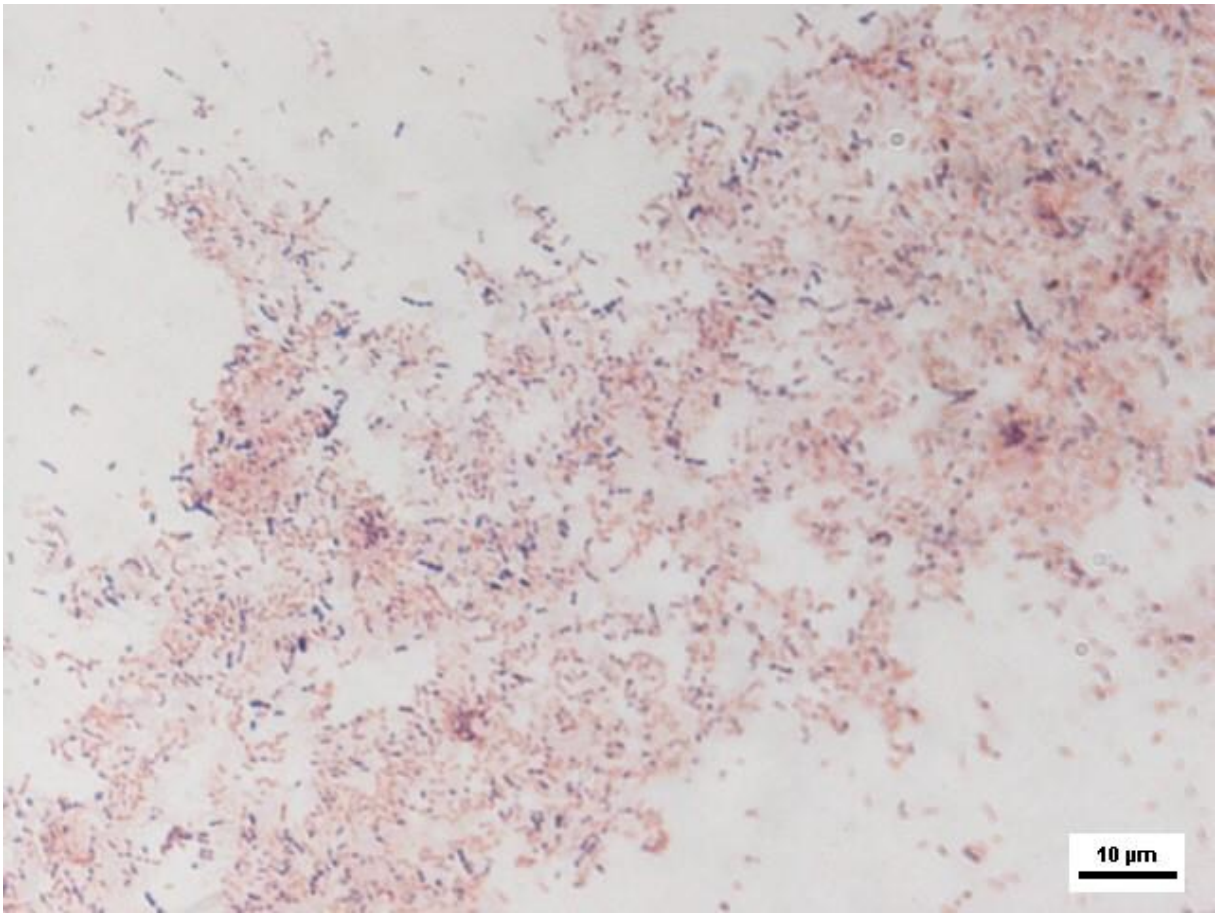


Figure 2: Microscopy features of *M. oralis* A. Gram coloration, *M. oralis* appears as Gram-variable coccobacilli, by pairs or short chains. **B.** Electron microscopy (SU5000 HITACHI, 10 KV, X 40.000), zoom on *M oralis* diplococcobacilli. **C.** Confocal microscopy, autofluorescence at 420 nm (LSM 900 (Carl Zeiss Microscopy GmbH).

A.



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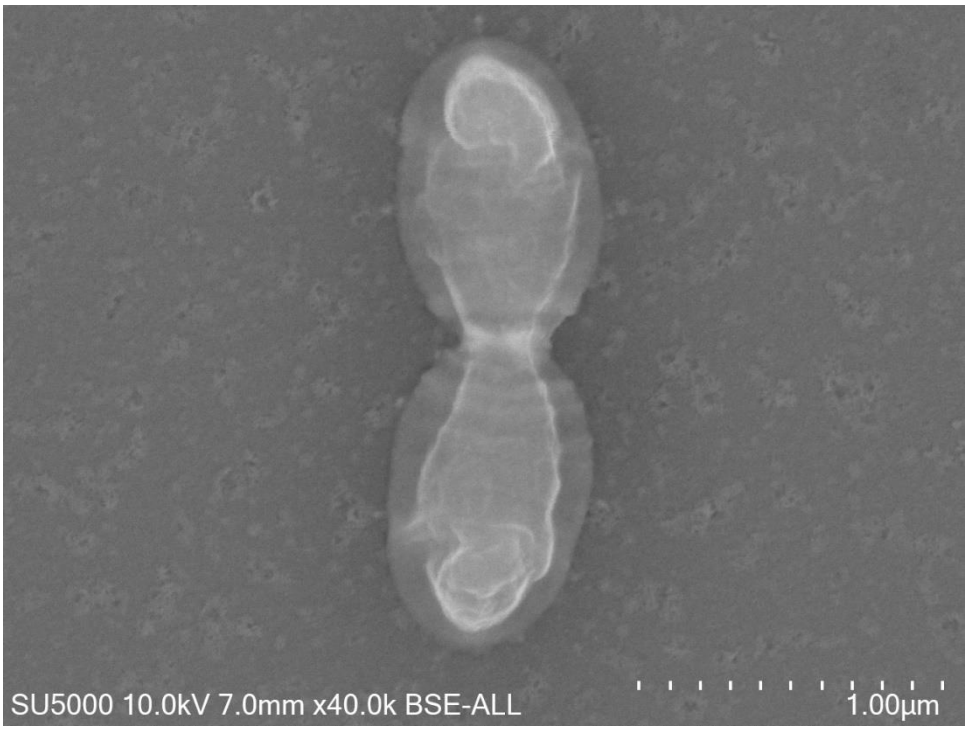


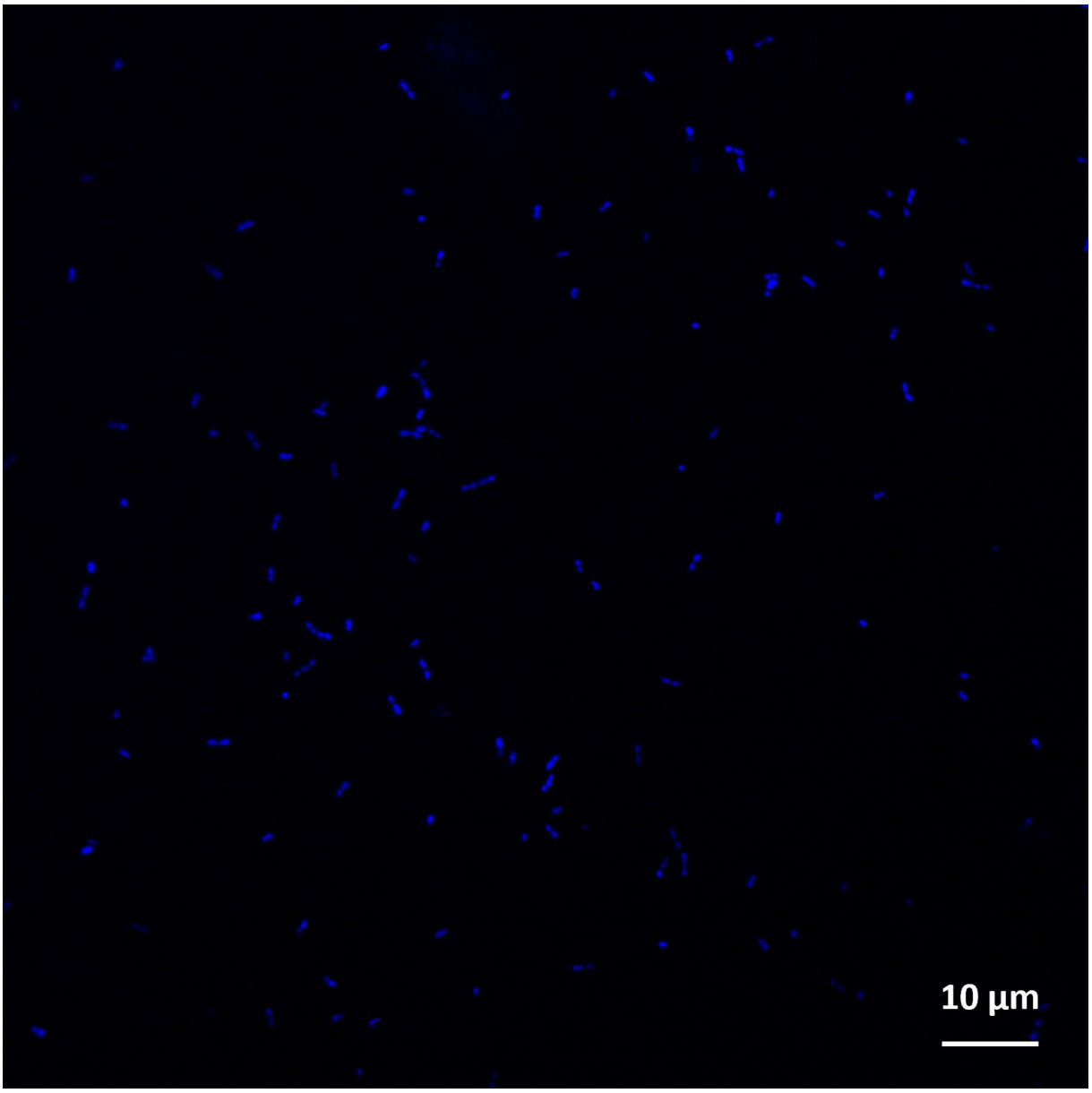
C. 

Figure 3: Localisation of *M. oralis* in microbiota.

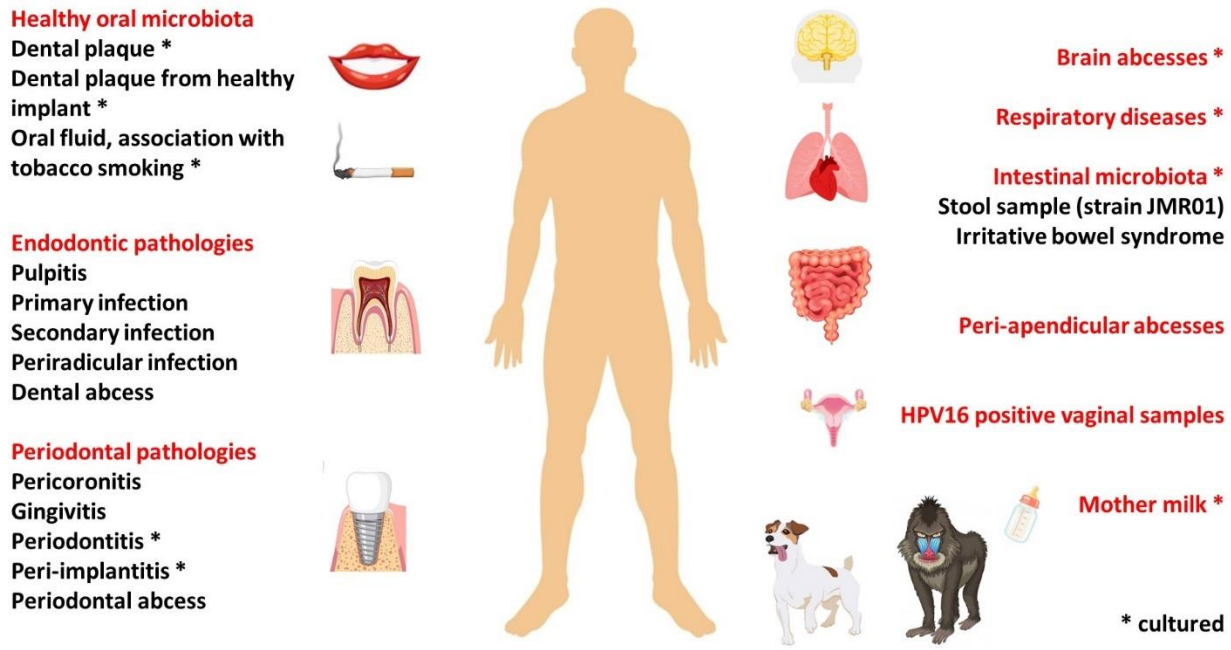


Figure 4: Past and present geographic distribution of *M. oralis.*



Legend

AMERICA

Buffalo, United States of America
- Sanitary indoors environnement

Columbus, United States of America
- Sanitary indoors environnement

Guarulhos, Brazil
- Peri-implantitis
- Periodontitis
- Healthy

Pennsylvania , United States of America
- Dental calculus (baboons)

Piracicaba, Brazil
- Endodontic pathologies

Pullman, United States of America
- Animals (slanzon rumen cow)

San Diego, United States of America
- Animals (dog)

San Fransisco, United States of America
- Periodontitis
- Healthy
- Ancient dental calculus

EUROPE

Aachen, Germany
- Periodontitis
- Endodontic pathologies
- Healthy

Aix-la-Chapelle, Germany
- Periodontitis
- Healthy

Avosnes, France
- Ancient dental calculus

Basel, Swiss
- Periodontitis

Cork, Ireland
- Feces

Douai, France
- Ancient dental calculus

El Salt, Spain
- Ancient dental calculus
- Ancient gut microbiota (coprolite)

Forbach, France
- Ancient dental calculus

Graz, Austria
- Sanitary indoors environnement

Les Fedons, France
- Ancient dental calculus

Łódź, Poland
- Endodontic pathologies
- Peri-implantitis

Lublin, Poland
- Periodontitis
- Peri-implantitis

Marseille, France
- Mother milk
- Periodontitis
- Healthy
- Peri-implantitis
- Saliva
- Respiratory diseases
- Brain abcesses
- Feces

Martigues, France
- Ancient dental calculus

Middenbeemster, Pays bas
- Ancient dental calculus

Milan, Italy
- First isolation from dental plaque

Saint-Gènes-Champanelle, France
- Animals (Cow Popova)

Saint-Mitre-les-Remparts, France
- Ancient dental calculus

Samsun, Türkiye
- Sanitary indoors environnement

San Teodoro, Italy
- Ancient dental calculus

South tyrol, Italy
- Ancient dental calculus

Spy cave, Belgium
- Ancient dental calculus

ASIA

Bagalkot, India
- Periodontitis
- Healthy

Japan
- Ancient dental calculus

Miyagi, Japan
- Sanitary indoors environnement

Okayama, Japan
- Periodontitis
- Healthy

Shangai, China
- Periodontitis

Tokyo, Japan
- Periodontitis
- Peri-implantitis

Zhejiang Sheng, China
- Vagina / cancer

AFRICA

Gabbarat el-Qurut, Egypt
- Ancient dental calculus (baboons)

Figure 5: *M. oralis* interactions with bacteria and other *Archaea*.

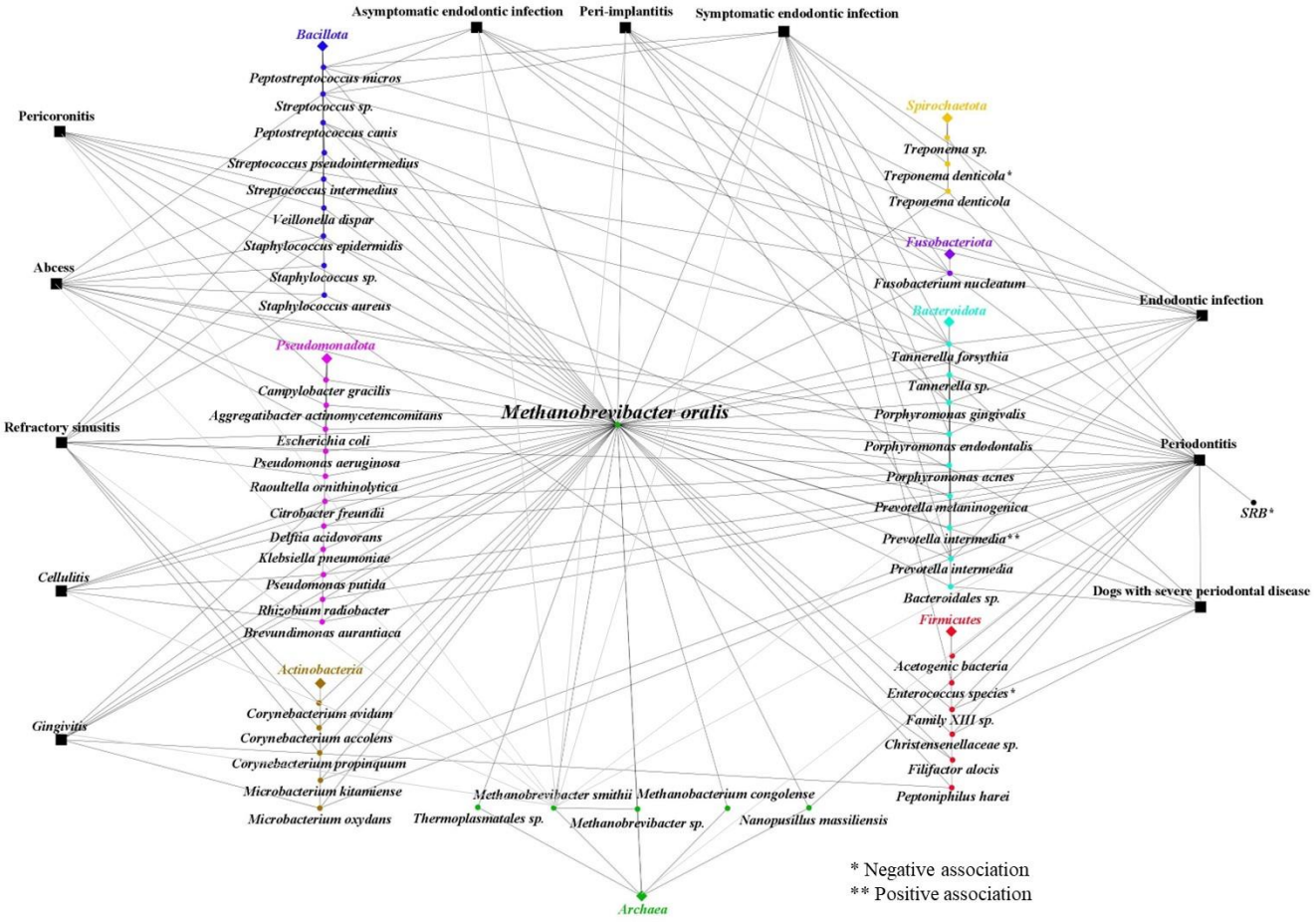


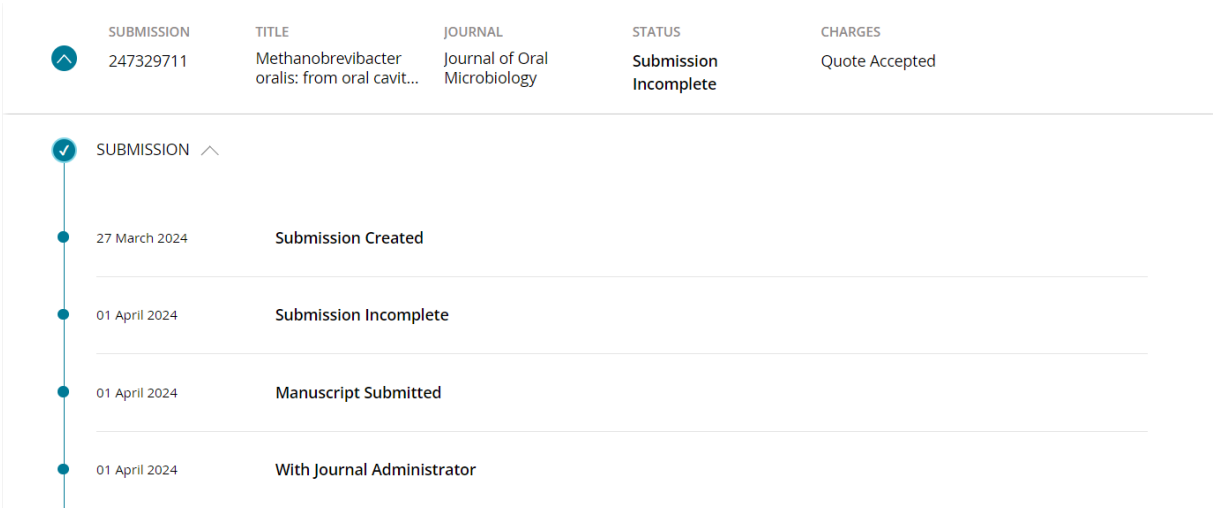
Table 1: The different PCR systems able to detect and amplify *M. oralis* sequences.

Primer pair	Sequences (5' - 3')	Target DNA	Amplicon size (approximative)	Temperature profile	Molecular analysis	Reference
300fEyAr	AGCJA/GIJA/GJGAGCCCGGAGATGG	16S rRNA	650	95° C (1 min), 35 cycles of 95° C (15s), 64° C (30s), 72° C (15s), and 72° C (7 min)	Standard PCR	Kulik *et al.*, 2001 (30); Faveri *et al.*, 2011 (71); Prakash *et al.*, 2020 (83)
954fEyAr	CGGCGTTGAA/GJTCCAATTAAAC					
SDArch0333a15	TCCAGGCCCTACGGG	16S rRNA	530	35 cycles of 94 °C (30s), 58° C (30s), and 72°C (3 min)	Standard PCR	Brans et al., 1994 (31); Liepp *et al.*, 2004 (4) ; Vickerman *et al.*, 2007 (93); Yamabe *et al.*, 2008 (80); Mansfield *et al.*, 2012 (87); Drancourt *et al.*, 2017 (13); Nkamga *et al.*, 2018 (14); Grine *et al.* (52); Togo *et al.* 2019 (50); Sogodogo *et al.*, 2019 (46,47); Guindo *et al.*, 2020 (66); Hassani *et al.*, 2020 (48), 2021 (12); Djemai *et al.*, 2021 (41), 2022 (42)
S*Univ0515a19	(FAM-)TTACCGCGGCKGCTGGCACTAMRA			95° C (10 min), 50 cycles of 95° C (30s), 55° C (30s), 60°C (45s), 65°C (15s), and 72°C (15s)	ABI Prism 7900HT Sequence Detection System (Applied Biosystems)	DeLong *et al.*, 1992 (32); Liepp *et al.*, 2004 (4)
A109F	ACKGCTCAGTAACACGT	16r RNA	798	95°C (10 min); 40 cycles of 95°C (10s), 65°C (10s), and 72°C (45s)	Standard PCR, Sybergreen RTQ-PCR and sequencing	Stahl *et al.*, 1991 (38); Grosskopf *et al.*, 1998 (37); Horz *et al.*, 2005 (39); Brzezinska-Blaszczyk *et al.*, 2018 (90); Vianna *et al.*, 2006 (34), 2008 (35), 2009 (36)
A934R	GTGCTCCCCCGCCAATTCCT					
Mbb279F	TGATCGGTACGGGGTTGTG	16r RNA	405	95° C (10 min); 35 cycles of 95° C (10 s), 58° C (10 s) and 72° C (25 s); fluorescence measurement at 78° C	Sybergreen RTQ-PCR and sequencing	Horz et al., 2005 (39)
Mbb709R	CAACAGGCGGTCCTCCCA					
Metha_16S_2_MBF	CGAACCGGATTAGATACCCG	16r RNA	/	50° C (2 min), 39 cycles of 95° C (5 min), 95° C (5s), and 60° C (30s)	RT-PCR	Drancourt *et al.*, 2020 (40); Guindo *et al.*, 2020 (66); Djemai *et al.*, 2021 (41), 2022 (42)
Metha_16S_2_MBR	CCCGCCAATTCCTTTAAGTT					
FAM_Metha_16S_2_MBP	FAM- CCTGGGAAGTACGGTCGCAAG					
ME1	GCMATGCARATHGGWATGTC	mcrA	760	30 cycles of 94° C (40s), 50° C (1 min and 30 s), 72° C (3 min), and 72° C (10 min)	Standard PCR	Hales *et al.*, 1985 (43); Scalan *et al.*, 2008 (44)
ME2	TCATKGCRTAGTTDGGRTAGT					
LuF	GGTGGTGTMGGATTCACACART AYGCWACAGC	mcrA	470	95° C (10 min); 40 cycles of 95° C (10s), 56° C (7s), and 72° C (25s)	Sybergreen RTQ-PCR and sequencing	Luton *et al.*, 2002 (45); Vianna *et al.*, 2006 (34), 2008 (35) and 2009 (36); Horz *et al.*, 2012 (115); Huynh *et al.*, 2015 (2), 2016 (21); Nkamga *et al.*, 2018 (14); Belkacemi *et al.*, 2018 (51); Grine *et al.*, 2018 (52)
LuR	TTCATTGGRTAGTTWGGRTAGTT					
mcrARev	GCTCTACGACCAGATMTGGCTTGG	mcrA	/	35 cycles of 94°C (30s), 58° C (30s), and 72°C (30s), and 72°C (3 min)	Standard PCR	Sogodogo *et al.*, 2019 (46,47)
mcrARev	CCGTAGTACGTGAAGTCATCCAGCA					
M. oralis -cnp602F	GCTGGTGTGTAATCGAACCTAAACG	Chaperonin gene cnp60	/	95° C (5 min), 40 cycles of 95° C (1s), 60° C (35s), and 45° C (30s)	RTQ-PCR	Bringuier *et al.*, 2013 (9); Huynh *et al.*, 2015 (2), 2016 (21); Drancourt *et al.*, 2017 (13); Togo *et al.*, 2019 (50); Guindo *et al.*, 2020 (66); Hassani *et al.*, 2020 (48), 2021 (12)
M. oralis -cnp602R	CACCCATACCCGGATCCATA					
M. oralis -cnp602P	FAM-AGCAGTGCACCTGCTGATATGGAAGG					
Arch915	GTGCTCCCCCGCCAATTCCT	16S rRNA	N/A	65° C (10 min), and 37° C (20h)	FISH	Amann *et al.*, 1990; Huynh *et al.*, 2016 (21); Grine *et al.*, 2018 (52); Sogodogo *et al.*, 2019 (46,47); Hassani *et al.*, 2020 (48), 2021 (12)
SBGA-1	Not available	16S rRNA	N/A	65 °C (8h)	FISH	Liepp *et al.*, 2004 (4)
LuR	TTCATTGGRTAGTTWGGRTAGTT	mcrA	N/A	80° C (5 min), and 46 °C (16h)	FISH	Luton *et al.*, 2002 (45); Sogodogo *et al.*, 2019 (46,47)

Table 2: *M. oralis* genomes features.

Organism Name	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*	*Methanobrevibacter oralis*
Organism Groups	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota	Archaea;Euryarchaeota
Strain	YH	DSM 7256	MGYG-HGUT-02162	M2 CSUR P5920	JMR01	N/A	N/A
BioSample	SAMEA9459756	SAMN04867341	SAMEA5851666	SAMEA104570764	SAMEA3138857	N/A	N/A
BioProject	PRJEB46774	PRJNA318760	PRJEB33885	PRJEB24872	PRJEB4880	PRJEB43389	N/A
Sample	Human dental plaque	Human dental plaque	Human feces	Human milk	Human feces	Ancient dental calculus from an Early Middle Age *Homo sapiens*	Ancient dental calculus from a Neanderthal
Assembly	GCA_912073625.1	GCA_001639275.1	GCA_902384065.1	GCA_900289035.1	GCA_000529525.1	N/A	N/A
Total Length (bp)	1,953,936	2,140,433	2,083,511	2,124,480	2,107,831	1,372,986	2,076,642
No. of Sequences	14	136	60	106	14	265	14
GC Content (%)	27.9	27.7	27.8	27.7	27.8	27.6	29.9
N50	308,900	45,270	88,069	45,266	36,2296	5,712	360,780
Gap Ratio (%)	0	0	0.00000096	0.00028713	0.01153888	0	68.406398
No. of CDSs	1,920	2,011	2,279	2,000	2,283	1,153	190
No. of rRNA	2	2	2	2	2	0	2
No. of tRNA	31	30	31	30	31	12	30
No. of CRISPRS	3	2	2	3	2	1	0
Coding Ratio (%)	84.9	80.7	82.1	81.8	81.2	69.5	4.7
Completeness(%)	100	100	95.33	100	95.33	52.41	24.3
Contamination(%)	0	0	0	0	0	0,028	0

Preuve de soumission du manuscrit dans Journal of Oral Microbiology.



SUBMISSION	TITLE	JOURNAL	STATUS	CHARGES
247329711	Methanobrevibacter oralis: from oral cavit...	Journal of Oral Microbiology	Submission Incomplete	Quote Accepted

SUBMISSION

27 March 2024	Submission Created
01 April 2024	Submission Incomplete
01 April 2024	Manuscript Submitted
01 April 2024	With Journal Administrator

Chapitre II: Optimisation des conditions de l'isolement par culture des méthanogènes

Préambule du chapitre II

Afin de confirmer leur présence effective dans un microbiote donné et de les isoler pour les étudier, les méthanogènes peuvent être cultivées à partir de divers échantillons cliniques. Toutefois, leur culture reste particulièrement fastidieuse pour plusieurs raisons. Elles sont aérointolérantes, sensibles au pH, elles ont des besoins spécifiques comme une source de carbone, des vitamines, des cofacteurs métalliques et, particulièrement pour les hydrogénotrophes telles que les espèces appartenant au genre *Methanobrevibacter* (comme *M. smithii* et *M. oralis*) et certaines méthyloptrophes notamment *M. stadtmanae*, une source d'hydrogène gazeux.

Dans les années 1960, Hungate a été l'un des pionniers dans ce domaine en mettant au point un milieu liquide strictement anaérobie suivi d'un milieu solide en roll-tube, permettant ainsi de contrôler l'atmosphère de culture et de doser précisément l'apport en hydrogène et en dioxyde de carbone. Depuis cette époque, divers milieux de culture adaptés aux besoins de chaque espèce connue ont été mis au point, répondant aux besoins spécifiques de chacune. Dans notre laboratoire, le milieu SAB® a été développé pour offrir une polyvalence permettant la culture et l'isolement de la plupart des espèces de méthanogènes d'intérêt clinique et avec une plus grande efficacité par rapport aux milieux de référence (26). L'introduction d'antioxydants dans le milieu SAB® solide a également permis la culture aérobie des méthanogènes en disposant les géloses dans la partie supérieure d'une double chambre dont la partie basse contenait une culture liquide de *Bacteroides thetaiotaomicron* produisant de l'hydrogène de manière continue (27). Plus récemment, Guindo et al. ont optimisé la technique de culture en double chambre en assurant la production d'hydrogène par réaction chimique entre l'acide acétique et des limailles de fer (28).

De manière générale dans notre laboratoire, les échantillons cliniques étaient collectés dans un milieu de transport spécifique puis transférés dès que possible dans un nouveau tube Hungate contenant du milieu SAB® liquide. L'enrichissement avait lieu sous atmosphère d'hydrogène à 80% et de dioxyde de carbone à 20%. Au bout de 7 jours, le méthane est mesuré par chromatographie en phase gazeuse et les échantillons positifs étaient déposés sur gélose SAB® contenant des antibiotiques et placés dans la partie haute de la double chambre dont la partie passe contient une source d'hydrogène, qu'elle soit biologique ou chimique. Les colonies microbiennes étaient généralement observées au bout de 9 jours. Toutefois, le recours quasi-systématique à l'hydrogène gazeux dans la

culture des méthanogènes représente une contrainte particulière pour un laboratoire de microbiologie. En effet, l'utilisation de l'hydrogène gazeux dans un laboratoire présente des risques d'incendie et d'explosion en raison de sa nature hautement inflammable et de sa capacité à former des mélanges explosifs avec l'air. Les fuites d'hydrogène peuvent créer des atmosphères explosives dans des espaces confinés, nécessitant une manipulation prudente et des mesures de sécurité telles que la ventilation adéquate et l'utilisation de détecteurs de gaz. Une formation sur les bonnes pratiques de manipulation et les procédures d'urgence est essentielle pour minimiser les risques pour la sécurité des personnes et des installations.

Ainsi, dans ce travail, nous avons souhaité optimiser les conditions de culture et d'isolement de la méthanogène la plus fréquente chez l'Homme, *M. smithii*. En se basant sur la capacité que possèdent la plupart des hydrogénotrophes à utiliser le formate comme source d'énergie et de carbone via la formate déshydrogénase tout en ayant besoin d'acétate (6), nous avons mis au point un nouveau milieu de culture, le milieu GG®, dérivé du milieu SAB® à la différence qu'une concentration augmentée en formate et en acétate a permis de se passer de l'hydrogène gazeux pour la culture de *M. smithii* à partir de selles humaines. Ceci a permis de faciliter grandement les conditions de culture de *M. smithii* en routine dans notre laboratoire.

Article 3: Culturing clinical *Methanobrevibacter smithii* using GG medium in a minimal anaerobe atmosphere

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Culturing clinical *Methanobrevibacter smithii* using GG medium in a minimal anaerobe atmosphere.

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ABSTRACT

Methanobrevibacter smithii (*M. smithii*), the most prevalent and abundant gut methanogen, detoxifies hydrogen into methane and is, therefore, of paramount importance for the equilibrium of the gut microbiota. The isolation by culture of *M. smithii* has routinely relied upon hydrogen-carbon dioxide-enriched, oxygen-deprived atmospheres. In this study, we developed a medium referred to as "GG", which allowed for *M. smithii* growth and isolation by culture in an oxygen-deprived atmosphere, with no supply of either hydrogen or carbon dioxide, making it easier to detect *M. smithii* by culture in clinical microbiology laboratories.

1. Introduction

Methanobrevibacter smithii (*M. smithii*), a methanogenic archaea referred to here simply as methanogen, is a key inhabitant of the gut microbiota and is the principal microorganism which reduces the hydrogen resulting from the fermentation of alimentary substrates by anaerobe bacteria (Lyu et al., 2018). The paramount importance of *M. smithii* in the gut microbiota equilibrium is further attested by the fact that it is not detected in stools collected from patients suffering from life-threatening severe malnutrition, illustrating the key role that this particular microorganism plays in the homeostatic regulation of the gut's physiology (Million et al., 2016).

Current protocols for the isolation by culture of *M. smithii* from clinical specimens of microbiota such as stools (Dridi et al., 2011), oral fluid (Grine et al., 2018), and clinical specimens collected from abscesses such as muscular abscesses (Nkamga et al., 2016) and cerebral abscesses (Drancourt et al., 2017), have all relied on supplying hydrogen and carbon dioxide into the culture atmosphere (Nkamga et al., 2016). Hydrogen can be supplied to the culture atmosphere as gaseous hydrogen from an external source (Khelaifia et al., 2013), as chemically produced hydrogen within the culture environment itself (Guindo et al., 2020), or as biotic hydrogen resulting from bacterial fermentation within the culture environment (Khelaifia et al., 2016).

In this study, we report the proof-of-concept that using a medium referred to as GG allows for the isolation by culture of *M. smithii* from clinical specimens without requiring either hydrogen or carbon dioxide in the culture atmosphere.

2. Materials and methods

***M. smithii* strain Q5488 investigation (Fig. 1).** *M. smithii* strain Q5488, a stool isolate preserved in the strains collection of the Unité des Rickettsies (CSUR Q5488 - Supplementary data 1) was inoculated into 30 different Hungate tubes (Dominique Dutscher, Brumath, France), each containing 200 µL of Dulbecco's Phosphate Buffered Saline (DPBS, Fisher Scientific, Waltham, Massachusetts, USA) in order to study six different experimental conditions, as detailed below (in other words, five Hungate tubes per experimental condition). Condition A1 included Hungate tubes seeded with an SAB medium, a versatile medium that allows the growth and the isolation of most of methanogen species as previously reported (Khelaifia et al., 2013), except that it did not contain acetate or formate (medium referred to as SAB 0). These tubes were degassed for three minutes with 2-bar nitrogen, inoculated with 200 µL of *M. smithii* strain Q5488 and degassed again for 30 s with 2-bar nitrogen. Condition A2 included five tubes and one control tube, similar to condition A1 with the difference that they were gassed with a mixture of 80% hydrogen and 20% carbon dioxide for 30 s at a pressure of 2 bars. Condition B1 included five Hungate tubes and one control Hungate tube,

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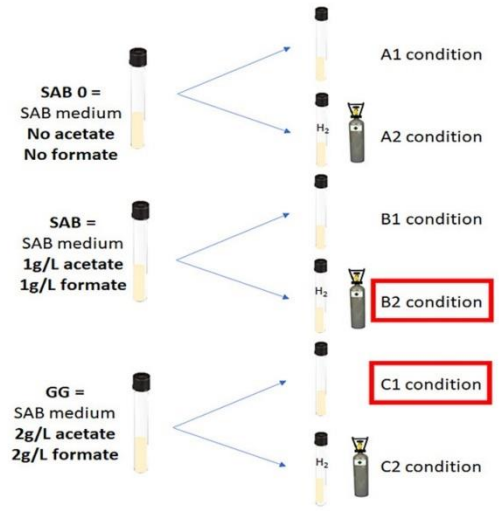

Fig. 1. Experimental design of the six studied conditions for the growth of *M. smithii* Q5488.The B2 and C1 conditions were also applied to other *M. smithii* strains and left-over stool samples.

all containing the SAB medium, as originally described, i.e. containing 1 g/L of acetate and formate (Khelaifia et al., 2013). This SAB medium was prepared from SAB 0 medium by adding 100 µL of a stock solution containing 50 g/L of acetate and formate (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France) buffered to pH 7.3 and filtered through a 0.2 µm filter (Merck, Darmstadt, Germany). These tubes were degassed for three minutes with nitrogen at a pressure of 2 bars and then degassed after inoculation with 200 µL of *M. smithii* strain Q5488, for 30 s with nitrogen, again at a pressure of 2 bars. Condition B2 was as above, with the difference that the five Hungate tubes and the control Hungate tube were gassed with a mixture of 80% hydrogen and 20% carbon dioxide for 30 s at a pressure of 2 bars. Condition C1 included five Hungate tubes and one control tube, all containing the SAB medium enriched with 2 g/L acetate and 2 g/L formate. This medium was referred to as the GG medium. GG medium was prepared from the SAB 0 medium (free of acetate and formate) by adding 200 µL of a 50 g/L stock solution of acetate and formate (Sigma-Aldrich Chimie) buffered to pH 7.3 and filtered to 0.22 µm (Merck). These tubes were degassed for three minutes with 2-bar nitrogen and degassed again for 30 s after having been inoculated with 200 µL of the *M. smithii* Q5488 strain suspension. Condition C2 was the same as condition C1, except that the five Hungate tubes and the negative control Hungate tube were gassed with a mixture of 80% hydrogen and 20% carbon dioxide for 30 s at a pressure of 2 bars. All the 30 tubes described here were incubated for seven days at 37 °C. The experimental design model of the six conditions studied for the growth of *M. smithii* Q5488 is presented in Fig. 1.

Investigation of additional *M. smithii* strains. A set of four additional *M. smithii* stool isolates preserved in the strains collection of the Unité des Rickettsies comprising *M. smithii* strain Q5710, *M. smithii* strain Q5711, *M. smithii* strain Q5713, *M. smithii* strain Q5717 and *M. smithii* strain Q5718 (Supplementary data 1), were investigated under conditions B1 and C2, as described above. For each of these *M. smithii* strains, ten Hungate tubes were inoculated with *M. smithii* and two negative control tubes were mock-inoculated with DPBS. Under condition B1, as described above, five *M. smithii*-inoculated Hungate tubes and one negative control tube containing 5 mL of SAB medium were degassed for three minutes with 2-bar nitrogen and degassed again for 30 s after inoculation with 200 µL of a *M. smithii* suspension or 200 µL of DPBS for the control tube. All these Hungate tubes were gassed with a mixture of 80% hydrogen and 20% carbon dioxide for 30 s at a pressure of 2 bars. As for condition C2 as described above, this included five tubes and one control tube all containing 5 mL of GG medium. Tubes were degassed for three minutes with 2-bar nitrogen and then degassed after inoculation of 200 µL of the *M. smithii* suspension or 200 µL of DPBS for the control tube, for 30 s with 2-bar nitrogen. No hydrogen and no carbon dioxide was added to these tubes. All the tubes were incubated at 37 °C for seven days.

Clinical specimens. A series of 20 anonymised left-over stool samples were collected from the clinical microbiology core-laboratory of the IHU Méditerranée Infection. Research on such anonymised left-over stool samples is not regarded as research on humans under current legislation in France, but rather as research on objects completely disconnected from humans. Therefore, the research reported here did not require the approval of any ethics committee. In practice, 0.5 g of stool suspended in 10 mL of DPBS 1× water was vortexed for a few seconds and 200 µL of this suspension was inoculated into two Hungate tubes (i.e., 100 µL per Hungate tube). One Hungate tube (tube A) containing SAB medium previously degassed for three minutes with 2-bar nitrogen, was gassed with 80% hydrogen mixed with 20% carbon dioxide for 30 s at 2-bar pressure (related to the B2 condition) and incubated under an anaerobic atmosphere. A second Hungate tube (tube B) containing GG medium previously degassed for three minutes with 2-bar nitrogen was incubated under an anaerobic atmosphere, without any added hydrogen (related to the C1 condition). Two negative control tubes, one mimicking tube A and the second mimicking tube B, were mock inoculated with sterile DPBS water and incubated in parallel to tube A and tube B. All the Hungate tubes were incubated at 37 °C for nine days. The experimental design for clinical samples culture is presented in Fig. 3.

Culture monitoring. The growth of methanogen was monitored by the detection and quantification of methane in the Hungate tubes by gas chromatography: *M. smithii* Q5488 strain cultures were monitored on D0, 48 h (D2), 5 days (D5) and 7 days (D7) while the other *M. smithii* strain cultures were monitored on D0 and D7, and cultures of stool samples were monitored on D0 and D9. As for gas chromatography, 100 µL was taken from the air in the tube and injected into a Clarus 580 chromatograph (Perkin Elmer, Waltham, Massachusetts, United States). The so-called "sensitive" method was used and resulting area under the curve (AUC) values were used as a semi-quantitative method to quantify methane production, as previously described (Khelaifia et al., 2013). Methanogen growth was then monitored using real-time PCR (RT-PCR). DNA was extracted from methane-positive cultures using the EZ1 Advanced XL Extraction Kit (QIAGEN, Hilden, Germany) with 200 µL as the sample volume and 200 µL as the elution volume; and *M. smithii* 16S rDNA was RT-PCR amplified, as previously described (Dridi et al., 2011).

2.1. Statistical analysis

The student's *t*-test (alpha risk 5%) was used to compare average methane production, according to the various conditions on the BiostaTGV website https://biostatgv.sentiweb.fr/.

3. Results

3.1. All results are reported in Supplementary data 5

Inoculation of *M. smithii* strain Q5488. Methane production was observed starting on D2 post-inoculation in all the Hungate tubes inoculated with *M. smithii* strain Q5488, while no methane was detected in the negative control tubes. Differences in methane production between the different conditions were observed on D7. The B2 condition (SAB medium under 80% hydrogen and 20% carbon dioxide

Table 1
Yield of culture of *M. smithii* Q5488 in six different culture conditions investigated in this study. *M. smithii* growth was assessed by calculating the *M. smithii* load (CFU/mL) after RT-PCR 16S rDNA *M. smithii* and methane production measured by gas chromatography (Area Under the Curve - AUC).

	A1 condition				A2 condition			
	RT-PCR 16S rDNA *M. smithii*		Methane measurment (AUC)		RT-PCR 16S rDNA *M.smithii*		Methane measurment (AUC)	
	Average CFU/mL	Standard deviation	Average AUC	Standard deviation	Average CFU/mL	Standard deviation	Average AUC	Standard deviation
Day 0	1.39E+04	7.99E+03	0	0	1.14E+04	4.25E+03	0	0
Day 2			34,190	12,711.71601			20,823.4	1374.958472
Day 5			63,617.4	12,568.43330			22,140.2	4168.101570
Day 7	1.75E+05	9.89E+04	132,983.4	102,092.91593	2.60E+05	1.39E+05	149,760.4	89,406.59687
	B1 condition				B2 condition			
	RT-PCR 16S rDNA *M.smithii*		Methane measurment (AUC)		RT-PCR 16S rDNA *M.smithii*		Methane measurment (AUC)	
	Average CFU/mL	Standard deviation	Average AUC	Standard deviation	Average CFU/mL	Standard deviation	Average AUC	Standard deviation
Day 0	3.58E+03	5.52E+02	0	0	3.66E+03	1.96E+03	0	0
Day 2			186,579.6	22,828.01886			184,583.2	51,381.52612
Day 5			595,903.4	229,905.3852			688,908	246,353.3748
Day 7	7.39E+04	1.04E+04	803,757.8	300,733.3666	1.02E+05	1.09E+05	3,877,982.8	739,873.7963
	C1 condition				C2 condition			
	RT-PCR 16S rDNA *M.smithii*		Methane measurment (AUC)		RT-PCR 16S rDNA *M.smithii*		Methane measurment (AUC)	
	Average CFU/mL	Standard deviation	Average AUC	Standard deviation	Average CFU/mL	Standard deviation	Average AUC	Standard deviation
Day 0	5.53E+03	2.07E+03	0	0	4.23E+03	3.22E+03	0	0
Day 2			223,468.6	40,933.95852			201,097.4	41,707.95075
Day 5			624,252.2	130,674.3317			860,322	135,693.1442
Day 7	8.01E+04	1.93E+04	2,949,185.2	1,075,892.909	1.15E+05	3.68E+04	4,067,917.6	1,216,245.189

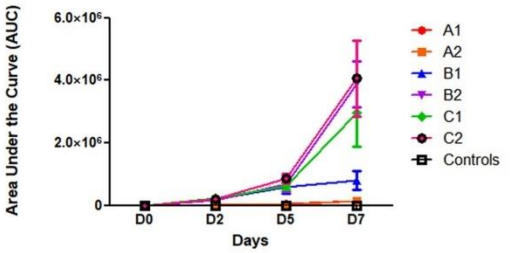

Fig. 2. Kinetics of methane concentration after inoculation of Hungate tubes with *M. smithii* Q5488 strain, in experimental conditions A1-C2 cross-differing by the culture medium composition and atmosphere for incubation. A1 condition corresponds to culture in SAB medium free of acetate and formate (SAB 0 medium) under nitrogen atmosphere; A2 condition corresponds to culture in SAB 0 medium under 80% hydrogen and 20% carbon dioxide atmosphere; B1 condition corresponds to culture in SAB medium under nitrogen atmosphere; B2 condition corresponds to culture in SAB medium under 80% hydrogen and 20% carbon dioxide atmosphere; C1 condition corresponds to culture in SAB medium enriched with 2 g/L acetate and 2 g/L formate (GG medium) under nitrogen atmosphere; C2 condition corresponds to culture in GG medium under 80% hydrogen and 20% carbon dioxyde atmosphere. The X axis presents an area under the curve of methane concentration derived from chromatographic measurement; the Y axis presents time in days. The points indicate the median and the vertical bars represent the standard deviation, resulting from five independent experiments.

atmosphere) and C2 condition (GG medium under 80% hydrogen and 20% carbon dioxide atmosphere) led to the greatest production of methane, with no significant difference between these two conditions (p-value C2/B2 = 0.15, Student's t-test). Interestingly, the methane production in condition C1 (GG medium under nitrogen atmosphere, no addition of hydrogen or CO2) was not significantly lower than that measured in conditions B2 and C2 (p-value C1/C2 = 0.16 and p-value C1/B2 = 0.16, Student's t-test) but three times higher than that measured in condition B1 (SAB medium under nitrogen atmosphere, no addition of hydrogen and carbon dioxide) (p-value C1/B1 = 0.003, Student's t-test). Accordingly, the two conditions A1 and A2, both lacking acetate or formate, were superimposed and showed the lowest production of methane (p-value A1/B2 = 0.0003, p-value A2/B2 = 0.0003, p-value A1/A2 = 0.79, Student's t-test). All these data are reported in Table 1 and Fig. 2. Examples of chromatograms are reported in Supplementary data 2 and 3.

RT-PCRs targeting the *M. smithii*-specific 16S rDNA showed a decrease in the *M. smithii* load between D0 (7.06E+03 (± 4.47E+03) CFU/mL) and D7 (1.34E+05 (± 7.15E+04) CFU/mL) corresponding in all the inoculated tubes (Table 1). However, there was no observable difference in the *M. smithii* load between D0 and D7 between the six conditions. RT-PCRs on the control tubes remained negative.

Investigation of additional *M. smithii* strains. Methane production was observed on D9 for all the tubes inoculated with additional *M. smithii* strains under investigation, both for the B2 condition (SAB medium under 80% hydrogen and 20% carbon dioxide atmosphere) and the C1 condition (GG medium under nitrogen atmosphere, no addition of hydrogen and carbon dioxide). There was no significant difference in the average methane production between the two conditions (p-value = 0.81, Student's t-test). No methane production was detected in any of the negative control tubes.

RT-PCRs targeting the *M. smithii*-specific 16S rDNA showed a decrease in the *M. smithii* load between D0 and D7 in all the inoculated tubes. The *M. smithii* load for the B2 condition was 1.56E+03 (± 2.13E+03) CFU/mL on D0 and 4.08E+04 (± 1.80E+04) CFU/mL on D7 and, for the C1 condition, the *M. smithii* load was 1.11E+04 (± 6.26E+03) CFU/mL on D0 and 6.17E+04 (± 3.01E+04) CFU/mL on D7. RT-PCRs of control tubes remained negative. All these data are reported in Table 2. Supplementary data 2 and 3 show chromatograms obtained for one tube of each *M. smithii* strains on D0 and D7 for the B2 and C1 conditions.

Clinical specimens. Nine of 20 (45%) stool samples investigated for both the B2 condition (tube A, SAB medium under 80% hydrogen and 20% carbon dioxide atmosphere) and the C1 condition (tube B, GG medium under nitrogen atmosphere, no addition of hydrogen or carbon dioxide) were culture-positive as assessed by a detectable production of methane (Fig. 3). The chromatograms obtained from stool samples 1 and 2 on D0 and D7 under the B2 and C1 conditions are presented in Supplementary data 4. There was no significant difference in methane production between the two B2 and C1 conditions (p-value = 0.2, Student's t-test) when inoculated with the same sample. Furthermore, RT-PCRs targeting the pan-methanogen 16S rDNA or the *M. smithii*-specific 16S rDNA showed a decrease in the number of Ct between D0 and D9 in all the methane-positive tubes. For the RT-PCRs targeting the pan-methanogen 16S rDNA, the methanogen load for the B2 condition was

Table 2
Culture of five *M. smithii* strains following the B2 protocol (SAB medium +80% hydrogen and 20% carbon dioxide) and the C1 protocol (GG medium under nitrogen atmosphere). *M. smithii* growth was assessed by calculating the *M. smithii* load (CFU/mL) after RT-PCR 16S rDNA *M. smithii* and methane production measured by gas chromatography (Area Under the Curve - AUC).

	RT-PCR 16S rDNA *M.smithii* (CFU/mL)				Methane measurment (Area Under the Curve - AUC)			
Strains	B2 condition D0	B2 condition D7	C1 condition D0	C1 condition D7	B2 condition D0	B2 condition D7	C1 condition D0	C1 condition D7
CSUR Q5488	3.35E+03	5.33E+04	5.21E+03	7.85E+04	0	3,877,982.8	0	2,949,185.2
CSUR Q5710	4.39E+03	1.01E+04	3.48E+03	1.63E+04	0	1,868,631.6	0	2,493,469.2
CSUR Q5711	2.86E+01	5.42E+04	1.67E+04	6.65E+04	0	2,370,773	0	2,040,190.4
CSUR Q5717	2.91E+01	4.41E+04	1.50E+04	5.16E+04	0	1,887,083.4	0	2,202,565.4
CSUR Q5718	2.86E+01	4.24E+04	1.52E+04	9.57E+04	0	2,039,885.2	0	1,821,646.4
Average	1.56E+03	4.08E+04	1.11E+04	6.17E+04	0	2,408,871.2	0	2,301,411.32
Standard deviation	2.13E+03	1.80E+04	6.26E+03	3.01E+04	0	756,308.4514	0	437,229.4856

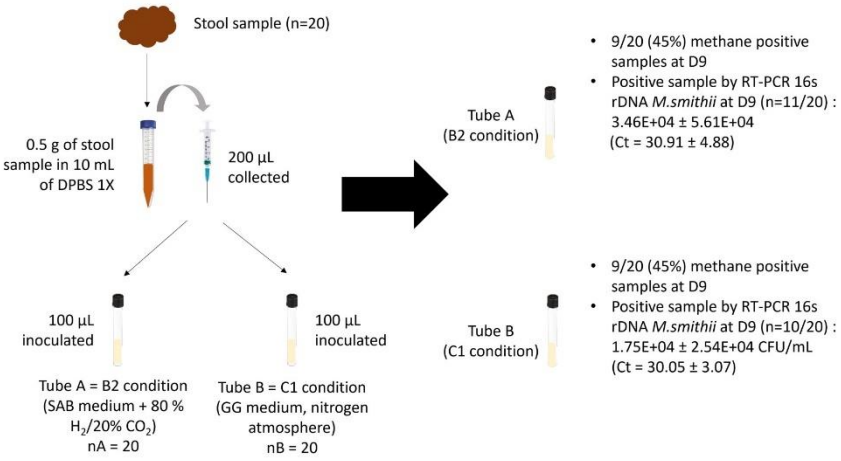

Fig. 3. Experimental design and results for attempts at anaerobic isolation by culture of *Methanobrevibacter smithii* from left-over stool samples, in the presence (Tube A, condition B2) and absence (Tube B, C1 condition) of hydrogen and carbon dioxide in the culture atmosphere.

4.50E+03 (± 7.79E+03) CFU/mL on D0 and 3.09E+06 (± 9.38E+06) CFU/mL on D9. The methanogen load for the C1 condition was 7.47E+03 (± 1.23E+04) CFU/mL on D0 and 5.94E+05 (± 1.57E+06) CFU/mL on D9. For the RT-PCRs targeting the *M. smithii* 16S rDNA, the *M. smithii* load for the B2 condition was 2.76E+02 (± 4.89E+02) CFU/mL on D0 and 3.46E+04 (± 5.61E+04) CFU/mL on D9, and for the C1 condition, the *M. smithii* load was 5.04E+02 (± 7.16E+02) on D0 and 1.75E+04 (± 2.54E+04) CFU/mL on D9. Conversely, RT-PCR-negative tubes produced no detectable methane and RT-PCR-positive tubes, with no decrease in the methanogen or *M. smithii* load, did not produce methane (Table 3).

4. Discussion

The data reported here, validated by the negativity of the negative controls introduced in every experiment and the reproducibility of the results over at least five independent experiments, indicated that *M. smithii* was isolated by culture and was growing without any source of hydrogen and carbon dioxide, using the GG medium. This GG medium was derived from the previously described SAB medium (Khelaifia et al., 2013), by enrichment in both acetate and formate, after *M. smithii* genome analysis indicated that *M. smithii* methanogenesis could rely on acetate catabolism and formate catabolism proceeded by *M. smithii* formate dehydrogenase (Lyu et al., 2018).

The observations reported here may be relevant to the homeostasis of the anaerobe microbiota in which *M. smithii* is detected, chiefly the gut microbiota. In the gut microbiota, both formate and acetate are by-products of bacterial fermentation, including the fermentation of *B. thetaiotaomicron* for acetate production (Fischbach and Sonnenburg, 2011). *Bacteroides, Bifidobacteria, Eubacteria, Lactobacilli, Clostridia, Ruminococci, Streptococci* and *Enterobacteria* produce acetate and formate in the human gut (Macfarlane and Macfarlane, 2012; Oliphant and Allen-Vercoe, 2019). Accordingly, most of the *Methanobrevibacter* species are able to achieve formate-based methanogenesis according to genome analysis (Chibani et al., 2020). *M. smithii* appears to be able to respond to the availability of formate by upregulating formate metabolism gene clusters (Samuel et al., 2007) and by expressing adhesin-like proteins (Hansen et al., 2011). Therefore, in such anaerobe microbiota, *M. smithii* inoculum, thought to be mainly driven by hydrogen, may be driven by a subtle balance between hydrogen, formate and acetate, all resulting from anaerobic fermentation, albeit by different bacterial species.

These observations also may provide insight into the currently inexplicable situation of *M. smithii* being detected in anaerobic samples devoid of hydrogenogenic bacteria. In a recent series of 27 *M. smithii*-positive anaerobic blood cultures, 21 (77%) of these blood cultures lacked any detectable hydrogenogenic bacterium but did present streptococci and staphylococci (Drancourt et al., 2020). It is notable that these bacterial species are recognised as being formate- and acetate-forming (Leibig et al., 2011). The same situation prevails in brain

Table 3
Isolation by culture of *M. smithii* from left-over stool samples following the B2 protocol (SAB medium +80% hydrogen and 20% carbon dioxide) and the C1 protocol (GG medium under nitrogen atmosphere). *M. smithii* growth was assessed by calculating the methanogen and *M. smithii* load (CFU/mL) after RT-PCR 16S rDNA *M. smithii* and methane production measured by gas chromatography (Area Under the Curve - AUC).

	RT PCR 16S rDNA methanogens (CFU/mL)				RT PCR 16S rDNA *M.smithii* (CFU/mL)				Methane measurment (Area Under the Curve - AUC)			
Samples	B2 condition D0	B2 condition D9	C1 condition D0	C1 condition D9	B2 condition D0	B2 condition D9	C1 condition D0	C1 condition D9	B2 condition D0	B2 condition D9	C1 condition D0	C1 condition D9
1	**1.11E+04**	**1.20E+06**	**6.21E+03**	**8.86E+05**	**3.31E+02**	**1.03E+05**	**2.61E+02**	**8.29E+04**	/	**1,751,082**	/	**4,191,411**
2	**1.61E+02**	**7.86E+04**	**6.39E+01**	**5.31E+04**	**3.83E+00**	**5.57E+03**	/	**3.01E+03**	/	**5,612,450**	/	**5,674,599**
3	/	/	/	7.76E+00	/	/	/	/	/	/	/	/
4	/	/	/	/	/	/	/	/	/	/	/	/
5	**1.84E+03**	**3.42E+07**	**1.70E+04**	**6.21E+06**	/	**3.03E+01**	/	**1.48E+02**	/	**3,690,910**	/	**5,781,839**
6	**1.38E+04**	**2.10E+06**	**3.62E+03**	**5.02E+05**	**4.02E+02**	**1.72E+05**	**8.82E+01**	**3.30E+04**	/	**4,143,458**	/	**4,680,609**
7	**1.90E+03**	**1.90E+05**	**7.22E+01**	**2.36E+05**	**3.48E+01**	**5.84E+03**	/	**1.81E+04**	/	**2,723,809**	/	**2,017,318**
8	**2.70E+04**	**1.29E+06**	**3.87E+04**	**3.83E+05**	**1.52E+03**	**3.64E+04**	**1.84E+03**	**1.60E+04**	/	**2,284,646**	/	**3,286,513**
9	/	/	/	4.97E+00	/	/	/	/	/	/	/	/
10	1.08E+01	5.89E+00	/	1.53E+01	/	/	/	/	/	/	/	/
11	**1.30E+03**	**9.95E+05**	**6.34E+01**	**4.37E+05**	**2.91E+01**	**5.47E+04**	/	**1.65E+04**	/	**2,044,792**	/	**3,736,573**
12	/	/	/	/	/	/	/	/	/	/	/	/
13	1.62E+01	/	1.09E+01	/	/	/	/	/	/	/	/	/
14	3.70E+01	9.83E+01	1.71E+02	1.44E+02	/	1.94E+00	5.20E+00	/	/	/	/	/
15	1.23E+03	9.08E+02	1.62E+03	2.30E+03	2.87E−01	2.96E+01	4.18E+01	8.11E−01	/	/	/	/
16	/	/	/	/	/	/	/	/	/	/	/	/
17	1.10E+01	1.15E+01	/	1.00E+01	/	/	/	/	/	/	/	/
18	**2.65E+02**	**6.14E+04**	**1.23E+02**	**5.91E+04**	**4.13E+00**	**1.77E+03**	/	**5.10E+02**	/	**2,894,389**	/	**4,606,048**
19	/	/	/	/	/	/	/	/	/	/	/	/
20	**4.29E+03**	**2.68E+04**	**2.19E+04**	**1.40E+05**	**1.31E+02**	**8.94E+02**	**7.84E+02**	**4.97E+03**	/	**2,598,671**	/	**1,488,917**
AVERAGE	4.50E+03	3.09E+06	7.47E+03	5.94E+05	2.76E−02	3.46E+04	5.04E+02	1.75E+04	/	3,272,201.3	/	4,160,256.8
STANDARD DEVIATION	7.79E+03	9.38E+06	1.23E+04	1.57E+06	4.89E−02	5.61E+04	7.16E+02	2.54E+04	/	1,225,274.617	/	1,482,525.105

abscesses where *M. smithii* has been co-detected with *Streptococcus intermedius*, a formate and acetate producer (Shain et al., 1996). These previous results align with the results of this study which shows that formate can be metabolised by methanogens as an energy source.

The implantation and use of the GG medium in clinical microbiology laboratories should make it easier to isolate *M. smithii* by culture from both microbiota and clinical samples collected from diseased tissues, by omitting the more specialised and complex gaseous mixes, particularly explosive hydrogen. Such routine use may also reveal whether other *Methanobrevibacter* methanogens of clinical interest, such as *Methanobrevibacter oralis* (Ferrari et al., 1994), could also be isolated by culture in the GG medium, under a limited anaerobe atmosphere.

Declaration of Competing Interest

VP, ET, GA, MD and GG are co-inventor of patent regarding this work entitled: "Milieu et procédé de culture des archaea methanogens".

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2023.106704.

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Chapitre III: *Candidatus* Methanosphaera massiliense sp. nov.

Préambule du chapitre III

La mise au point du milieu GG® a permis l'optimisation des conditions de culture de cinq souches de *M. smithii* et de cultiver *M. smithii* à partir d'échantillons de selles humaines par l'augmentation des concentrations en acétate et en formate, permettant d'éviter le recours à l'hydrogène gazeux, gaz dangereux dans son utilisation en laboratoire de microbiologie.

A l'instar du milieu SAB®, le milieu GG® contient également du méthanol, nous espérions à ce moment-là que le milieu GG® pouvait être presque aussi polyvalent que le milieu SAB®. Ainsi, nous avons souhaité poursuivre ce travail de culture et d'isolement des méthanogènes à partir d'autres échantillons cliniques. Grâce au milieu GG®, nous avons pu découvrir, cultiver et isoler une nouvelle espèce de méthanogène à partir d'une selle humaine appartenant au genre *Methanosphaera*, *Cand.* M. massiliense. Il s'agit seulement de la dixième espèce cultivée chez l'Homme depuis près de 40 ans et la deuxième appartenant au genre *Methanosphaera* après *M. stadtmanae*. Ce travail a également permis de développer la version solide du milieu GG® abandonnant la technique de la double chambre, la source d'hydrogène n'étant plus nécessaire. La gélose GG® était simplement disposée dans un sachet hermétique avec un générateur d'atmosphère anaérobie.

Par la suite, notre précédent travail de revue de littérature nous ayant permis de lister toutes les facettes à étudier d'une méthanogène, nous avons entrepris la description de *Cand.* M. massiliense incluant notamment sa description phénotypique, l'obtention de son génome et de ses caractéristiques, la recherche de ses substrats préférentiels et de sa sensibilité aux antibiotiques. Particulièrement, les analyses phylogénétiques, tout d'abord à partir du gène de l'ARNr 16S puis du génome complet ont permis de mettre en évidence sa proximité avec d'autres espèces appartenant au genre *Methanosphaera* retrouvées chez d'autres espèces animales. Après la mise au point d'un système de PCR spécifique, nous avons pu détecter la présence de *Cand.* M. massiliense dans des fèces de kangourous roux et de porcs destinés à la consommation humaine. Nous avons donc émis l'hypothèse que la source d'acquisition de cette méthanogène pouvait être la consommation de viande.

Article 4: *Candidatus* Methanosphaera massiliense sp. nov., a methanogenic archaeal species found in a human fecal sample and prevalent in pigs and red kangaroos

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Candidatus Methanosphaera massiliense sp. nov., a methanogenic archaeal species found in a human fecal sample and prevalent in pigs and red kangaroos

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ABSTRACT *Methanosphaera stadtmanae* was the sole *Methanosphaera* representative to be cultured and detected by molecular methods in the human gut microbiota, further associated with digestive and respiratory diseases, leaving unknown the actual diversity of human-associated *Methanosphaera* species. Here, a novel *Methanosphaera* species, *Candidatus* Methanosphaera massiliense (*Ca*. M. massiliense) sp. nov. was isolated by culture using a hydrogen- and carbon dioxide-free medium from one human feces sample. *Ca*. M. massiliense is a non-motile, 850 nm Gram-positive coccus autofluorescent at 420 nm. Whole-genome sequencing yielded a 29.7% GC content, gapless 1,785,773 bp genome sequence with an 84.5% coding ratio, encoding for alcohol and aldehyde dehydrogenases promoting the growth of *Ca*. M. massiliense without hydrogen. Screening additional mammal and human feces using a specific genome sequence-derived DNA-polymerase RT-PCR system yielded a prevalence of 22% in pigs, 12% in red kangaroos, and no detection in 149 other human samples. This study, extending the diversity of *Methanosphaera* in human microbiota, questions the zoonotic sources of *Ca*. M. massiliense and possible transfer between hosts.

IMPORTANCE Methanogens are constant inhabitants in the human gut microbiota in which *Methanosphaera stadtmanae* was the only cultivated *Methanosphaera* representative. We grew *Candidatus* Methanosphaera massiliense sp. nov. from one human feces sample in a novel culture medium under a nitrogen atmosphere. Systematic research for methanogens in human and animal fecal samples detected *Ca*. M. massiliense in pig and red kangaroo feces, raising the possibility of its zoonotic acquisition. Host specificity, source of acquisition, and adaptation of methanogens should be further investigated.

KEYWORDS methanogens, *Methanosphaera*, *Candidatus* Methanosphaera massiliense, hydrogen-free culture, human gut microbiota, host transfer

Human gut methanogens comprised of cultured representatives for *Methanobrevibacter smithii* (*M. smithii*) (1), *Methanobrevibacter oralis* (*M. oralis*) (2), *Methanobrevibacter arboriphilicus* (3), *Methanomassiliicoccus luminyensis* (*M. luminyensis*) (4), and *Methanosphaera stadtmanae* (*M. stadtmanae*) (5), *Candidatus* Methanomethylophilus alvus (6), *Candidatus* Methanomassiliicoccus intestinalis (7), and *Candidatus* Methanobrevibacter intestini (8–10), with a combined prevalence of 97.5% for *M. smithii* and 30% for *M. stadtmanae* (11) which indicated these various methanogens were overlapping constant gut inhabitants (12). An exception is the dysbiotic depletion of *M. smithii* and its absence in children with deadly severe acute malnutrition (13).

Since the detection of *Methanosphaera* spp. and *Methanobrevibacter* spp. in the bioaerosols from poultries, dairy farms, and piggeries (14–16), studies were conducted

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V.P., M.D., G.A., G.G., and E.T. are co-inventors of a patented culture medium referred to as GG culture medium in this report (patent FR 23 01404).

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in animal models to investigate the role of methanogens in hypersensitivity diseases. The administration of *M. stadtmanae* extract aerosol but not *M. smithii* aerosol in the airways of mice yielded a hypersensitivity response with an accumulation of eosinophils and neutrophils in the lungs (17, 18), laying the groundwork for the potential pathogenic role of *M. stadtmanae*. The pro-inflammatory capacity of *M. stadtmanae* was next used to induce hypersensitivity pneumonitis disease in mice models (19, 20). Moreover, further studies focused on human cells' response exposition to methanogens, *M. smithii* and *M. stadtmanae* are both able to activate mononuclear cells but *M. stadtmanae* induced a higher release of pro-inflammatory cytokines than *M. smithii* (21, 22). *M. stadtmanae* phagocytosis is crucial for cell activation (21). At the molecular level, *M. stadtmanae* RNA was identified as the microbe-associated molecular pattern (MAMP) that can trigger an NLRP3 inflammasome activation through the toll-like receptor 8 (TLR8) (23). Interestingly, *M. stadtmanae* was more prevalent in patients with inflammatory bowel syndrome which developed a specific IgG response to this methanogen (22). More recently, a decrease in the *Methanosphaera* abundance was noticed in healthy to long-time diabetic patients, whereas an increase in *M. smithii* was observed (24).

Although *M. stadtmanae* was for a long time the sole *Methanosphaera* member cultured from human feces (5) and further detected by PCR-based methods in human dental plaque (25), a new human isolate, *Methanosphaera* sp. PA5 was provided by Hoedt et al. in 2018 (26). Furthermore, *Methanosphaera* has been cultured from mammal guts including *Methanosphaera cuniculi* (*M. cuniculi*) in rabbits (27), *Methanosphaera* sp. WGK6 in Western grey kangaroo (*Macropus fuliginosus*) (28), and *Methanosphaera* sp. BMS in cow guts (26). Recently, Hoedt et al. (26) and Chibani et al. (9) obtained, respectively, 7 and 20 *Methanosphaera* spp. genomes from metagenomic data of mammals (including humans), some of them not corresponding to known isolates. Particularly, among the 20 *Methanosphaera* spp. genomes provided by Chibani et al. from the human gut, 17 were related to *M. stadtmanae*, two to *M. cuniculi*, and one to a genome obtained from cow rumen, suggesting possible host transfers and adaptation from animals to human gut.

These cumulative data suggested that human health interest, host-associated, and yet-uncultured *Methanosphaera* members remained to be discovered by isolation and culture in the human gut microbiota, subject to the opportunity to apply renewed culture approaches to suitable clinical materials. Using a recently designed hydrogen-free and carbon dioxide-free medium named GG medium (29, patent FR 23 01404), combined with molecular approaches, our investigations of mammal and human feces led to the discovery of a novel *Methanosphaera* representative isolate.

RESULTS

Isolation and microscopic observations

Methane was detected from one methanogen 16S rRNA gene PCR-positive stool sample culture. Successive transfers and subcultures on a solid medium yielded colonies forming a translucent carpet (Fig. S1), which was further subcultured in a liquid medium and produced methane after a 15-day incubation at 37°C. Colonies comprised of Gram-positive diplococci and clumps, with the individual diameter measured at 850 nm using electron microscopy. Gram variability was observed after 15 days of culture. These non-motile cocci showed auto-fluorescence at 420 nm using confocal microscopy. The purity of the final culture was established as only autofluorescent cocci were observed in confocal microscopy from several fields as well and only cocci were observed in electron microscopy. Furthermore, we checked that no bacteria grew on a COS agar plate (Fig. 1)

Genome assembly and analysis

The methanogen 16S rRNA gene sequence determined after PCR sequencing exhibited 100% coverage and 95.77% identity with the homologous sequence of *M. cuniculi* (NCBI accession number: NR_104874.1), and 100% coverage and 95.6% identity with the homologous sequence of *M. stadtmanae* (NCBI accession number: NR_074323.1),

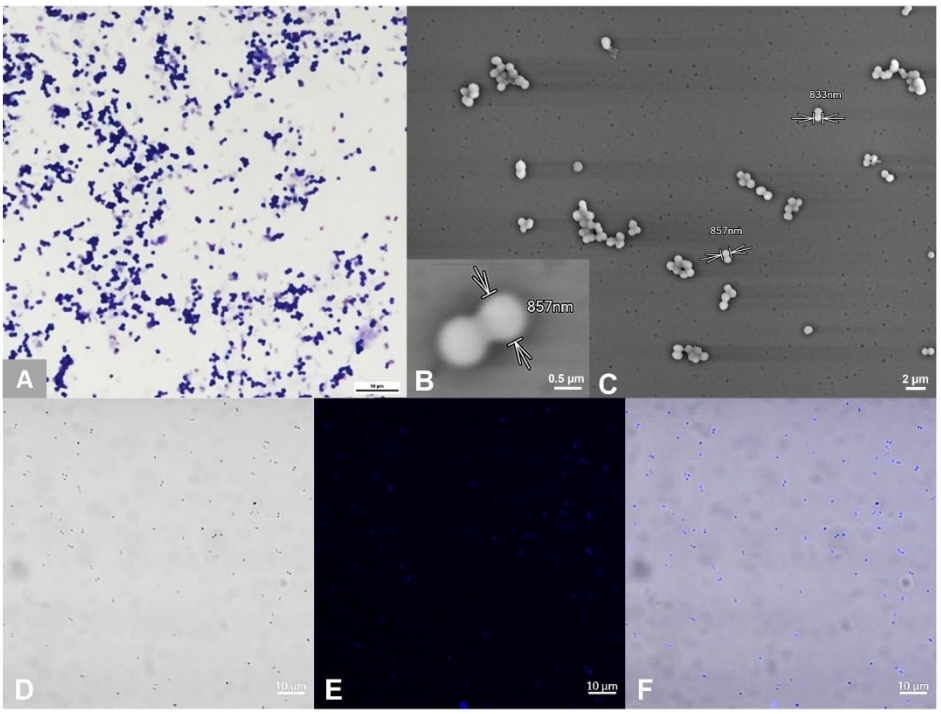

FIG 1 Microscopy features of *Candidatus* methanosphaera massiliense sp. nov. (A) Gram coloration, *Ca.* M. massiliense appears as Gram-positive cocci, diplococci, or clump. (B) Electron microscopy (TM4000 HITACHI, 10 KV, ×10,000), zoom on *Ca.* M. massiliense diplococcus. (C) Electron microscopy (TM4000 HITACHI, 10 KV, ×2,500), showing a large view of *Ca.* M. massiliense culture, the methanogen appears as cocci, diplococci, and clumps. (D) Confocal microscopy, brightfill mode view of *Ca.* M. massiliense culture (LSM 900 [Carl Zeiss Microscopy GmbH]). (E) Confocal microscopy, autofluorescence at 420 nm: all the cocci, diplococci, and clumps are autofluorescent (LSM 900 [Carl Zeiss Microscopy GmbH]). (F) Confocal microscopy merged view of autofluorescence at 420 nm and bright fill mode (LSM 900 [Carl Zeiss Microscopy GmbH]).

suggesting a novel *Methanosphaera* species whose genome was then sequenced. The combined assembly of Illumina and Nanopore data generated two contigs totaling 1,785,773 base pairs (bp) close to the 1,767,403 bp genome sequence size of *M. stadtmanae* (NCBI accession number: GCA_000012545.1) whose dDNA-DNA Hybridization (dDDH) was 23.5%. CheckM completeness was 97.6% without contamination. Genome annotation yielded an 84.5% coding ratio for 1,690 protein-encoding genes with 1,232 assigned to Clusters of Orthologous Groups (COGs) (Table S1), 42 tRNA-encoding genes, 4 rRNA, and 2 CRISPRs (Table 1; Fig. 2). The nucleotide composition yielded 29.7% GC content with 0% gaps. The 16S rRNA gene sequence from the whole genome shared 97.22% sequence similarity (100% coverage) with *M. stadtmanae*, 96.95% (100% coverage) with *Methanosphaera* sp. BMS, and 96.55% (99% coverage) with *M. cuniculi* and 98.9% (92% coverage) with *Methanosphaera* sp. WGK6. The 16S rRNA sequence-based phylogenetic tree showed that the new methanogen clustered with only host-associated sequences and not with environmental ones, it also first clustered with uncultured archaeon clones from pig feces (accession n°: HM573447.1 and HM573413.1) and *Methanosphaera* sp. WGK6 16S rRNA (accession n°: KF697728.1) (Table S2; Fig. 3). Whole-genome sequence (WGS)-based phylogeny incorporating 37 genomes including *Methanosphaera* genomes recovered from Chibani et al. and Hoedt et al. (Table S3) indicated the methanogen was distant from the cultured species, *Methanopshaera* sp.

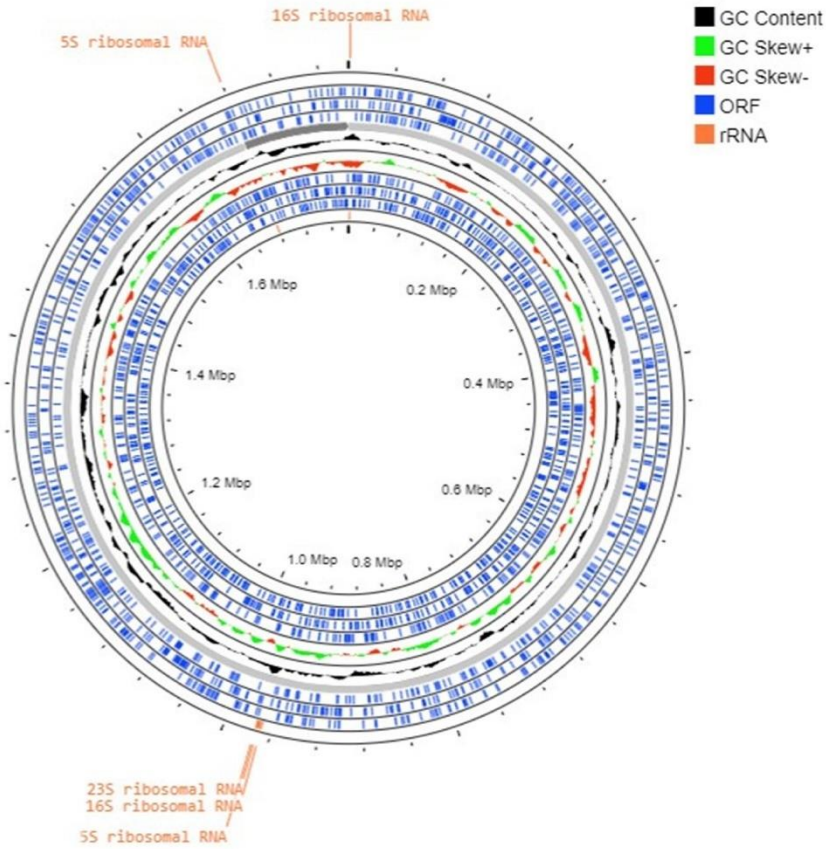

FIG 2 Circular representation of *Candidatus* methanosphaera massiliense sp. nov. genome. Genome representation was performed using the Proksee platform with standard parameters (version 1.0.0) (https://proksee.ca/). The two contigs are presented with color-coded highlights to distinguish important genomic features and provide information about the composition and structure of the *Candidatus* Methanosphaera massiliense sp. nov. genome. ORF regions are indicated in blue, rRNA genes in orange, GC content in black, and positive and negative GC skew in green and red, respectively. This analysis reveals the distribution of protein-coding genes and the variation in GC content across the genome.

WGK6 (average nucleotide identity [ANI], 79%) isolated from Western grey kangaroo gut, followed by *M. stadtmanae* (ANI, 78%) isolated from human gut, *M. cuniculi* (ANI, 75%) isolated from rabbit gut and *Methanosphaera* sp. BMS, a bovine species (ANI, 75%) (Fig. 4). However, it clustered with *Methanosphaera* sp. RUG761 from bovine (ANI, 99%) and *Methanosphaera* sp. M5 from humans with colorectal cancer (ANI, 99%), two genomes that clustered together (ANI, 99%), and with *Methanosphaera* sp. SHI1033 from sheep (ANI, 97%) (Fig. 4). The dDDH values with these three species were above 70% whatever the DDH calculation method (Table S3). Following these phylogeny data and culture isolation, we introduced a new methanogen species named *Candidatus* Methanosphaera massiliense (*Ca.* M. massiliense) sp. nov. (strain: BB6), "massiliense" is for "Marseille," the city where it was isolated. It was deposited into the international public collection CSUR WDCM 875 (Collection Souches Unité des Rickettsies, WDCM 875, Marseille, France) under the reference Q7470 and sent to the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures.

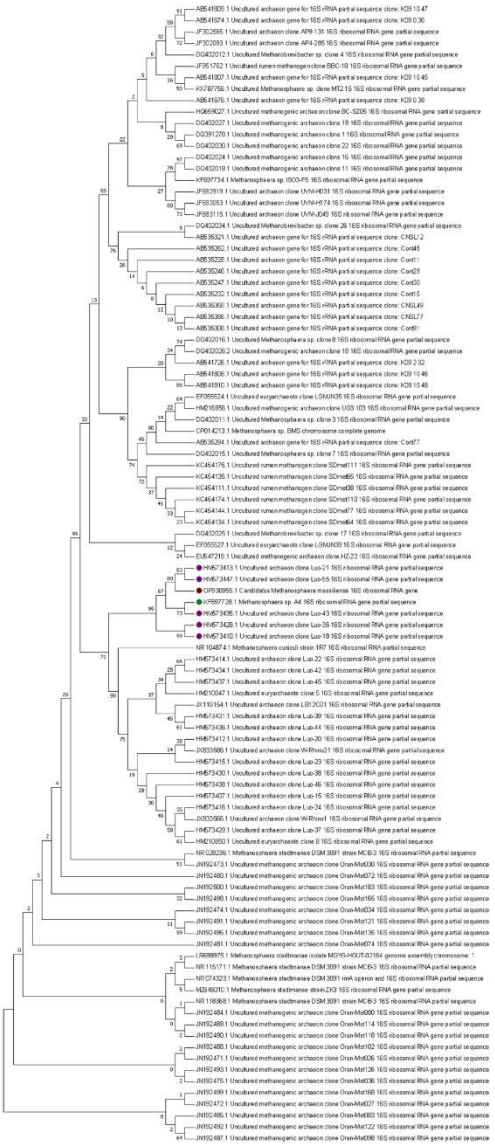

FIG 3 Maximum likelihood phylogenetic tree based on 16S rRNA sequence analysis of *Candidatus* Methanosphaera massiliense sp. nov. and the first 100 hit blasts downloaded from NCBI GenBank database (03-01-2023). The 16S rRNA sequence-based phylogenetic tree showed that *Candidatus* Methanosphaera massiliense sp. nov. (red) first clustered with uncultured archaeon clones from pig feces (green) and *Methanosphaera* sp. from western grey kangaroo (purple).

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry yielded reproducible peptide spectra but no identification by spectra comparison with our bacterial database. The cell mass was first confirmed through PCR sequencing to match with BB6 isolate before MALDI-TOF analysis. Comparison with *M. stadtmanae* DSM3091 peptidic profile did not show significant differences within the two spectra (Fig. S2).

Antibiotic susceptibility

Antibiotic susceptibility testing by macrodilution indicated that the methanogen was *in vitro* resistant to amoxicillin (minimum inhibitory concentration [MIC] > 100 mg/L], imipenem (MIC > 100 mg/L), fusidic acid (MIC > 4 mg/L), and bacitracin (MIC > 4 mg/L), and *in vitro* susceptible to metronidazole (MIC <1 mg/L) and chloramphenicol (MIC < 4 mg/L); while all antibiotics were active as expected against the appropriate *Escherichia coli* and *Staphylococcus aureus* controls (Table S4).

pH susceptibilily

pH susceptibility testing showed that isolate BB6 is also able to grow at pH 6 and pH 7.3 but not out of this range. Subcultures from these two pH conditions yielded to methane production after a 7-day culture. The pH of 7.3 in the GG medium was considered the reference pH for the methanogen culture (Table S4).

Substrate affinity testing and methanogenesis pathways

Ca. M. massiliense was able to grow on GG0 medium (basal GG medium) with methanol under $H_2/CO_2/N_2$ atmosphere, GG0 medium with methanol and ethanol under nitrogen atmosphere, and GG medium (containing acetate, formate, and methanol) supplemented with ethanol as the reference condition (Table S4). Proxi-growth curves of methane production and optical density from day 0 to day 7 are also available in Table S4. Slow growth was observed on methanol alone. No methane was detected with no substrate, under the $H_2/CO_2/N_2$ atmosphere and no other substrate, on formate, acetate, ethanol alone, or ethanol under the $H_2/CO_2/N_2$ atmosphere (Table S4). Genome annotation revealed the presence of a NADPH-butanol dehydrogenase and an aldehyde dehydrogenase closely related to *Methanosphaera* sp. SHI1033 and *Methanosphaera* sp. WGK6 (Figures S3a through d), which allows growth in a hydrogen-free atmosphere in a medium containing methanol and ethanol. The absence of formate dehydrogenase and the presence of the incomplete enzymatic pathway for acetoclastic methanogenesis, only the acetyl-CoA synthetase is encoded, are consistent with the previous experimental results.

TABLE 1 *Candidatus* Methanosphaera massiliense sp. nov. genome sequence characteristics (accession number: JARBXM000000000)

Total sequence length (bp)	1,785,773
Number of sequences	2
Longest sequences (bp)	1,679,591
N50 (bp)	1,679,591
Gap ratio (%)	0
GC content (%)	29.7
Number of CDSs	1,690
Average protein length	297.6
Coding ratio (%)	84.5
Number of rRNAs	4
Number of tRNAs	42
Number of CRISPRs	2
CheckM completeness (%)	97.6
CheckM contamination (%)	0

Feces screening by specific RT-PCR and amplicon sequencing

The specificity of the *Ca.* M. massiliense RT-PCR system was confirmed as all methanogen strains including *Methanobrevibacter* and *Methanosphaera* species and negative controls remained negative but was positive for *Ca.* M. massiliense, while the *Methanobrevibacter* and *Methanosphaera* strains yielded positivity for the 16S rRNA gene (Table S5). Moreover, the RT-PCR system was *in silico* positive for *Ca.* M. massiliense, *Methanosphaera* sp. Mb5 and RUG761 but not for *Methanosphaera* sp. SHI1033 and WGK6 nor *M. stadtmanae*. Then, a total of 150 leftover human stool samples and 313 ground-collected feces from six mammal species located in four French departments (Fig. S4) were investigated using a *Ca.* M. massiliense -specific RT-PCR system, with a Ct value <37 considered as positive. One of 150 human fecal samples (0.67%), 14/64 (22%) pig fecal samples, and 6/50 (12%) red kangaroo fecal samples were positive by RT-PCR for *Ca.* M. massiliense while the other mammal samples remained negative (Table S6a). The one

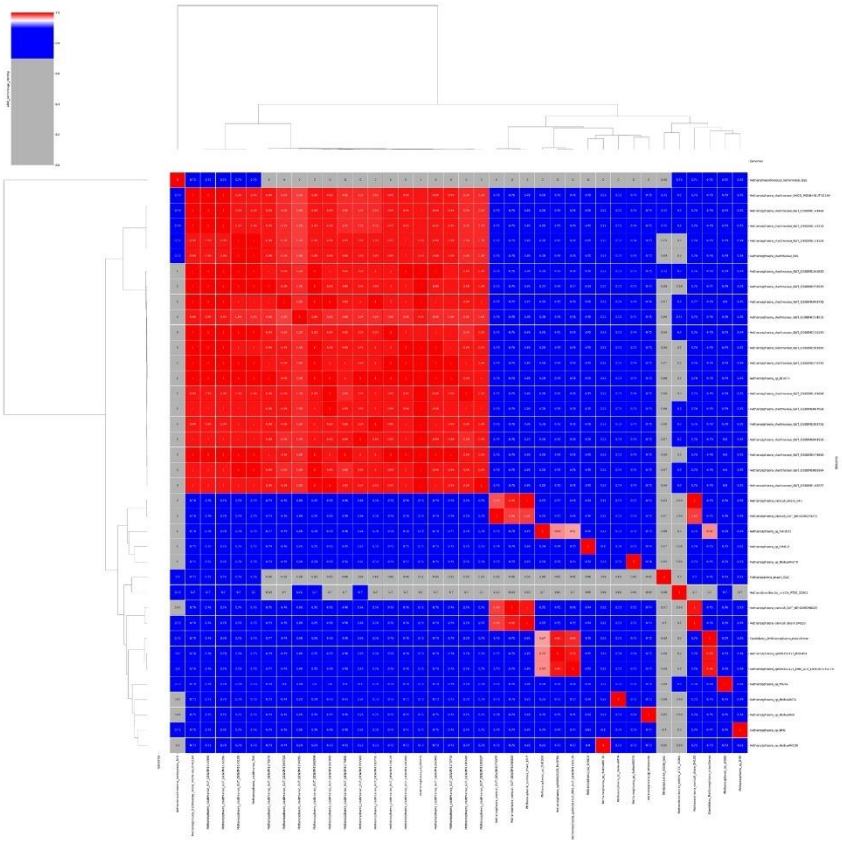

FIG 4 Heatmap phylogeny of *Candidatus* Methanosphaera massiliense sp. nov. and closely related methanogen species generated with ANI values calculated using PYANI software version (0.2.7) with standard parameters. *Ca.* M. massiliense was distant from the cultured *Methanosphaera* species, *Methanopshaera* sp. WGK6 (Average Nucleotide Identity (ANI), 79%) isolated from Western grey kangaroo gut, followed by *M. stadtmanae* (ANI, 78%) isolated from human gut, *M. cuniculi* (ANI, 75%) isolated from rabbit gut and *Methanosphaera* sp. BMS, a bovine species (ANI, 75%) but it clustered with *Methanosphaera* sp. RUG761 (ANI, 99%) detected in bovine and *Methanosphaera* sp. M5 (ANI, 99%) obtained from a man with colorectal cancer, the two genomes clustered together (ANI, 99%), and with *Methanosphaera* sp. SHI1033 (ANI, 97%), from sheep.

positive human feces sample was the same as *Ca*. M. massiliense had been isolated by culture as above. *Ca*. M. massiliense identity in all positive samples was confirmed as the sequences showed an identity average of 96.28% ± 0.82 and 100% coverage with *Ca*. M. massiliense whole-genome sequence and no other correspondence was found in the GenBank database (Table S6b). Moreover, *Ca*. M. massiliense load was comparable in the human sample (3,77E+07 CFU/mL) and in pig and kangaroo samples (average load 2.68E+07 CFU/mL ± 1.25E+07) (Table S6a).

DISCUSSION

Ca. Methanosphaera massiliense is the second *Methanosphaera* species isolated in humans and described using a polyphasic approach combining morphology by light and electron microscopy, peptide fingerprinting by mass spectrometry, antibiotics, pH susceptibility, substrate affinity testing, and whole-genome sequence-derived analyses, with the negative results of controls introduced in all experiments confirming the authenticity of the results.

Whole-genome sequence and 16S rRNA gene sequence data confirmed the originality of *Ca*. M. massiliense's identity among isolated species but *Ca*. M. massiliense might be the cultured representative of *Methanosphaera* sp. RUG761 and *Methanosphaera* sp. M5, a methanogen detected in bovine and a man with colorectal cancer (9).

Among isolated species, *Ca*. M. massiliense was found to be most closely metabolically related to *Methanosphaera* sp. WGK6, a methanogen isolated by culture in one Western grey kangaroo (28). Both methanogens can grow on methanol and hydrogen but also in a hydrogen-free medium containing methanol and ethanol, as they share NADPH-butanol dehydrogenase and aldehyde dehydrogenase (28), GG medium supplemented with ethanol should be suitable for growing these methanogens. A distinctive feature of *M. stadtmanae*, *M. cuniculi,* and *Methanosphaera* sp. BMS is that they have only a hydrogen-dependent methanol-fueled metabolism (26, 27, 30).

The enzymes identified through genome sequencing and annotation play a crucial role in characterizing the population susceptible to the presence of *Ca*. M. massiliense in the gut microbiota. This population may consist of individuals who have had exposure to ethanol and methanol, both of which can originate from either bacterial and fungal sources or consumable items like beverages and food products (31–33). Indeed, ethanol and methanol are both chemical compounds found in various sources that can be consumed by humans. Ethanol is primarily found in alcoholic beverages such as beer, wine, and spirits (31). In addition, ethanol can be used as an additive or solvent in certain foodstuffs (32). Methanol can be present in foods due to natural fermentation processes or the breakdown of pectin in certain fruits and vegetables (33). Alcoholic beverages containing ethanol may also contain low levels of methanol, although these are generally considered safe for human consumption (34, 35). Also, the fact that *Ca*. M. massiliense only remained alive in a narrow neutral pH further suggesting that individuals lacking gastric acidity may favor the implantation of *Ca*. M. massiliense in their gut, as well as individuals exposed to large *Ca*. M. massiliense inoculum (36).

Considering the findings from this study, we propose a hypothesis suggesting that potential sources of *Ca*. M. massiliense may be of mammalian zoonotic origin, as indicated by the observed prevalence of *Ca*. M. massiliense in pigs destined to slaughterhouse. This species has also been identified in other animals, including those with rumen, cow, sheep, and human hosts (9). Fundamentally, our research suggests that this methanogenic archaeal species is not confined to a single host type and does not exhibit host-specific lineages. This implies that food consumption and other contact with animals could serve as pathways for the transmission of these microorganisms to the human intestinal microbiota (37). The success of crossing implantation in individuals of other mammal species, including humans, may depend on the density of contact between species and individual-specific factors. Notably, other methanogens, particularly *Methanobrevibacter* spp. and *Methanomassiliicoccus* spp., have been detected in both humans and other mammals (38–40), such as pigs: *M. smithii*, *Methanobrevibacter*

millerae (*M. millerae*), and *M. luminyensis* (38). This finding underscores the intricate nature of microbial interactions within diverse host environments, emphasizing the need for additional research to comprehend the dynamics of microbiota exchange and its potential implications for human and animal health.

In conclusion, we are reporting the second isolation by culture of a *Methanosphaera* species from human feces referred to as *Ca*. M. massiliense sp. nov., which was obtained using a home-made specific culture GG medium under a nitrogen atmosphere. It is genetically more closely related to mammalian animals *Methanosphaera* than to *M. stadtmanae*. Our findings raise the question of potential zoonotic sources and the conditions of adaptation to a minority of individuals of *Ca*. M. massiliense and methanogens in general and their possible consequences to human health.

MATERIALS AND METHODS

Specimen collection

This prospective study was conducted from August 2021 to September 2021 at the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in Marseille, France. In this study, only leftover stool samples were studied after the microbiological diagnosis laboratory work was completed and after the investigators had verified that no patient objected to the use of leftover clinical samples for research purposes (Article L1211-2 of the French Public Health Code). According to French law, the research on anonymized leftover stool samples used in this study was considered non-interventional research (Article L1221-1.1 of the French Public Health Code) and did not require any ethics committee approval. In addition, we used animal fecal samples collected on the ground. No ethical approval was required for these samples, for which the mammal owners' oral consent was obtained by two of us (B.D. and B.L.). Samples were screened for methanogens presence using RT-PCR (primers: Metha_16S_2_MBF, 5′-CGAACCGGATTAGATACCCG-3′ and Metha_16S_2_MBR, 5′-CCCGCCAATTCCTTTAAGTT-3′; probe: FAM_Metha_16S_2_MBP, [FAM]-5′-CCTGGGAAGTACGGTCGCAAG-3′) as previously described (41).

Culture and isolation of the new methanogen species

A 0.5 g 16S rRNA RT-PCR-positive stool sample was suspended in 10 mL of Dulbecco's phosphate-buffered saline (DPBS) 1× water (Thermo Fisher Scientific, Waltham, MA, USA) and vortexed for a few seconds. A 200 µL volume of this suspension was inoculated into a Hungate tube containing GG medium (29, patent FR 23 01404) that is derived from SAB medium (42), containing acetate, formate, and methanol as standards, previously degassed for 3 minutes with 2 bar nitrogen. The inoculated tube was incubated for 7 days at 37°C. On day 7, methane was detected by gas chromatography using a Clarus 580 chromatograph (Perkin Elmer, Waltham, MA, USA) confirming methanogen growth as previously described (42) and optical density was measured by putting the Hungate tubes in a spectrophotometer cell density meter model 40 (Thermo Fisher Scientific, Waltham, MA, USA). Then, 200 µL of growing culture was inoculated into a fresh GG medium containing fresh antibiotics, 100 mg/L amoxicillin, 100 mg/L vancomycin, 100 mg/L imipenem, 50 mg/L daptomycin, and 50 mg/L amphotericin B (Sigma Aldrich, Saint Quentin Fallavier, France) and supplemented with ethanol at 2% (vol/vol). Then, 100 µL of the growing culture was inoculated on the same home-made solid GG medium obtained by adding 15 g/L agar (Condalab, Madrid, Spain) to the broth formulation of the GG medium with ethanol which was then incubated at 37°C in an anaerobic atmosphere using BD Pouch Gaspak Ez Anaerobe System (Becton, Dickinson and Company, Franklin Lakes, USA) for 9 days. Negative controls consisted in the inoculation of a solid medium with PBS, the liquid medium previously inoculated with PBS for 7 days (Fig. 1). The isolate was sub-cultured 10 times to ensure the purity of the culture, checked by autofluorescence using a confocal light microscope LSM 900 (Carl Zeiss Microscopy GmbH, Jena,

Germany) at the 63× objective with immersion oil as previously described (43), electron microscopy with the Scanning Electron Microscope TM4000 plus (Hitachi, Tokyo, Japan), and inoculation of 100 µL on Columbia agar 5% sheep blood (COS) media (bioMérieux, Marcy-Étoile, France) plate.

Light microscopy

Gram staining was performed as previously described (44). For confocal microscopy, a 10 µL volume from the pure culture was placed on a glass slide and sealed with a coverslip under an anaerobic chamber. Auto-fluorescence was observed under a confocal light microscope LSM 900 (Carl Zeiss Microscopy GmbH, Jena, Germany) at the 63× objective with immersion oil as previously described (43).

Electron microscopy

A 100 µL volume of inoculated culture broth was mixed with 100 µL of 4× glutaraldehyde and then stirred for 30–60 minutes at room temperature. A 20 µL volume of 10% phosphotungstic acid was added and stirred again for 5 minutes, deposited on a Cytospin (Thermo Fisher Scientific, Waltham, MA, USA), and centrifugated for 7 minutes at 800 rpm. Slides were read with the Scanning Electron Microscope TM4000 plus (Hitachi, Tokyo, Japan). Micrographs were acquired at high magnifications ranging from 2,500× to 10,000× with an accelerating voltage of 10 kV using the Backscatter Electron detector under the high vacuum mode.

Whole genome sequencing

DNA was extracted from 200 µL of culture as previously described (45) using the EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France) after overnight incubation at 56°C with 20 µL proteinase K (Qiagen). Glass powder was added to the mix, incubated for 20 minutes at 100°C and immediately vortexed 90 s at 6.5 m/s with the FastPrep instrument (MP Biomedical Europe, Illkirch, France). The mix was centrifuged for 5 minutes at 17,000 × *g* and DNA was extracted from 200 µL of supernatant and eluted in a 50 µL volume. The methanogen identification was first done by partial 16S rRNA gene PCR-amplification followed by Sanger sequencing (primers: SDArch0333aS15, 5′-TCCA GGCCCTACGGG-3′ and SDArch0958aA19, 5′-YCCGGCGTTGAMTCCAATT-3′) as previously described (46). Generated reads were assembled by ChromasPro software (version 1.34), and then aligned to the NCBI GenBank database using the Blast platform. DNA was then engaged for WGS using the MiSeq Illumina pair-end protocol (Illumina, San Diego, CA, USA) and Oxford Nanopore single-long reads (Oxford Nanopore Technologies, Oxford, UK) platforms as previously described (47, 48). Quality control of NGS data was done using the fastQC command on the Galaxy Europe online platform (https://usegalaxy.eu/). Illumina and Nanopore reads were concatenated and *de novo* assembled using Spades software version (version 3.15.4) and generated contigs were blasted against the NCBI GenBank database. The quality of genome assemblies was checked using CheckM (49). The ANI analysis was performed with the 38 genomes included in this study (Table S3) using PYANI software version (0.2.7) with standard parameters, and genome sequences with >95% identity corresponding to the same species (50). dDDH (DNA-DNA hybridization) values were calculated using the TYGS [Type (Strain) Genome Server] online tool (51) based on the 38 included genome sequences. The threshold for DDH values indicating membership in the same species was above 70% (52). Phylogenetic analysis based on WGS-derived 16S rRNA gene sequences with the 100 hit-blasts was performed using Neighbor-Join and BioNJ algorithms standard parameters on MEGA software (version 7.0.26). The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model and 1,000 replicates bootstrap consensus. Branches corresponding to partitions reproduced in less than 90% of bootstrap replicates are collapsed. The initial tree was automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated

using a JTT model, then selecting the topology with the superior log likelihood value. All positions containing gaps and missing data were eliminated, and a total of 1138 positions were included in the final data set (Fig. 3). Genome representation was performed using the Proksee platform with standard parameters (version 1.0.0; https://proksee.ca/). The genome was annotated on the DDBJ Fast Annotation and Submission Tool online platform (https://dfast.ddbj.nig.ac.jp/) and putative enzymes involved in the utilization of different substrates for methanogenesis were sought. Phylogenetic trees of NADPH-dependent butanol dehydrogenase and aldehyde dehydrogenase gene sequences and protein sequences were obtained using Neighbor-Join and BioNJ algorithms standard parameters on MEGA software (version 7.0.26). The evolutionary history was inferred using the maximum likelihood method based on the JTT matrix-based model (Figures S3a through d). Blastp was used against the COG database to find putative encoded protein functions for *Ca*. M. massiliense and other methanogens (Table S1).

MALDI-TOF mass spectrometry

Protein extraction was performed as previously described (53) from 1 mL of pure liquid cultures of the new isolate and *M. stadtmanae* DSMZ3091 (CSUR P9634). Two deposits were made for each sample and peptide profiles were determined using an Autoflex II mass spectrometer equipped with a 337 nm nitrogen laser (Brüker Daltonics, Bremen, Germany). Protein extract of *E. coli* DH5α (Brüker Daltonics) was used as a positive control and non-inoculated medium as a negative control. Spectra were recorded in the positive linear mode for masses ranging from 2 to 20 kDa (parameter settings: ion source 1 [IS1], 20 kV; IS2, 18.5 kV; lens, 7 kV). A spectrum was obtained after 675 shots with a variable laser power. The software tool AutoXecute acquisition control was applied for the automated data acquisition. Peptidic spectra analysis and comparison were performed using FlexAnalysis 2.4 software (Brüker Daltonics).

Antibiotic susceptibility testing

Antibiotic susceptibility was tested using a previously described macrodilution technique and based on *M. stadtmanae* susceptibility (54). Amoxicillin, imipenem, metronidazole, bacitracin, fusidic acid, and chloramphenicol (Sigma Aldrich, Saint Quentin Fallavier, France) were dissolved in sterile water and added separately to the broth medium to obtain a final concentration of 100 mg/L for amoxicillin and imipenem, 1 mg/L for metronidazole, 4 mg/L for chloramphenicol and 4 mg/L for bacitracin and fusidic acid. For each antibiotic, two Hungate tubes containing 4.5 mL of liquid medium were inoculated with 10^5 colony-forming units (CFU)/mL of methanogen, and one control tube was inoculated with 500 µL of PBS. Two Hungate tubes containing 4.5 mL of liquid medium without antibiotics inoculated with 10^5 CFU/mL of methanogen were used as positive controls. Culture tubes were incubated for 5 days at 37°C in an anaerobic atmosphere and were monitored using gas chromatography at day 0 (D0) and day 5 (D5). 100 µL was sampled from the headspace of the tube and injected into a Clarus 580 chromatograph (Perkin Elmer, Waltham, MA, USA). The so-called "sensitive" method was used and the resulting area under the curve values and their conversion to molar concentration (mol/L) were used to quantify methane production. The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration that inhibited methane production. The following controls were performed for each antibiotic to verify its activity: *E. coli* CSUR (Collection Souches Unité des Rickettsies, Marseille, France) Q6942 and *S. aureus* CSUR Q7280 whose antibiotic susceptibility was previously determined, were each seeded on two Columbia agar 5% sheep blood (COS) media (bioMérieux, Marcy-Étoile, France) and two additional COS media were inoculated with the same antibiotic solution used for each experiment, two non-seeded COS media were inoculated with the antibiotic solution and two others with PBS.

pH susceptibility testing

Five pH conditions were tested after the pH of the liquid culture medium was adjusted to pH 2, 4, 6, 8, and 10 using HCl or KOH (Sigma Aldrich, Saint Quentin Fallavier, France). For each condition, two Hungate tubes containing 4.5 mL of liquid culture medium were inoculated with 10^5 CFU/mL of the methanogen, and one Hungate tube was inoculated with PBS as a negative control. Positive controls consisted of two Hungate tubes containing liquid medium at pH 7.3 inoculated with 10^5 CFU/mL. The culture tubes were incubated for 7 days at 37°C in an anaerobic atmosphere and were monitored using gas chromatography at day 0 (D0) and day 7 (D7) as described above. The methanogen was considered susceptible to a pH value when no methane was detected after a 7-day incubation. Methane-positive cultures were then subcultured in GG medium at pH 7.3 and 37°C for 7 days to ensure the viability of the methanogen.

Substrate affinity testing

A basal GG medium (GG0) derived from the original GG medium but without acetate, formate, methanol or ethanol was used, and each substrate or substrate combinations were added separately in the medium GG0. Nine conditions were tested using GG0 medium, six under nitrogen atmosphere: no substrate, acetate (2 g/L), formate (2 g/L), methanol (2% vol/vol), ethanol (2% [vol/vol]), methanol and ethanol (2% [vol/vol]), and three under 5% H_2/20% CO_2/75% N_2 atmosphere without any substrate, with methanol or with ethanol. For each condition, three Hungate tubes containing 4.5 mL of liquid culture medium were inoculated with 10^5 CFU/mL of the methanogen, and one Hungate tube was inoculated with PBS as a negative control. The culture tubes were incubated for 7 days at 37°C and were monitored using gas chromatography at day 0 (D0) and day 7 (D7) as described above.

Human and animal faeces molecular screening

The prevalence of the methanogen was assessed in 150 human and 313 animal stool samples using a real-time (RT) PCR assay, with the fecal samples coming from 52 dogs, 20 sheep, 75 horses, 52 cows, 50 red kangaroos, and 64 pigs (Fig. S4). DNA was extracted as described above from 0.5 g put in 500 µL of G2 buffer (Qiagen, Courtaboeuf, France). We used phylogeny and annotation of the methanogen genome sequence to investigate and identify a candidate sequence for RT-PCR screening of a large stool panel from humans and animals. Because the 16S rRNA-encoding gene was not informative for the screening, the DNA polymerase encoding gene was targeted for primer design. Using the Primer3 program (https://primer3.org/), an in-house RT-PCR system was designed, targeting 124 bp of *Ca.* M. massiliense polymerase encoding gene including a 6-carboxyfluorescein [FAM]- 5′-AGCACGTATGACTACAGGACA-3′ probe, forward primer (5′-AACACCGCTTACTGTTGCAC-3′) and reverse primer (5′-ACTCGTCCGTAT CTGGCTTTA-3′). RT-PCR specificity was *in silico* tested by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) using each primer alone and *in-silico* amplified using both primers and the specific probe, particularly against *Methanosphaera* sp. Mb5, RUG761, SHI1033, WGK6 genome sequences, using Amplify4 software (version 1.0, Bill Engels, University of Wisconsin). The RT-PCR amplification program was 95°C for 15 minutes, followed by 39 cycles of 95°C for 30 s and 60°C for 1 minutes, and a final step of 40°C for 30 s. RT-PCR validation was done in a 20 µL volume containing 5 µL DNA, 20 µM of each primer, and 10 µM of the specific probe, using the Roche Master Mix according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). To disclose any cross-contamination, 5 µL of DNase/RNase-free distilled water was used as a negative control. The specificity was tested with *M. smithii* Q5488, *M. oralis* Q6268 preserved in the CSUR, and *M. stadtmanane* DSM3091 (CSUR P9634). As a supplementary control, the methanogens 16S rRNA gene was also amplified as previously described to ensure the presence of methanogens (38). A calibration curve was obtained using serial dilution

from 10^8 CFU/mL (0.5 McFarland turbidity standard) to 10^1 CFU/mL and was used to quantify the load of *Ca*. M. massiliense in each sample.

Amplicon sequencing

The amplicons obtained were sequenced according to Sangers' method using the BigDye Terminator cycle sequencing kit DNA (Applied Biosystems, Foster City, USA) with the forward primer (5′-AACACCGCTTACTGTTGCAC-3′) and reverse primer (5′-ACTCGTCCG TATCTGGCTTTA-3′) from our RT-PCR system described above. Sequences were assembled with Chromas Pro software, version 1.7 (Technelysium Pty Ltd., Tewantin, Australia) and compared with sequences available in the GenBank database using the online NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

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DATA AVAILABILITY

All genomic data were submitted to the GenBank NCBI database under accession number OP930955 (16S rRNA sequence) and JARBXM000000000 (genome). The amplicon sequences were also deposited under accession numbers: OX456088; OX456151; OX456152; OX456153; OX456154; OX456155; OX456156; OX456157; OX456158; OX456157; OX456158; OX456159; OX456160; OX456161; OX456162; OX456163; OX456164; OX456165; OX456166; OX456167; OX456168; OX456169; OX456170.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (Spectrum05141-22-s0001.jpg). Hydrogen-free and carbon dioxide-free culture protocol in GG medium for isolation of *Candidatus* Methanosphaera massiliense sp. nov.
Figure S2 (Spectrum05141-22-s0002.jpg). *M. stadtmanae* DSM3091 and *Candidatus* Methanosphaera massiliense sp. nov. MALDI-TOF mass spectrometry spectra.
Figure S3 (Spectrum05141-22-s0003.pdf). Phylogenetic tree based on NADPH-dependent butanol dehydrogenase protein and nucleotide sequence and aldehyde dehydrogenase analysis protein and nucleotide sequence.
Figure S4 (Spectrum05141-22-s0004.jpg). Map of France locating the sources of animal and human fecal samples investigated.
Table S1 (Spectrum05141-22-s0005.xlsx). Clusters of Orthologous Groups (COGs) functional categories assignation and representation of *Ca*. M. massiliense and other methanogens.
Table S2 (Spectrum05141-22-s0006.xlsx). *Candidatus* Methanosphaera massiliense sp. nov. and the first 100 hit blasts downloaded from NCBI GenBank Database.
Table S3 (Spectrum05141-22-s0007.xlsx). Methanogen genomes used in this study.
Table S4 (Spectrum05141-22-s0008.xlsx). Data for pH susceptibility testing, antibiotic susceptibility testing, and substrate affinity testing.
Table S5 (Spectrum05141-22-s0009.xlsx). Testing specificity of the designed RT-PCR system for *Ca*. M. massiliense sp. nov.
Table S6 (Spectrum05141-22-s0010.xlsx). Prevalence of *Ca*. M. massiliense in human and animal faecal samples and amplicon sequencing.

Open Peer Review

PEER REVIEW HISTORY (review-history.pdf). An accounting of the reviewer comments and feedback.

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Chapitre IV: *Methanobrevibacter massiliense*

Préambule du chapitre IV

À la suite de la découverte de *Cand.* M. massiliense grâce au milieu GG® dans un échantillon de selle humaine, nous avons souhaité ré-utiliser le milieu GG® pour cultiver une autre espèce particulièrement fastidieuse appartenant au genre *Methanobrevibacter*, *M. massiliense*.

M. massiliense avait été co-cultivée pour la première fois dans notre laboratoire avec la bactérie *P. piscolens* à partir d'un échantillon de plaque dentaire d'un individu atteint de parodontite sévère (17). Une description sommaire avait été faite et la méthanogène avait été perdue faute de moyen de culture et de conservation durable. Nous sommes donc partis à la recherche de *M. massiliense* dans une nouvelle situation clinique, l'infection endodontique. Notre objectif premier était d'en apprendre plus sur l'implication des méthanogènes dans cette condition pathologique par la culture, les études précédentes n'étant basées que sur des méthodes de biologie moléculaire.

Ayant détecté *M. massiliense* dans 3/20 (15%) pulpes nécrotiques, nous nous sommes finalement concentrés sur l'isolement de cette méthanogène. Toutefois, nos tentatives d'isolement en culture pure se sont révélées infructueuses mais ont montré qu'elle était particulièrement dépendante de *P. piscolens*. En co-cultivant *M. massiliense* avec *P. piscolens* dans le milieu GG® à partir de deux échantillons différents, nous avons réussi de manière reproductible, à maintenir cette méthanogène. Cela nous a permis de l'étudier de manière plus approfondie et donc d'obtenir de plus amples informations sur sa morphologie grâce à son autofluorescence caractéristique des méthanogènes, ainsi que deux génomes de haute qualité grâce au séquençage de nouvelle génération, nous éclairant un peu plus son métabolisme et sa relation avec *P. piscolens*. Particulièrement, nous avons pu obtenir un spectre protéique spécifique de la co-culture qui pourrait servir à identifier plus facilement et plus rapidement *M. massiliense* dans nos cultures.

Par la suite, nous nous sommes intéressés à l'antiquité de la relation entre *M. massiliense* et *P. piscolens*. Nous savions déjà que *M. massiliense* était l'espèce prédominante entre le 14ème et le 18ème siècle, ayant été supplanté par la suite par *M. oralis*. Pour ce travail, nous avons repris les données de métagénomiques utilisées dans une autre étude publiée en 2021 (29). Cette étude relatait l'existence de deux nouveaux phylotypes appartenant au genre *Methanobrevibacter*, appelés TS-1 et TS-2. La similitude de l'arbre phylogénétique de TS-2 et de *M. massiliense* nous a tout d'abord conduit à nous demander si TS-2 et *M. massiliense* correspondaient à la même espèce. Après avoir confirmé cette hypothèse par comparaison des génomes, nous avons réalisé des analyses statistiques de corrélation entre le nombre de reads associés à *M. massiliense* et le nombre

de reads associé à *P. piscolens* sur un total de 97 échantillons de tartre ancien. Là encore notre hypothèse s'est confirmée démontrant l'association durable entre *M. massiliense* et *P. piscolens,* peut être depuis le Néolithique. La compréhension du métabolisme de *P. piscolens*, utilisant uniquement des substrats protéiques et pas les sucres (30), nous éclaire quant à la raison possible du déclin de *M. massiliense* au profit de *M. oralis* au 18ème siècle, période correspondant à la large distribution et consommation du sucre en Europe lors de la révolution industrielle.

Article 5: *Methanobrevibacter massiliense and Pyramidobacter piscolens Co-Culture Illustrates Transkingdom Symbiosis*

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Methanobrevibacter massiliense and *Pyramidobacter piscolens* Co-Culture Illustrates Transkingdom Symbiosis

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Abstract: Among oral microbiota methanogens, *Methanobrevibacter massiliense* (*M. massiliense*) has remained less studied than the well-characterised and cultivated methanogens *Methanobrevibacter oralis* and *Methanobrevibacter smithii*. *M. massiliense* has been associated with different oral pathologies and was co-isolated with the *Synergistetes* bacterium *Pyramidobacter piscolens* (*P. piscolens*) in one case of severe periodontitis. Here, reporting on two additional necrotic pulp cases yielded the opportunity to characterise two co-cultivated *M. massiliense* isolates, both with *P. piscolens*, as non-motile, 1–2-µm-long and 0.6–0.8-µm-wide Gram-positive coccobacilli which were autofluorescent at 420 nm. The two whole genome sequences featured a 31.3% GC content, gapless 1,834,388-base-pair chromosome exhibiting an 85.9% coding ratio, encoding a formate dehydrogenase promoting *M. massiliense* growth without hydrogen in GG medium. These data pave the way to understanding a symbiotic, transkingdom association with *P. piscolens* and its role in oral pathologies.

Keywords: *Archaea*; methanogen; *Methanobrevibacter massiliense*; *Synergistetes*; *Pyramidobacter piscolens*; hydrogen-free culture; dental pulp

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1. Introduction

Methanogenic archaea (methanogens) are constant inhabitants of the human digestive tract microbiota [1], are regularly detected in the oral cavity [2], neonatal meconium, gastric fluid [3–5] and faeces [6], and have been detected in ancient dental calculus samples collected as far back as the Neolithic period [7–10]. The association between methanogens and oral pathologies is ambiguous, as methanogens have been detected in saliva collected from apparently healthy individuals and, more specifically, from tobacco smokers [11] as well as in oral cavity samples collected from patients diagnosed with pericoronitis [12], gingivitis, periodontitis [13–16], peri-implantitis [17–19], endodontic infections [20–24], and dental abscesses [25]. It has been hypothesised that methanogens may contribute to microbial community dynamics, particularly through syntrophic interactions with bacteria. In the context of endodontic conditions, molecular methods have revealed the presence of methanogens, showing a higher prevalence in inflamed pulp [23] compared to necrotic pulp in both primary and secondary infections [20–22,24]. Notably, one study suggested a closer association between methanogens and symptomatic cases [21]. Furthermore, root canal treatment seems to be effective in eliminating methanogens [26]. Notably, none of these studies reported the successful cultivation of methanogens from pulp samples.

Methanobrevibacter smithii (*M. smithii*) and *Methanobrevibacter oralis* (*M. oralis*) are the two main methanogens detected by molecular methods and isolated by culture among the

oral microbiota, with *M. oralis* being the most prevalent [13–16]. However, several studies have reported *M. oralis* like-species, leaving the diversity of the *Methanobrevibacter* genus in the oral microbiota [20,22,23,27] unknown.

Further studies revealed a third oral *Methanobrevibacter* phylotype [13,27], possibly interacting with a *Synergistetes* spp. in periodontitis and endodontic infections [27]. This was further detected as *Methanobrevibacter* sp. N13 in 45% of ancient dental calculus samples, while *M. oralis* was detected in only 20% of the same samples. Thus, *M. oralis* is less prevalent than *Methanobrevibacter* sp. N13 in historical populations than in modern populations [7]. *Methanobrevibacter* sp. N13 was later named *Methanobrevibacter massiliense* (*M. massiliense*) after it was co-isolated with the *Synergistetes Pyramidobacter piscolens* (*P. piscolens*) during an 18-month culture of a leftover dental plaque sample collected from a patient diagnosed with severe periodontitis [28]. Additionally, *M. massiliense* has been identified in Malian patients with oral pathologies [29] in cases of peri-implantitis [17], and in a challenging sinusitis case, where it was the sole microorganism identified [30]. These instances collectively underscore its potential relevance in various human pathological conditions. *M. massiliense*, however, remains an uncharacterised methanogen that may have implications in oral pathologies.

In this study, our aim was first to recover then to cultivate and isolate *M. massiliense* in a novel oral condition, endodontic infections. This provided an opportunity for comprehensive characterisation, including genomic and microscopic features, contributing to a more thorough understanding of this challenging methanogen. Additionally, we sought to assess the prevalence of *M. massiliense* compared to other *Methanobrevibacter* species and to explore potential associations with putative partners, notably among *Synergistetes* spp.

2. Materials and Methods

2.1. Ethical and Regulatory Statement

In this study, teeth were extracted as part of routine clinical practice. No teeth were specifically extracted for the present work. The extracted teeth were leftover clinical material, classified as "waste from healthcare activities", in accordance with the French Public Health Code (Article L1211-2). Investigators orally verified that no patient objected to the use of leftover clinical samples, including extracted teeth, for research investigations. To ensure patient confidentiality and privacy, strict anonymisation procedures were implemented throughout the study so that any personal identifying information was removed from the extracted teeth, which were assigned a unique identification code. Teeth were extemporaneously examined for cavities and pulp vitality. The research team had no access to any patient-related data or information that could potentially compromise the anonymity of involved individuals. Accordingly, this study was approved by the Institut Méditerranée Infection (IHU) Ethics Committee No. 2022-14.

2.2. Sample Collection

A total of 32 molars and premolars were collected from 32 patients as part of routine practice by dentists and dental students under the supervision of a graduate dentist at the Pôle Odontologie Timone (Assistance Publique des Hôpitaux de Marseille) between September 2022 and February 2023. Twenty-two teeth had to be extracted for carious reasons and ten teeth were extracted as wisdom teeth. None were in the state of root debris or were fractured during the extraction (Supplementary Data S1). Each tooth was stored in a 50 mL Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) containing 10 mL of Dulbecco's Phosphate Buffered Saline (DPBS) 1× water (Thermo Fischer Scientific, Waltham, MA, USA) at 4 °C for a maximum of 24 h. Manipulations were carried out under anaerobiosis in an anaerobic chamber to avoid exposing the dental pulp to oxygen (Don Whitley, Bingley, UK). Each tooth was immersed in a 10 mL 20% chlorhexidine digluconate solution (Gilbert Healthcare, Hérouville-Saint-Clair, France) for 15 min, rinsed with DNase/RNase-free distilled water (Thermo Fischer Scientific, Waltham, MA, USA), re-immersed in a 10 mL 70% alcohol solution (Gilbert Healthcare, Hérouville-Saint-Clair,

France) for one minute, rinsed with DNase/RNase-free distilled water, and cleaned with sterile gauze soaked with 3% sodium hypochlorite and then rinsed again before being wiped with a new sterile gauze. Each tooth was then partially and longitudinally sectioned using a sterile diamond disk and fractured using a sterile syndesmotome (Hu-Friedy, Frankfurt am Main, Germany), and the dental pulp was extirpated using a sterile excavator (Hu-Friedy, Frankfurt am Main, Germany), one half being put in an Eppendorf tube containing 200 µL of DPBS 1× for molecular analysis and one half for methanogen isolation by culture.

2.3. Methanogen DNA Detection

DNA was directly extracted from the dental pulp with 200 µL of G2 buffer added, as previously described [31]. The EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France) was used after sample pretreatment, consisting of overnight incubation at 56 °C with 20 µL proteinase K (Qiagen, Courtaboeuf, France), the addition of glass powder, incubation for 20 min at 100 °C, vortexing for 90 s at 6.5 m/s with FastPrep (MP Biomedical Europe, Illkirch, France) and, finally, centrifugation for five minutes at 17,000× *g*. Methanogen was first detected by real-time PCR (RT-PCR) based on the methanogen 16S rRNA gene, as previously described [32]. PCR-positive samples were sequenced by Sanger sequencing of the partial archaeal 16S rRNA gene (primers: SDArch0333aS15, 5′-TCCAGGCCCTACGGG-3′ and SDArch0958aA19, 5′-YCCGGCGTTGAMTCCAATT-3′), as previously described [20]. Generated sequences were assembled using the ChromasPro software (version 1.34), then aligned against the NCBI GenBank database using the Blast platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 March 2023). Only *M. massiliense*-positive samples were selected for further culture and isolation attempts. The presence of *M. massiliense* in the corresponding culture was also confirmed by the same methods.

2.4. Culturing M. massiliense

Dental pulps which tested positive by PCR sequencing for *M. massiliense* were cultured in a Hungate tube containing 5 mL of SAB medium [33] consisting of 100 mg/L amoxicillin, 100 mg/L vancomycin, 100 mg/L imipenem, 50 mg/L daptomycin and 50 mg/L amphotericin B (Sigma Aldrich, Saint Quentin Fallavier, France), in a 5% hydrogen, 10% CO_2, 85% nitrogen atmosphere at 37 °C for 15 days. Culture was monitored by methane detection with gas chromatography using a Clarus 580 chromatograph (Perkin Elmer, Waltham, MA, USA), as previously described [33]. *M. massiliense*-positive cultures were subcultured using two conditions: in one condition, 200 µL was inoculated in a Hungate tube containing 5 mL of SAB medium incubated under a 5% hydrogen, 10% CO_2, 85% nitrogen atmosphere, and in the other condition, 200 µL was inoculated in liquid GG medium under a nitrogen atmosphere [34]. Both tubes were incubated at 37 °C for 15 days. Methane was detected at days 5, 10 and 15 as described above, and cultures were finally established in GG medium, as above. In parallel, 200 µL of PBS 1× water was inoculated under the same conditions as the negative controls. To identify bacteria co-culturing with *M. massiliense*, 100 µL of the culture was inoculated on a Columbia agar 5% sheep blood (COS) medium plate (bioMérieux, Marcy-Étoile, France), incubated under an anaerobic atmosphere using GasPak (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37 °C for 15 days. At 3-, 5-, 7-, 10- and 15-day time points, cultures were observed for colonies, which were subsequently cultured and identified using MALDI-TOF mass spectrometry.

2.5. Culture Microscopy

M. massiliense-P. piscolens co-cultures were examined for autofluorescence under a confocal epifluorescence microscope, LSM 900 (Carl Zeiss Microscopy GmbH, Jena, Germany), at the 63 × objective with immersion oil, as previously described [35]. Slides were prepared with 10 µL volume deposit and sealed under an anaerobic atmosphere. For scanning electron microscopy, 100 µL of each culture was added to 100 µL of 4× glutaraldehyde and stirred for 30 min at room temperature. Then, 20 µL of 10% phosphotungstic acid

was added, stirred again for five minutes, and finally was cytocentrifugated for seven minutes at 800 rpm onto a glass slide (Cytospin®, Thermo Fischer Scientific, Waltham, MA, USA). The slides underwent analysis using the TM4000 plus Scanning Electron Microscope (Hitachi, Tokyo, Japan). Micrographs were obtained at high magnifications, ranging from ×1000 to ×2500, employing an accelerating voltage of 10 kV and using the Backscatter Electron (BSE) detector in a high vacuum environment. As a comparison control, *P. piscolens* strains P2428, Q3403, Q3381, Q8451 and Q8452, from both liquid and solid cultures, were microscopically observed, as described above. Moreover, fluorescent in situ hybridization (FISH) was performed directly on pulp sample No. 74 using the archaea-specific Arch915 probe Alexa 488 (5′-GTGCTCCCCCGCCAATTCCT-3′) [36] and the partial 16S rRNA bacterial domain specific EUB 338 probe Alexa 647 (5′-GCTGCCTCCCGTAGGAGT-3′) [37], as previously described [11]. Negative controls consisted of the same manipulation on methanogen 16S rRNA gene PCR-negative dental pulp and on DPBS water 1×. The universal DNA stain DAPI was also added. Slides were also observed with a confocal epifluorescence microscope LSM 900 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at appropriate wavelengths.

2.6. MALDI-TOF Mass Spectrometry Culture

One millilitre of *M. massiliense* co-cultures Q8282 and Q8283 and 1 mL of the five *P. piscolens* strains (P2428, Q3381, Q3403, Q8451 and Q8452, Collection Souches Unité des Rickettsies, CSUR, WDCM 875, Marseille, France), cultured in GG medium, as well as one 5 µL loop resuspended in 1 mL of DPBS 1× water (Thermo Fischer Scientific, Waltham, MA, USA) of the five *P. piscolens* strains (P2428, Q3381, Q3403, Q8451 and Q8452), cultured in COS medium, were collected for protein extraction, as previously described [38]. Two deposits were made for each sample and the protein extract of *Escherichia coli* (*E. coli*) DH5α™ (Brüker Daltonics, Bremen, Germany) was used as a positive control and free matrix as a negative control. Peptide profiles were obtained using an Autoflex II mass spectrometer with a 337 nm nitrogen laser (Brüker Daltonics, Bremen, Germany). The AutoXecute software acquisition control tool (Flex control 3.0, Brüker Daltonics, Bremen, Germany) was applied for automated data acquisition and spectral comparisons were performed using FlexAnalysis 2.4 software (version 2.4, Brüker Daltonics, Bremen, Germany).

2.7. P. piscolens Antibiotic Susceptibility Testing and M. massiliense Isolation Attempts

P. piscolens strains Q8451, Q8452, P2428, Q3403 and Q3381 were tested for tetracycline susceptibility using the tetracycline E-test (bioMérieux, Marcy-Étoile, France). Tetracycline was selected with a view to isolating *M. massiliense* in pure culture from *P. piscolens* co-culture, as methanogens are resistant to tetracycline (MIC > 100 mg/L) [39], whereas *P. piscolens* is susceptible to tetracycline [40]. In detail, *P. piscolens* suspensions (1 McFarland) were subcultured on one COS plate without antibiotic as a growth positive control and one COS plate with tetracycline E-test. A third COS plate was mock-inoculated with 100 µL of DBS 1× water as a negative control. Plates were incubated under an anaerobic atmosphere using the GasPak anaerobic generator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for five days at 37 °C. Furthermore, each *P. piscolens* strain was subcultured (in duplicate) in one Hungate tube containing 5 mL of GG medium under nitrogen atmosphere in addition to 50 mg tetracycline and another containing 100 mg of tetracycline. One additional Hungate tube, mock-inoculated with 100 µL of DBS 1× water, was used as a negative control. Tubes were incubated for five days at 37 °C. In an attempt to isolate *M. massiliense* in pure culture, the *M. massiliense-P. piscolens* co-culture Q8282 was subcultured (in duplicate) in five distinct conditions: 100 µL of a growing culture was inoculated in GG medium as a positive control; in GG medium supplemented with 100 mg tetracycline; in GG medium supplemented with 100 mg tetracycline and 3 g/L of acetate; in GG medium supplemented with 100 mg tetracycline with volatile fatty acids solution [33]; and in GG medium supplemented with 100 mg tetracycline with 3 g/L of acetate and volatile fatty acids solution. All tubes were incubated for three weeks at 37 °C. Negative

controls encompassed the same five conditions, but with the inoculation of 100 µL of DPBS 1× water.

2.8. Whole Genome Sequencing and Analysis

DNA was extracted from 200 µL of each co-culture using the EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France), as previously described [41]. The mixture was incubated overnight at 56 °C with 20 µL of proteinase K (Qiagen), followed by the addition of glass powder and incubation for 20 min at 100 °C. The mixture was then immediately vortexed for 90 s at 6.5 m/s using the FastPrep instrument (MP Biomedical Europe, Illkirch, France). Subsequently, the mixture was centrifuged for five minutes at 17,000× *g* and DNA was extracted from 200 µL of supernatant, eluted in a 50 µL volume. Whole genome sequencing (WGS) was carried out using the MiSeq Illumina pair-end protocol (Illumina, San Diego, CA, USA) and Oxford Nanopore single-long reads (Oxford Nanopore Technologies, Oxford, UK) platforms, as previously described [42,43]. The fastQC command on the Galaxy Europe online platform (https://usegalaxy.eu/, accessed on 1 March 2023) was used to conduct quality control of NGS data. Reads were mapped against the *P. piscolens* reference genome (NCBI accession number: GCF_022846135.1) using CLC software (version 3.15.4, CLC Genomics Workbench QIAGEN Bioinformatics). Mapped reads were assembled to obtain *P. piscolens* genomes. For *M. massiliense* genomes, Illumina and Nanopore reads not mapped on the *P. piscolens* reference genome were de novo assembled using Spades software (Version 3.15.4), and the generated contigs were checked by Blast against the NCBI GenBank database. The quality of genome assemblies was checked using CheckM [44]. Whole genome comparisons were made using PYANI software version (0.2.7) with standard parameters, genome sequences with >95% identity corresponded to the same species [45] (Supplementary Data S4, Table S4.1). The TYGS (Type (Strain) Genome Server) online tool [46] was used to calculate dDDH (DNA-DNA hybridization) values based on the 25 genome sequences included. The threshold for DDH values indicating membership in the same species was above 70% [47]. The obtained genomes were annotated on the DDBJ Fast Annotation and Submission Tool online platform (https://dfast.ddbj.nig.ac.jp/, accessed on 1 March 2023) (Supplementary Data S3, Table S3.2), as well as *M. oralis* and *P. piscolens* reference genomes (accession numbers GCF_912073625.1 and GCF_022846135.1, respectively) and *P. piscolens* genomes from this study (accession numbers CAUZXV01000000 and CAUZXU01000000). Phylogenetic analysis based on WGS-derived 16S rRNA gene sequences with the 32 hit blasts was performed using the Neighbor-Join and BioNJ algorithms standard parameters on MEGA software (version 7.0.26) (Supplementary Data S4, Tables S4.3 and S4.4). The Maximum Likelihood method based on the JTT matrix-based model and 1000 replicates bootstrap consensus was used to infer the evolutionary history. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The initial tree was automatically obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, then selecting the topology with the higher log likelihood value. The final data set comprised 1180 positions.

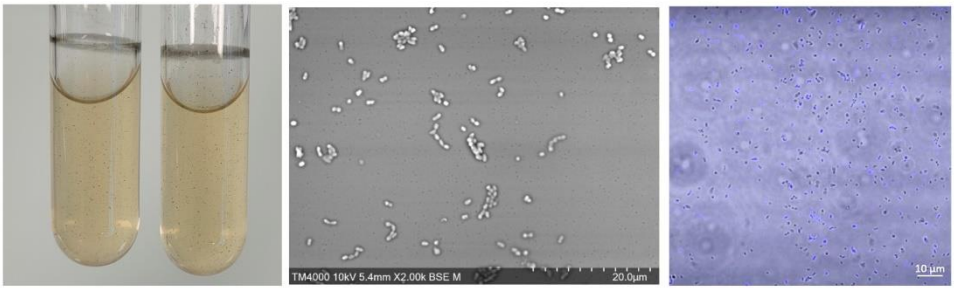
3. Results

3.1. Molecular Identification

RT-PCR detection of methanogens was positive in 11 of the 22 (50%) dental pulp samples collected from carious teeth, but in none of the ten wisdom teeth used as negative controls. Sequencing identified *M. oralis* in 7/11 positives (64%), *M. smithii* in one positive (1/11, 9%), and *M. massiliense* in three samples (3/11, 27%). No. 14, No. 73 and No. 74 first hit blasts, respectively, yielded 98% coverage and 98.78% identity (sample No. 14), 99% coverage and 96.9% identity (sample No. 73), and 99% coverage and 98.29% identity (sample No. 74) with *M. massiliense* (Supplementary Data S1).

3.2. Co-Culture of *M. massiliense* with *P. piscolens*

M. massiliense was successfully cultured from dental pulp samples No. 14 and No. 74 but not from sample No. 73 or negative controls, using the SAB medium as well as the GG medium under nitrogen atmosphere, with methane being increasingly detected from day 0 to day 20 (Supplementary Data S2, Table S1). Subculture in GG medium was achieved in a minimum of three weeks when cultures turned black with black deposits (Figure 1) and methane was detected. In contrast, culturing *P. piscolens* in GG medium under a nitrogen atmosphere yielded no methane, no hydrogen was detected, and no black deposits were observed (Supplementary Data S3, Figure S3.1). In parallel, subculturing GG broth inoculated with samples No. 14 and No. 74 on COS yielded colonies within days, with MALDI-TOF identification of *P. piscolens* (scores > 2). *P. piscolens* was the only bacterium identified at days 3, 5, 7, 10, and 14. Therefore, two *P. piscolens* isolates were deposited in the CSUR under references Q8451 (from sample No. 14) and Q8452 (from sample No. 74) (Table 1). In addition, although the five *P. piscolens* isolates (including Q8451 and Q8452 here reported) were susceptible in vitro to tetracycline on COS medium (MIC = 20 µg/mL) and in GG medium (50 mg/L < MIC < 100 mg/L), nevertheless attempts to isolate *M. massiliense* in pure culture by incorporation of tetracycline proved unsuccessful under all tested conditions (Supplementary Data S2, Table S2.2). Finally, two co-cultures of *M. massiliense* and *P. piscolens* from dental pulp samples No. 14 and No. 74 were added to our international public collection CSUR WDCM 875 (Collection Souches Unité des Rickettsies, WDCM 875, Marseille, France) under the references Q8282 and Q8283, respectively.



(**a**) (**b**) (**c**)

Figure 1. Macroscopic and microscopic features of *Methanobrevibacter massiliense*. (**a**) Macroscopic observation of *M. massiliense* and *P. piscolens* co-cultures, dark deposits are visible. (**b**) Electron microscopy (TM4000 HITACHI, 10 KV, × 1.500), coccobacilli are visible, but no morphologic features made it possible to distinguish between *M. massiliense* and *P. piscolens*. (**c**) Confocal microscopy merged view of autofluorescence at 420 nm and brightfill mode; autofluorescent coccobacilli are visible as well as non-autofluorescent coccobacilli (LSM 900 (Carl Zeiss Microscopy GmbH)).

Table 1. Characteristics of *Methanobrevibacter massiliense* and *Pyramidobacter piscolens* genomes retrieved in this study.

	***M. massiliense* Q8282**	***M. massiliense* Q8283**	***P. piscolens* Q8451**	***P. piscolens* Q8452**
Total sequence length (bp)	1,834,388	2,299,297	2,662,074	2,638,206
Number of sequences	19	15	11	4
Longest sequences (bp)	384,479	551,700	846,238	1,351,082
N50 (bp)	325,131	400,054	616,914	1,351,082
Gap Ratio (%)	0	0	0.001503	0.078728
GC content (%)	31.3	30.6	59.6	59.8

Table 1. *Cont.*

	***M. massiliense* Q8282**	***M. massiliense* Q8283**	***P. piscolens* Q8451**	***P. piscolens* Q8452**
Number of CDSs	1903	2332	2512	2438
Average protein length	275.8	277.9	310.9	315.8
Coding ratio (%)	85.9	84.5	88	87.5
Number of rRNAs	2	4	5	12
Number of tRNAs	28	32	55	57
Number of CRISPRs	3	2	2	3
CheckM completeness (%)	100	97.67	100	100
CheckM contamination (%)	0	4.72	0	0

3.3. Microscopy

FISH detected coccobacilli archaea and bacteria in the *M. massiliense*-positive dental pulp sample No. 74, while bacteria but not archaea were detected in the methanogen-negative sample, and the DPBS 1× water negative control yielded no fluorescence (Figure 2). Confocal microscopy observations of *M. massiliense-P. piscolens* Q8282 and Q8283 co-cultures revealed autofluorescent 1–2-µm-long and 0.5–0.8-µm-wide coccobacilli corresponding to *M. massiliense*, but no fluorescent coccobacilli of the same size at 420 nm (Figure 1). Furthermore, no *P. piscolens* strain was autofluorescent and no autofluorescence was observed in the negative control (Supplementary Data S3, Figure S3.2). Further electron microscopy observation of Q8282 and Q8283 co-cultures and of *P. piscolens* cultured either in liquid or solid medium showed coccobacilli (0.5–0.8 µm wide and 1–2 µm long) often featuring diplo-coccobacilli (Figure 1 and Supplementary Data S3, Figure S3.3). Therefore, morphological observations alone were not sufficiently discriminative to definitively identify coccobacilli as *M. massiliense* or *P. piscolens*. However, we observed dark crystal structures probably corresponding to the previously observed black deposits in both Q8282 and Q8283 co-cultures and not in *P. piscolens* cultures (Figure 1 and Supplementary Data S3, Figure S3.3).

3.4. MALDI-TOF Mass Spectrometry

No interpretable MALDI-TOF spectra were obtained from negative controls, while *E. coli* (positive control) was correctly identified with an identification score of 2.57. Five *P. piscolens* strains cultivated on COS medium were identified with identification scores > 2 and yielded MALDI-TOF-MS spectra identical to the ones derived from culture in liquid GG medium culture, except for one peak which was lacking (average m/z = 8074.28 m/z +/−1.64; average relative intensity = 50.68 arbitrary unity (a.u.) +/−14.29). Further, Q8282 and Q8283 co-cultures yielded identical MALDI-TOF-MS spectra, which were superimposable with those derived from *P. piscolens* cultured on COS and GG broth media, except for two additional peaks (average m/z = 6883.35 m/z +/−1.04; average relative intensity = 93.2 a.u. +/−5.38 and average m/z = 6946.40 m/z +/−1.03; average relative intensity = 79.14 a.u. +/−20.41) and the missing peak reported above, suggesting that these two additional peaks were specific to *M. massiliense* presence in the co-culture (Figure 3).

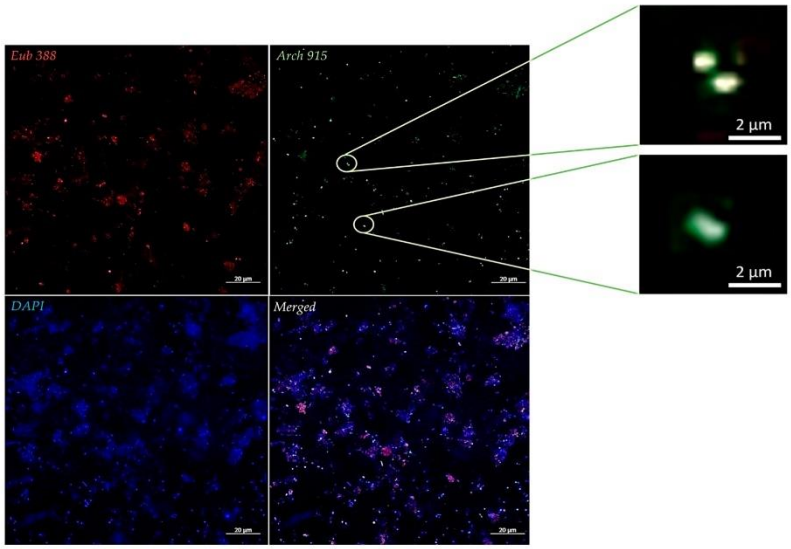
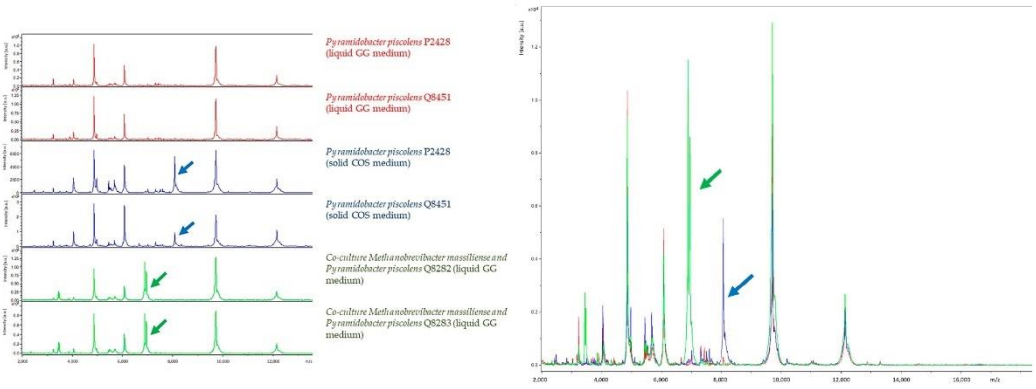


Figure 2. Fluorescence in situ hybridization (FISH) representative detection of *M. massiliense* and bacteria in a direct necrotic pulp sample. Eub 388 probe staining bacteria 16S rRNA gene in red. Arch 915 probe staining the archaeal 16S rRNA gene in green. Universal DNA DAPI staining in blue. Merged. Scale bar, 20 µm.



(**a**) (**b**)

Figure 3. MALDI-TOF spectra and superposition of spectra from co-cultures Q8282 and Q8283 with spectra from *P. piscolens* cultivated on COS and in liquid GG medium. (**a**) MALDI-TOF spectra of *P. piscolens* cultivated on COS (in red), MALDI-TOF spectra of *P. piscolens* cultivated in liquid GG medium (in blue), MALDI-TOF spectra of co-cultures Q8282 and Q8283 (in green), smoothed and baseline substrated. (**b**) Superposition of the six spectra. All the spectra are superimposable, with one missing peak in the co-culture of *M. massiliense* and *P. piscolens*, the same as that absent in the liquid medium spectra (blue arrow), and two additional peaks in the co-cultures with *M. massiliense* (green arrow). The superposition of the spectra reveals a significant correlation between co-cultures Q8282 and Q8283 with strains of *P. piscolens* cultivated on COS, as well as in liquid GG medium. These observations suggest a specific spectrum associated with *M. massiliense* in the co-cultures.

3.5. Whole Genome Sequencing and Analysis

Mapping llumina and Nanopore data against a *P. piscolens* reference genome made it possible to reconstruct two *P. piscolens* genomes derived from Q8282 (Q8451, accession number CAUZXV01000000) and Q8283 (Q8452, accession number CAUZXU01000000) co-cultures. Both genomes are complete without contamination according to CheckM and share similar characteristics together and with the *P. piscolens* reference genome in terms of length, GC% and coding ratio (Table 1). Then, combined assembly of Illumina and Nanopore data yielded two *M. massiliense* genomes derived from Q8282 (GenBank accession number CAUJAM01000000) and Q8283 (GenBank accession number CAUJAN01000000) co-cultures. The former *M. massiliense* genome, here chosen as the reference genome, consisted of a gapless 1,834,388 base pair (bp) molecule displaying a 31.3% GC content. The completeness of this genome, as determined by CheckM, was 100%, without contamination. Genome annotation indicated an 85.9% coding ratio and 1903 protein-encoding genes, 28 tRNA-encoding genes, two rRNA genes, and three CRISPRs (Table 1, Figure 4, Supplementary Data S4).

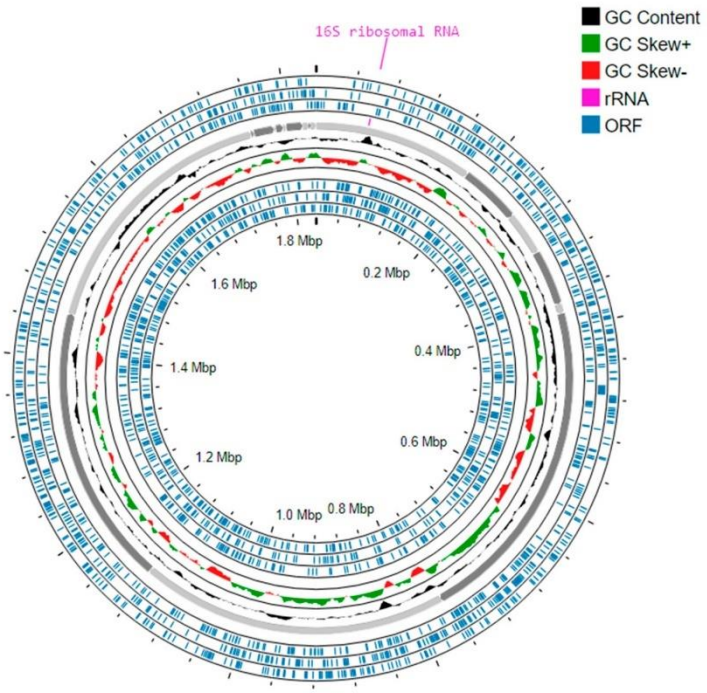


Figure 4. Circular representation of the *Methanobrevibacter massiliense* reference genome. The genome was visualised using the Proksee platform with default settings (version 1.0.0), available at https://proksee.ca/, accessed on 1 March 2023. The 19 contigs are displayed with colour-coded highlights, allowing for the identification of key genomic elements, and providing insight into the composition and structure of the *M. massiliense* genome. Open-reading frames are shown in blue, GC content is represented in black, while positive and negative GC skew are depicted in green and red, respectively. The rRNA regions are highlighted in pink. This analysis provides an overview of the distribution of protein-coding genes and reveals variations in GC content throughout the genome.

The 16S rRNA gene sequence extracted from WGS exhibited a 99.66% sequence similarity (100% coverage) with the *M. massiliense* Q8283 genome, 99.84% similarity (85%

coverage) with *Methanobrevibacter* sp. N13 (*M. massiliense*), 98.44% sequence similarity (100% coverage) with *Methanobrevibacter* sp. YE315, a methanogen isolated from pooled rumen of zebus (*Bos indicus*), 97.85% (91% coverage) with *M. smithii*, 97.82% (90% coverage) with *Methanobrevibacter gottschalkii* (*M. gottschalkii*) isolated from horse and pig faeces [48], 97.47% (85% coverage) with *Methanobrevibacter thaueri* (*M. thaueri*) isolated from cow faeces [48] and 97.09% (99% coverage) with *M. oralis* (Supplementary Data S4, Tables S4.3 and S4.4). Accordingly, the 16S rRNA gene sequence-derived phylogenetic tree clustered *M. massiliense* Q8282 and Q8283 with *Methanobrevibacter* sp. N13, then with *Methanobrevibacter* sp. YE315, *M. thaueri* and *M. gottschalkii*, and *Methanobrevibacter millerae* (*M. millerae*) (Figure 5).

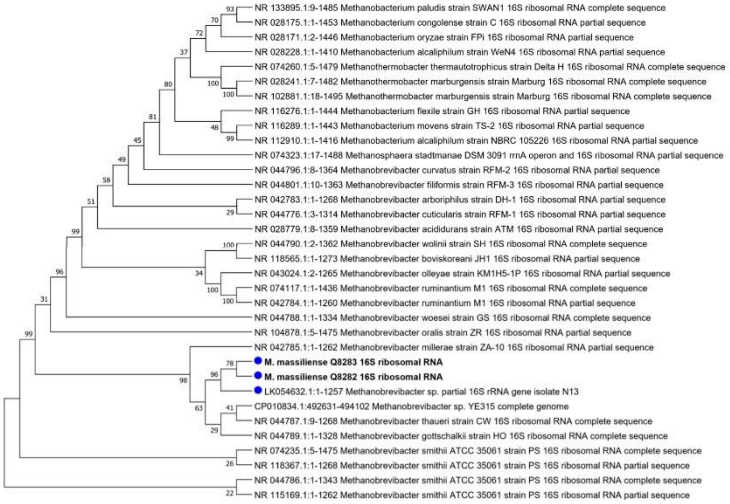


Figure 5. Maximum Likelihood phylogenetic tree based on 16S rRNA sequence analysis of *M. massiliense* and the first 35 hit blasts downloaded from NCBI GenBank Database (3 January 2023).

Average Nucleotide Identity (ANI), including 15 genomes, indicated that the two *M. massiliense* genomes share 99% identity but were distant from any other cultured *Methanobrevibacter* species. Indeed, the reference genome of *M. massiliense* displayed an ANI of 80% with *M. gottschalkii*, followed by *Methanobrevibacter* sp. YE315 (ANI, 79%), *M. thaueri* (ANI, 78%), and *M. oralis* (ANI, 78%) (Figure 6). The dDDH values of the two genomes with other methanogen species confirmed these previous results (Supplementary Data S4, Table S4.2). The *M. massiliense* genome annotation uncovered features absent from the *M. oralis* reference genome, including malate dehydrogenase (encoded by LOCUS_01990 and LOCUS_13560), molybdate permease (*modB*, encoded by LOCUS_01740 and LOCUS_19870), and nicotianamine synthase (encoded by LOCUS_00800 and LOCUS_19110). Additionally, both *P. piscolens* genomes (LOCUS_18390, LOCUS_05780, and LOCUS_15000) and *M. massiliense* genomes (LOCUS_01130 and LOCUS_18360), contained pyridoxamine kinase involved in vitamin B6 synthesis, while *M. oralis* possessed PDX genes (LOCUS_15160 and LOCUS_18540). Furthermore, *P. piscolens* genomes exhibited various metal ion dehydrogenases.

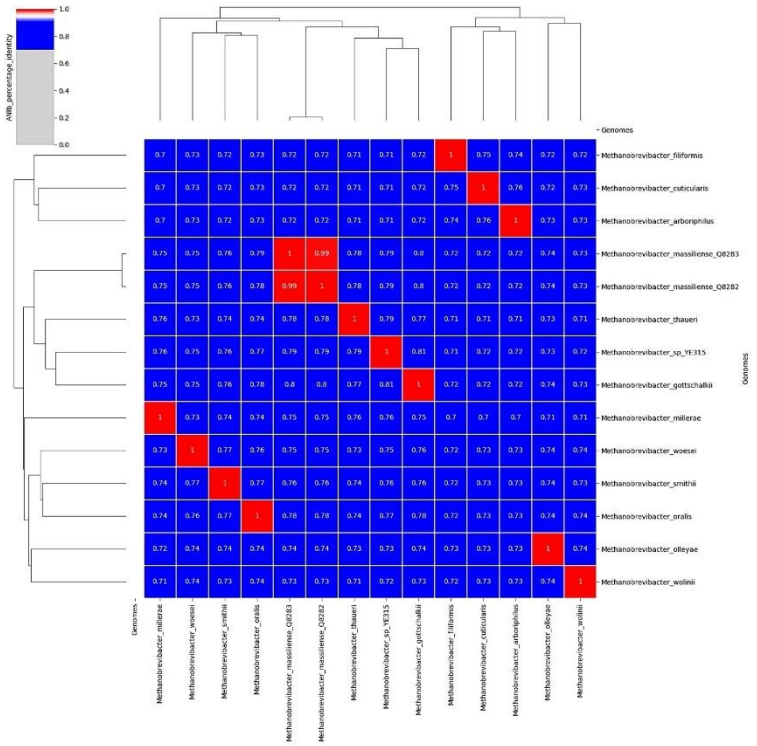


Figure 6. Heat map phylogeny generated with ANI values using PYANI software version (0.2.7) with standard parameters of *M. massiliense* and closely related methanogen species.

4. Discussion

In this study, methanogens were identified in half of the pulp samples extracted from carious teeth, consistent with findings from a prior study [24]. *M. oralis* emerged as the predominant species, followed by *M. massiliense* and *M. smithii*. This outcome aligns with some observations in periodontitis and peri-implantitis within dental plaque [16,17]. However, in contrast to earlier reports of methanogens in saliva and dental plaque from healthy individuals and around healthy implants [11,17,18], no methanogens were detected in healthy dental pulp. This absence is unsurprising, given the enclosed nature of the pulp chamber, which is secluded from the oral cavity. This observation leads to the hypothesis that methanogens may colonise microorganisms involved in the endodontic infection process. Notably, *M. massiliense* was the sole methanogen cultured from endodontic sites, leading to two *M. massiliense–P. piscolens* co-cultures obtained by culturing necrotic pulp samples collected from carious teeth in two different patients. The comprehensive characterisation of this fastidious methanogen was achieved through various techniques, including microscopy, genomic analysis, and phylogenetic investigation, bolstered by the implementation of negative controls to ensure data validity. Both co-cultures successfully subcultured in GG medium [34] were deposited in the public collection CSUR, and their genomes were made available in the GenBank public database.

Interestingly, we detected the presence of the same associated bacterium, *P. piscolens*, in two separate individuals, which aligns with previous findings in a case of severe periodontitis [28]. *P. piscolens* belongs to the lesser-explored phylum Synergistetes and is a Gram-negative coccobacillus originally isolated from oral samples [40]. While the molecular

detection of *P. piscolens* in endodontic samples was previously established, our culture-based approach provides additional evidence of its potential involvement in endodontic infections. *P. piscolens* has also been identified in diverse human specimens, including the gut [49,50], stool samples, and the sinuses [51]. Furthermore, it has been detected in animals, such as the gut microbiota of pigs [52], cattle rumen [53], and subgingival microbiota of horses [54]. Investigating the presence of *M. massiliense* in animals could shed light on new oral and also non-oral ecological niches for this methanogen, potentially uncovering zoonotic sources and transmission pathways.

Notably, *M. massiliense* appeared to be more challenging to cultivate than other methanogens, as we were unable to eliminate *P. piscolens* without affecting the viability of *M. massiliense*, indicating a strong dependency of methanogen on its partner. Despite attempts to enhance *M. massiliense* growth by increasing acetate and fatty volatile acids typically produced by *P. piscolens* [40], no significant improvement was observed. However, our hypothesis is that hydrogen may not be required for *M. massiliense* growth, as it grew in GG medium under a nitrogen atmosphere, and as *P. piscolens* did not produce hydrogen in our cultures in GG medium, as previously described [40]. Genome annotation also revealed the presence of a formate dehydrogenase, confirming this possibility.

The association between *M. massiliense* and *P. piscolens* in a co-culture revealed a transkingdom dependency (Archaea and Bacteria). Interestingly, while *P. piscolens* was isolated independently, *M. massiliense* was not, leading us to hypothesise that *M. massiliense* is reliant on *P. piscolens* for sustenance, supporting a unidirectional dependency. *M. massiliense* possesses a malate dehydrogenase that is not present in *M. oralis*, which has been isolated in pure culture [55]. Malate may play a crucial role, potentially serving as a source for acetyl-CoA, which is essential for various metabolic reactions, including the biosynthesis of the coenzyme methylcobalamin (Methyl-COB), an important cofactor in methanogenesis [56]. *P. piscolens* may have the capacity to produce malate through fumarate hydratase, suggesting a cooperative relationship, where one provides the required malate for important metabolic reactions in the other. However, fumarate hydratase is widespread in bacteria, and its presence in *P. piscolens* may not be sufficient to explain the uniqueness of *M. massiliense* dependency. However, the high metal requirement of *M. massiliense*, evident as it possesses nicotianamine synthase and a molybdate transporter absent in *M. oralis*, raises intriguing questions. Particularly, nicotianamine is a type of metallophore, a small organic molecule used to chelate metals, aiding in their absorption and transport within cells [57]. Dark crystal structures observed in co-culture may corroborate these findings. Metal ions are crucial co-factors in various metabolic pathways, including methanogenesis [58], and *P. piscolens* harbours several metal-ion-dependent dehydrogenases, possibly involved in electron transfer between the two species. Both share the same vitamin B6 biosynthesis pathway through pyridoxal kinase, while *M. oralis* employs an alternative pathway via PDX1 and PDX2 [10]. This suggests shared essential co-factors for diverse metabolic functions between *M. massiliense* and *P. piscolens*.

This intricate association underscores the importance of cooperation between different species, spanning domains, in microbial ecosystems. Sharing essential metabolites and co-factors enables these organisms to thrive together but also highlights the importance of studying metabolic pathways, testing substrate affinity, and making systematic efforts to enhance *M. massiliense* culture. Investigating the production and consumption of metabolites over time has become crucial to understanding how they interact. This knowledge can be valuable in finding ways to obtain a pure culture of *M. massiliense*. While the precise mechanisms of this association remain unresolved, delving into these interactions may provide valuable insights into their roles in human health and disease. Understanding these microbial relationships not only advances our fundamental knowledge of the microbiome but also opens avenues for potential therapeutic interventions and preventive strategies in oral health, promising advancements in both oral and systemic health landscapes.

5. Conclusions

In conclusion, our study not only reinforces the potential involvement of methanogens in endodontic infections, as indicated by an observed prevalence of 50% in infected pulps, but also emphasises the rediscovery of *M. massiliense* in a novel pathological context. Notably, *M. massiliense* emerges as the second prevalent methanogen, coexisting with *P. piscolens*. This unique association demonstrates a one-way dependency, where *M. massiliense* relies on *P. piscolens* for growth and survival. Genomic analysis highlighted possible metabolic cooperation between these species. Nevertheless, further research, encompassing in-depth exploration of metabolic pathways, production and consumption of metabolites, extensive large-scale testing of substrate affinity, and persistent isolation efforts, is imperative for a more comprehensive understanding. This intricate dependence underscores the importance of cooperation in microbial ecosystems. Gaining insights into these interactions would be useful for unravelling the complexities of human health and disease. Although the exact mechanisms require further investigations, this study opens avenues for future research on possible therapeutic interventions and preventive strategies in oral health, promising improvements in both oral and overall health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12010215/s1, Supplementary Data S1: Characteristics of samples used in this study; Supplementary Data S2: Table S2.1. Culture follow-up of the co-cultures *M. massiliense*/*P. piscolens*; Table S2.2. *M. massiliense* Q8282 isolation attempts; Supplementary Data S3: Figure S3.1: (**a**) Macroscopic observation of *M. massiliense*/*P. piscolens* Q8282 and Q8283 co-cultures, dark deposits are visible (**b**). Macroscopic observation of *P. piscolens* Q8451 and Q8452 cultures, dark deposits are not visible. (**c**) Macroscopic observation of negative controls (GG medium inoculated with DPBS 1× water); Figure S3.2: Confocal microscopy of *M. massiliense*/*P. piscolens* Q8282 (line 1), of *P. piscolens* cultured in liquid GG medium (line 2) and *P. piscolens* cultured on COS medium (line 3): brightfill mode view (column 1): observation of diplo-coccobacilli, autofluorescence at 420 nm (column 2): autofluorescent coccobacilli are visible for co-culture but no autofluorescent coccobacilli are visible for *P. piscolens* culture in GG medium or on COS medium, merged view of autofluorescence at 420 nm and brightfill mode (column 3): autofluorescent coccobacilli are visible as well as no autofluorescent coccobacilli for co-culture but no autofluorescent coccobacilli are visible for *P. piscolens* culture in GG medium or on COS medium (LSM 900 (Carl Zeiss Microscopy GmbH)); Figure S3.3: Electron microscopy (TM4000 HITACHI, 10 KV, X 1.500). (**a**) Observation of *M. massiliense* and *P. piscolens* co-culture, coccobacilli are visible, but no morphologic features allow distinction between *M. massiliense* and *P. piscolens*. (**b**) and (**c**) Observation of deposits in *M. massiliense* and *P. piscolens* co-culture. (**d**) Observation of *P. piscolens* culture in GG medium. (**e**) Observation of *P. piscolens* culture in COS medium; Supplementary Data S4: Table S4.1. Accession number of genomes used in this study; Table S4.2. DDDH analysis of *M. massiliense*; Table S4.3. *M. massiliense* from Q8282 co-culture first hit blasts; Table S4.4. *M. massiliense* from Q8283 co-culture first hit blasts.

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Conflicts of Interest: V.P., G.A., E.T. and G.G. are co-inventors of a patented culture medium referred to as GG culture medium in this report (patent FR 23 01404).

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Article 6: Dental calculus *Methanobrevibacter massiliense-Pyramidobacter piscolens, a millennium-old association*

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Dental calculus Methanobrevibacter massiliense-Pyramidobacter piscolens, a millennium-old association

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Short Report

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Abstract

Background

The metagenomic study of ancient dental calculus yielded two novel *Methanobrevibacter* species, TS-1 and TS-2. TS-2 was phylogenetically related to *Methanobrevibacter massiliense* (*M. massiliense*), a methanogen previously cultivated in close association with the bacterium *Pyramidobacter piscolens* (*P. piscolens*) in the oral cavity.

Results

Comparative genomics, including Average Nucleotide Identity, digital DNA-DNA hybridisation, and genome sequence-derived 16S rRNA gene sequence analysis revealed that TS-2 was confused with *M. massiliense*. Quantifying ancient and modern dental calculus metagenomic read counts yielded a positive correlation between *Methanobrevibacter* sp. YE315, the proxy-methanogen for *M. massiliense* in the Kraken2 database and *P. piscolens* (Pearson correlation test, p = 0.23, *P*-value = 0.026); whereas no such correlation was found with *Fretibacterium fastidiosum* (p = -0.04, *P*-value = 0.69) and *Synergistetes* phylum (p = -0.0072, *P*-value = 0.94) used as negative controls. Similar results were obtained with unclassified *Methanobrevibacter*, and the combined read counts of unclassified *Methanobrevibacter* and *Methanobrevibacter* sp. YE315.

Conclusion

Genomic comparisons provide evidence that *M. massiliense* is the named and cultured representative of Neolithic-old TS-2, suggesting a long-standing association between *M. massiliense* and *P. piscolens*. The nature, determinants, and evolutionary significance of this association remain unknown.

Background

Among the methanogens recovered from the human oral cavity microbiota, *Methanobrevibacter massiliense* (*M. massiliense*), which we discovered in severe periodontitis and necrotic dental pulp specimens, is unique in being isolated in indispensable coculture with *Pyramidobacter piscolens* (*P. piscolens*), an anaerobe bacterium belonging to the *Synergistetes* phylum (1) and in microscopically observed strict association with the methanogen (2, Pilliol *et al*., submitted for publication). Interestingly, we previously showed that *M. massiliense* dated at least from the 14th century. Its comparative prevalence with another oral cavity methanogen, *Methanobrevibacter oralis* (*M. oralis*) shifted in 18th century dental calculus samples, possibly correlating with changes in diets at that time, yet leaving the antiquity of *M. massiliense-P. piscolens* association unknown (3).

Recently, a dental calculus metagenomic study covering a time span from the Neolithic era to the present day was published in the journal *Microbiome* and provided a unique opportunity to investigate the antiquity of the association between *M. massiliense* and *P. piscolens* (4).

By analysing datasets from that study, we were able to confirm that this association between dental calculus dated back to the Neolithic period and continues to endure in contemporary populations.

Materials and methods

Data collection. Comparative genomics included the *M. massiliense* reference genome (GenBank accession number: GCA_963082475) and TS-1, TS-2 and *M. oralis* metagenomic assembled genomes (MAGs) kindly provided by Granehäll *et al.* (4). Twelve closely related methanogen genomes, including *Methanobrevibacter* sp. YE315 as well as *P. piscolens* whole genome sequence (GenBank accession number: GCF_022846135.1), were also included as outgroups (Additional file 1: Table S1). Correlation analyses incorporated a dataset of sequence data extracted from 97 modern and ancient human dental calculuses used in the study by Granehäll *et al.* (4) (Additional file 2: Table S1); 20 data sequences from dental calculus aDNA extracted from 20 individuals exhumed in Trentino, South Tyrol, Italy and covering the Neolithic to the Early Middle Ages, deposited in the European Nucleotide Archive (ENA) browser (https://www.ebi.ac.uk/ena/browser/view/PRJEB43389) (4), and 77 raw data sets from both ancient and modern human calculus samples retrieved from various studies, including 43 data sets from (5), 25 from (6) and nine from (7). The data originated from Europe (n = 93), Guadeloupe (n = 2), Nepal (n = 2) and Mongolia (n = 1) (4). We did not select samples from soil, skin, dental plaque, tongue dorsa or stools to achieve a valuable comparison between samples, as the oral cavity harbours different ecological niches with specific compositions (8, 9), and because the presence of *P. piscolens* in the gut microbiota (10, 11) contrasts with *M. massiliense* which has never been reported in the gastrointestinal tract (12). This distinction could potentially obscure any correlations when considering both gut and oral microbiota together.

Comparative genomic analysis: Average nucleotide identity (ANI) was determined on 14 genomes and 11 MAGs using PYANI software version 0.2.7 with standard parameters. Genomic sequences exhibiting > 95% identity were deemed to represent the same genomic species (13). DNA-DNA hybridization (dDDH) values calculated using Type (Strain) Genome Server (TYGS) online tool (https://tygs.dsmz.de/) (Additional file 1: Table S2) delineated species using a 70% DDH value threshold (14). 16S rRNA gene sequences were retrieved from *M. massiliense*, TS-1 and TS-2 MAGs after genome annotation using the DDBJ Fast Annotation and Submission Tool online platform (https://dfast.ddbj.nig.ac.jp) and BLASTed against NCBI GenBank database. A 16S rRNA gene sequence-based tree incorporated the first 32 top hits and was designed following a two-step approach: sequences were first aligned using MEGA's alignment tool to ensure proper alignment of homologous regions, then a neighbour-joining algorithm with standard parameters was used to generate the tree (Additional file 1, Tables S3, S4, S5). The evolutionary history of *M. massiliense* and TS-2 was inferred using the maximum likelihood method based on the JTT matrix-

based model and tree reliability was challenged by performing 1000 bootstrap replications, collapsing branches with < 50% bootstrap, for clarity.

Taxonomic assignation. To determine the microbial composition of each sample data set, a taxonomic assignment was performed with metagenomic paired-end reads including blank sequencing data on https://www.bv-brc.org/, using Kraken2 (15), which allowed taxonomic classification of metagenomic data based on the k-mer approach (Additional file 2: Table S1, S2, S5 to S11). Resulting taxonomic compositions were visualised in a Krona pie chart from the converted Kraken2 data and the report.txt outputs were used for interactive analysis of metagenomics and comparison using Pavian (16) (Additional file 2: Table S1, S2, S5 to S11). As the Kraken2 database does not include *M. massiliense* and *M. oralis* representatives, taxonomic assignment based on these two species reference genomes (accession numbers GCA_963082475 and GCF_912073625.1, respectively) identified the *Methanobrevibacter* species to which *M. massiliense* and *M. oralis* reference genome-sequences were assigned. We also confirmed the accurate taxonomic assignment of the *P. piscolens* genome (accession number: GCF_022846135.1).

Correlation analysis. The Pearson correlation test was used to estimate the strength of association in the number of *Methanobrevibacter* sp. Y315, unclassified *Methanobrevibacter*, and combined sum of reads assigned to *Methanobrevibacter* sp. Y315 and unclassified *Methanobrevibacter* read counts to match *M. massiliense*/TS-2 with *P. piscolens* read counts, as well as the association with the *Synergistetes* phylum and *Fretibacterium fastidiosum* (*F. fastidiosum*) reads, used as negative controls (Additional file 2: Table S2). Further, the same tests incorporated reads assigned to *M. smithii* to match *M. oralis* according to the Kraken2 taxonomic assignment of *the M. oralis* reference genome (accession number: GCF_912073625.1). We also conducted a range of statistical analyses using diverse combinations of read counts from various *Methanobrevibacter* species, using a 5% cut-off value for alpha significance level. To interpret the Pearson correlation coefficient, we considered its value in relation to its direction and magnitude. Statistical analyses were performed using R software with appropriate functions for calculating the Pearson correlation coefficient and its associated *P*-value (Additional file 2: Tables S3 and S4).

Results and discussion

Antiquity of M. massiliense in Neolithic dental calculus. TS-2 and *M. massiliense* were representative of the same species as indicated by a ANI value > 95% derived from TS-2 MAGs (4) and *M. massiliense* genome comparison, a conclusion further supported by a > 90% dDDH value between both taxa while dDDH value between *M. massiliense* and TS-1 was only 19.1% (Fig. 1; Additional file 1: Table S2). Two 16S rRNA gene sequences from TS-2_calc_2084__bin.6 and from TS-2_calc_2095__bin.8 exhibited 100% sequence similarity over 1,473 base pairs (bp) while no further 16S rRNA gene sequence was found in the other TS-1 and TS-2 MAGs. The *M. massiliense* 16S rRNA gene sequence exhibited 99.53% sequence similarity (100% coverage) with TS-2 16S rRNA gene sequences. A statistically supported phylogenetic tree based on 16S rRNA gene sequences placed *M. massiliense* within the TS-2 cluster (Additional file 1:

Tables S3, S4, S5 and Fig. 2). Thus, genomic comparisons and phylogenetic analyses concurred to provide conclusive evidence that *M. massiliense* is the current named, cultured representative of TS-2. Therefore, considering that the oldest TS-2 MAG retrieved by Granëhall *et al.* originates from Neolithic dental calculus, we can reasonably infer the presence of *M. massiliense* during this prehistorical period (4).

Antiquity of the M. massiliense-P. piscolens association in Neolithic dental calculus. To assess the association between *M. massiliense* and *P. piscolens*, we tested the strength of the correlating genomic reads of both microorganisms in the presence of controls. According to the taxonomic assignment of the *M. massiliense* and *M. oralis* reference genomes, *Methanobrevibacter* sp. YE315 and *M. smithii* were used as proxies for *M. massiliense* and *M. oralis*, respectively, in the Kraken2 database (Additional file 2: Tables S3 and S4). In other words, we conducted correlation analyses incorporating *Methanobrevibacter* sp. YE315 reads in place of *M. massiliense*, and unclassified *Methanobrevibacter* reads and combined reads of *Methanobrevibacter* sp. YE315 plus unclassified *Methanobrevibacter*. A significant positive correlation was established between *Methanobrevibacter* sp. YE315 and *P. piscolens* ($p = 0.23$, *P*-value = .026), while no correlation was observed with the *Synergistetes* phylum or *F. fastidiosum* ($p = -0.0072$, *P*-value = 0.94 and $p = -0.04$, *P*-value = 0.69, respectively). These findings were reproduced when analysing both unclassified *Methanobrevibacter* reads and unclassified *Methanobrevibacter* reads combined with *Methanobrevibacter* sp. YE315 reads (Additional file 2: Table S3). The same statistical analyses were performed using read counts assigned to *M. smithii* as a proxy for *M. oralis* (Additional file 2: Table S4) and no statistical correlation was observed with *P. piscolens*, *Synergistetes* phylum or *F. fastidiosum* (Additional file 2: Table S4). Further statistical analyses did not uncover any significant correlation (Additional file 2: Tables S3 and S4).

The data reported here underscore the enduring nature of the association between *M. massiliense* and *P. piscolens* back to the Neolithic era. Previous studies concur, reporting a shift from the predominant *M. massiliense* to *M. oralis* in the oral cavity around the 18th century. This coincides with the widespread consumption of sugar in Europe, where paleomicrobiology studies were conducted (3, 4). The combined data lead us to refine the hypothesis that increased sugar consumption prompted a shift from a longstanding association between *M. massiliense* and *P. piscolens*, which does not use sugar (1), to the dominance of *M. oralis*, suggesting the possibility of an unknown sugar-consuming “ghost” bacterium accompanying *M. oralis*.

Conclusions

Genomic comparisons and phylogenetic analyses have provided strong evidence indicating that *M. massiliense*, which had been previously co-isolated with *P. piscolens*, is indeed the current named, cultured representative of TS-2. Statistical correlation analysis, based on a substantial dataset of metagenomic information, suggests that the association between *M. massiliense* and *P. piscolens* in dental calculus dated back to the Neolithic era, and that this association persists in a minority of modern European populations today.

Abbreviations

MAGs = Metagenome Assembled Genomes

ANI = Average Nucleotide Identity

dDDH = digital DNA-DNA hybridisation

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All accession numbers of the data analysed during this study are included in this article and its supplementary information files. The MAGs from Granehäll *et al.* study have been provided by the authors on our request.

Competing interests

Not applicable.

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Authors' contributions

VP: data analysis, drafting the manuscript. MB: data analysis, drafting the manuscript. LT: drafting. MD: supervision, drafting the manuscript. GA: supervision, drafting the manuscript. GG: supervision, drafting the manuscript. ET: supervision, drafting the manuscript, funding.

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Figures

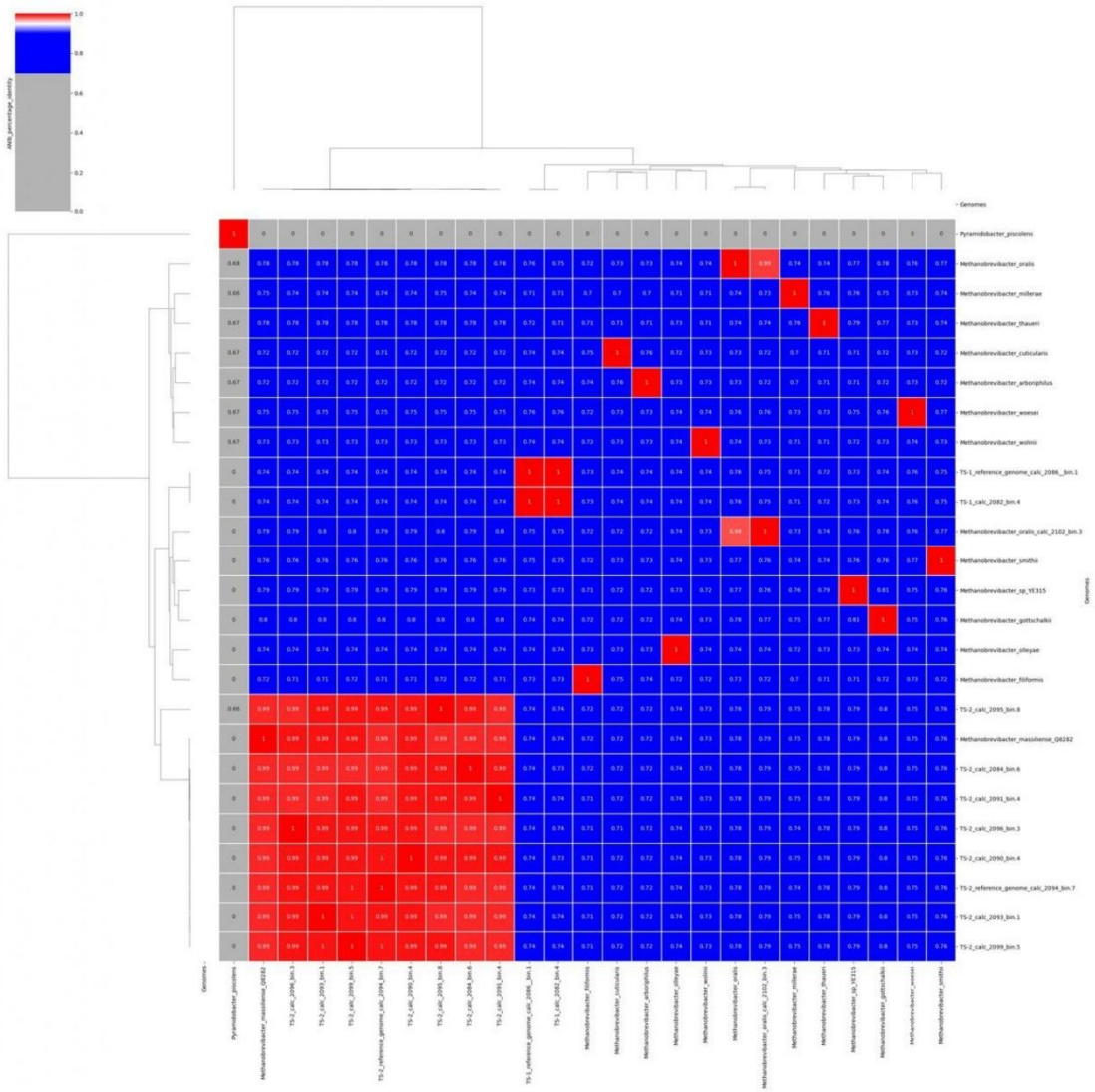

Figure 1

Heatmap phylogeny of *Methanobrevibacter massiliense*, TS-2 and closely related methanogen species generated with ANI values calculated using PYANI software version (0.2.7) with standard parameters.

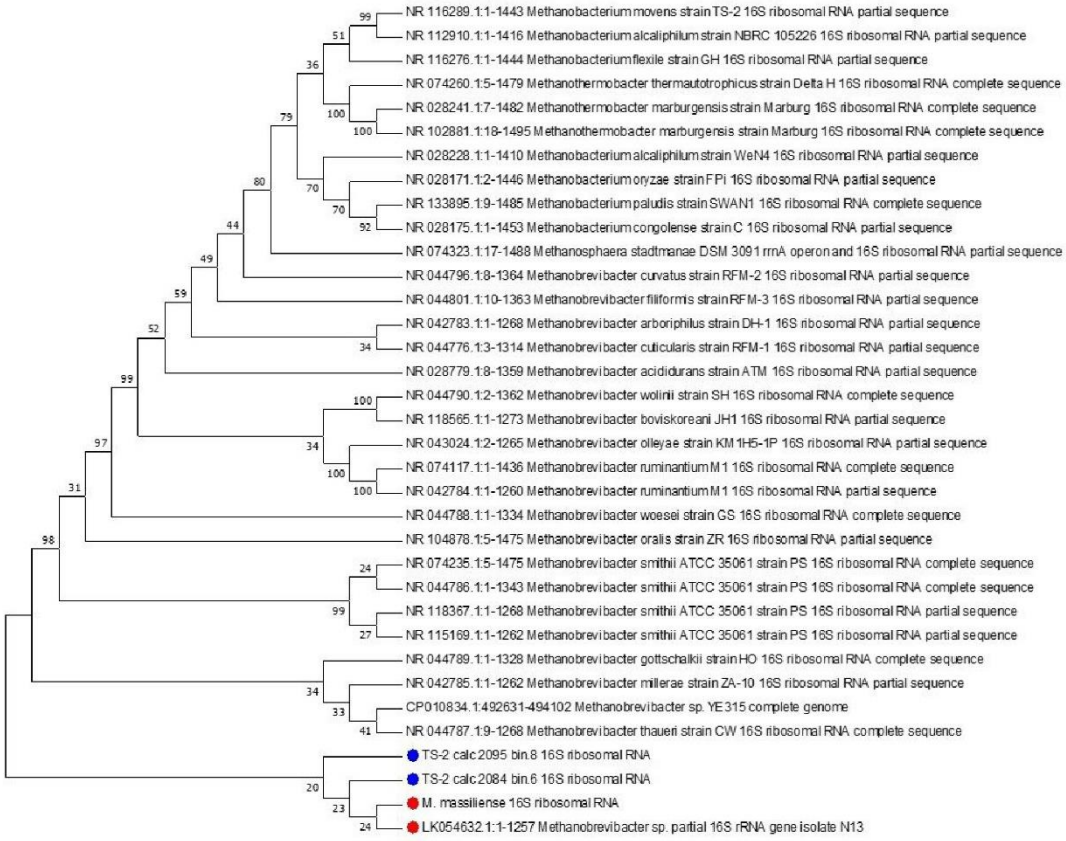

Figure 2

Maximum likelihood phylogenetic tree based on 16S rRNA gene sequence analysis of *Methanobrevibacter massiliense*, TS-2 and the first 32 hit blasts downloaded from NCBI GenBank Database (30-06-2023). The 16S rRNA gene sequence-based phylogenetic tree showed that *Methanobrevibacter massiliense* (blue) clustered with TS-2 (red).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
- Additionalfile2.xlsx

Conclusion et perspectives

Ainsi, ce travail de thèse s'inscrit tout d'abord dans la continuité du regain d'intérêt pour la culture afin d'étudier la composition en micro-organismes vivants au sein d'un microbiote donné (31). En effet, la culture est le point de départ pour l'étude approfondie de tous les micro-organismes. Les analyses de métagénomiques ont montré qu'il existait encore de nombreux micro-organismes inconnus et non cultivés (31), c'est pourquoi il est indispensable de continuer à innover dans les milieux et les techniques de culture, notamment en ce qui concerne les méthanogènes.

Ce travail de thèse a donc également permis de faire le point sur les différentes méthodes de culture des méthanogènes et les innovations les plus récentes. Il nous est toutefois apparu que le protocole de culture des méthanogènes restait long et fastidieux et toutes les modifications proposées avaient déjà cherché à simplifier ces procédures. Plusieurs de ces innovations concernaient la source et la disponibilité de l'hydrogène gazeux. L'hydrogène posait un problème quant à la dangerosité et les contraintes réglementaires associées à son utilisation. La technique de coculture avec une bactérie productrice d'hydrogène s'est révélée tout à fait efficace mais présentait des inconvénients tels que la nécessité de conditions de croissance spécifiques pour chaque micro-organisme, une complexité accrue du processus de culture, une difficulté à contrôler précisément les interactions entre les micro-organismes, ainsi qu'un coût potentiellement plus élevé en termes de temps et de ressources matérielles. La production d'hydrogène a pu être facilitée par sa production chimique lors de l'action d'un acide sur de la limaille de fer mais restait tout de même délicate en raison des risques liés à la manipulation de l'acide acétique et également des difficultés à contrôler précisément l'atmosphère de culture. La mise au point du milieu GG® dans ce travail de thèse a permis de faciliter grandement la culture des méthanogènes. En effet, l'augmentation des concentrations en acétate et en formate a permis de se passer de l'hydrogène gazeux que ce soit en milieu liquide ou en milieu solide pour l'isolement de *Cand.* M. massiliense. Particulièrement, cette dernière technique nous a permis d'abandonner l'utilisation complexe des doubles chambres en disposant simplement la gélose GG® dans un sachet hermétique avec un générateur anaérobie. L'utilisation aérobie du milieu GG® est une évolution tout à fait envisageable, le milieu contenant déjà des antioxydants.

L'amélioration des techniques de culture des méthanogènes marque une avancée significative vers l'intégration de leur culture dans la routine des laboratoires de microbiologie clinique. En effet, la capacité à cultiver ces micro-organismes revêt une importance croissante car ils pourraient jouer un rôle non négligeable dans les dysbioses (14), les infections abcédées (9) et même les endocardites infectieuses (13). L'optimisation de la culture des méthanogènes offre ainsi de nouvelles perspectives pour mieux comprendre leur rôle dans la santé humaine et les maladies associées, ouvrant la

voie à de futures applications diagnostiques et thérapeutiques dans différents domaines de la médecine. Il reste à déterminer si ce protocole peut être étendu à d'autres méthanogènes d'intérêt clinique. Dans le cadre de cette thèse, nous avons entrepris de répondre à cette question en isolant avec succès une nouvelle espèce, *Cand.* M. massiliense, et co-cultivant à nouveau *M. massiliense* avec *P. piscolens* grâce au milieu GG®. Notre objectif a été de procéder à une description et à une caractérisation approfondie de ces micro-organismes nouvellement cultivés.

Tout d'abord, le travail de revue de littérature sur *M. oralis* a été une première étape essentielle pour synthétiser les différents aspects clés nécessaires à la description et l'étude des méthanogènes. Ce processus nous a permis de réunir des informations sur les caractéristiques phénotypiques de *M. oralis*, notamment grâce à nos propres observations microscopiques, ainsi que sur les diverses méthodes de détection et d'identification disponibles. De plus, nous avons exploré les divers microbiotes où *M. oralis* a été détectée, ainsi que les conditions pathologiques potentiellement associées à sa présence. Un aspect crucial de cette revue a été la recherche des micro-organismes ayant pu être associés à *M. oralis*, qu'ils soient *Archaea* ou bactéries. Nous avons pu observer que *M. oralis* avait pu être retrouvée avec une large diversité de bactéries et que ces bactéries étaient différentes entre le microbiote oral et les situations extra-orales. Au sein de la cavité orale, *M. oralis* a pu être associée de manière significative à la bactérie *Prevotella intermedia*.

Par la suite, en nous appuyant sur les différents paramètres étudiés dans notre revue de littérature, nous avons entrepris une description détaillée de *Cand.* M. massiliense. Cette démarche incluait une caractérisation phénotypique, un séquençage complet de son génome ainsi qu'une analyse approfondie de ses réactions face à différents substrats et à divers antibiotiques. En parallèle, le développement d'un système de PCR spécifique nous a permis de détecter la présence de *Cand.* M. massiliense dans les fèces de porcs destinés à la consommation humaine, suggérant ainsi une éventuelle source d'acquisition de cette méthanogène via la consommation de viande. L'étude de la distribution de *Cand.* M. massiliense dans les populations consommant ou non du porc pourrait être un sujet d'étude intéressant nous permettant d'avancer dans la confirmation de cette hypothèse. Par ailleurs, le génome de *Cand.* M. massiliense avait déjà été documenté dans la littérature, ayant été détecté dans les selles d'un unique individu atteint de cancer colorectal. L'intérêt clinique de *Cand.* M. massiliense dans le contexte du cancer colorectal devient particulièrement intrigant. Bien que le rôle des méthanogènes dans le cancer demeure largement méconnu, une étude a suggéré une association entre une déplétion en méthanogènes et le cancer colorectal (32), tandis qu'une autre a identifié une corrélation positive entre *M. smithii* et ce type de cancer (33). Cependant, le potentiel rôle de *Cand.* M. massiliense dans cette pathologie reste encore à élucider.

Enfin, la redécouverte de *M. massiliense* dans une nouvelle situation clinique, l'infection endodontique, a confirmé sa viabilité dans cette pathologie et révélé sa forte dépendance à la bactérie *P. piscolens*. Malgré des caractéristiques similaires observées en microscopie et dans le spectre protéique entre les deux cocultures obtenues, les difficultés rencontrées dans la culture n'ont pas permis d'isoler *M. massiliense*, ce qui souligne la nécessité de

poursuivre les efforts dans ce domaine. Une analyse méticuleuse des voies métaboliques à partir des deux génomes ainsi que des expériences à grande échelle pour identifier les substrats nécessaires sont essentielles pour atteindre cet objectif. En outre, une étude approfondie de *P. piscolens* est également cruciale pour comprendre pleinement cette relation symbiotique. Par ailleurs, l'observation d'une corrélation positive entre *M. massiliense* et *P. piscolens* au cours du temps à partir de l'étude de données de métagénomique a suscité l'hypothèse que les modifications du régime alimentaires pourraient être responsable du déclin de *M. massiliense* au profit de *M. oralis* au 18ème siècle. Étant asaccharolytique (30), *P. piscolens* aurait pu être remplacé par d'autres bactéries favorisant le métabolisme de *M. oralis* à partir de la révolution industrielle correspondant à la large diffusion et consommation du sucre en Europe. La recherche d'une "ghost" bactérie (bactérie fantôme) associée à *M. oralis*, susceptible de dégrader préférentiellement les sucres, est donc envisagée, et *P. intermedia*, capable de dégrader les sucres et les protéines, apparaît comme un candidat intéressant à explorer.

Finalement, notre travail de thèse offre de nouvelles perspectives sur l'évolution du microbiote humain à travers deux exemples, l'un à l'échelle de l'individu, l'autre à l'échelle de l'évolution humaine. D'abord, en examinant l'acquisition potentielle de *Cand.* M. massiliense par le biais de l'alimentation carnée par un individu. Des précédents travaux avaient identifié quatre modes potentiels d'acquisition des méthanogènes, notamment l'acquisition intra-utérine (34), la transmission par le lait maternel (35) et la transmission environnementale via les produits laitiers (36) ou le contact avec les animaux (37). Notre travail suggère que la consommation de viande pourrait être un nouveau mode d'acquisition des méthanogènes et influencer leur diversité tout au long de la vie. Cependant, les mécanismes précis restent à élucider, notamment en ce qui concerne les facteurs qui ont permis et favorisé l'implantation de cette espèce. Ensuite, en examinant l'évolution du microbiote à l'échelle de l'histoire de l'Homme avec le déclin de *M. massiliense* que nous savons associée à *P. piscolens* au profit de *M. oralis*. Il y a environ 10 000 ans, le Néolithique a été marqué par un changement majeur avec la transition vers l'agriculture, favorisant des communautés microbiennes adaptées à un nouveau régime alimentaire différent de celui des chasseurs-cueilleurs (38). Cependant, depuis la révolution industrielle, l'introduction d'aliments transformés et l'utilisation répandue d'antibiotiques ont induit d'autres modifications dans le microbiote humain (38). Des études futures sur l'évolution de la diversité des méthanogènes, de *P. piscolens* dans l'histoire humaine et sur les partenaires potentiels de *M. oralis* pourraient éclairer davantage la dynamique d'évolution des méthanogènes et des microbiotes en général, tandis que des expériences sur l'utilisation des sucres pourraient apporter des éclaircissements sur les mécanismes sous-jacents à ces évolutions. D'autres paramètres tels que le tabac ou les antibiotiques pourraient être également étudiés.

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Annexes

Brevet FR2301404. Déposé le 15/02/2023; Autorisation de divulgation le 02/05/2023.

Notification de la date de dépôt.

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CABINET BEAU DE LOMENIE
MADAME FLIJANE STEPHANIE
TOUR MEDITERRANEE
65 AVENUE JULES CANTINI
13006 MARSEILLE
FRANCE

Courbevoie, le 17/02/2023

OBJET : Notification de la date de dépôt

J'ai l'honneur de vous faire connaître que votre demande référencée ci-dessus a reçu la date de dépôt du **15/02/2023** attestant de sa recevabilité.

L'instruction de votre dossier va se poursuivre et le Ministre chargé de la Défense est habilité à prendre connaissance de votre demande, à titre confidentiel.

J'attire votre attention sur le fait que l'invention faisant l'objet de cette demande ne peut être divulguée et exploitée librement, tant qu'une autorisation ne vous aura pas été accordée à cet effet. Le refus d'une telle autorisation intervient dans un délai maximum de 5 mois à compter du dépôt.

Veuillez agréer l'expression de ma considération distinguée.

Pour le Directeur général
de l'Institut national de la propriété industrielle
Joris REPPERT

Directeur du département administratif

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Page 1/1

Autorisation de divulguer et d'exploiter l'invention FR2301404 (Milieu GG).

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13006 MARSEILLE
FRANCE

Courbevoie, le 02/05/2023

OBJET : Demande de brevet d'invention ou de certificat d'utilité N°FR2301404

J'ai l'honneur de vous faire connaître qu'après avis du Ministre chargé de la Défense, habilité à prendre connaissance, à titre confidentiel, des demandes de brevet ou de certificat d'utilité auprès de l'Institut National de la Propriété Industrielle, l'autorisation de divulguer et d'exploiter l'invention, objet de la demande ci-dessus désignée, vous est accordée.

Cette autorisation a pour seul effet de vous délier de l'obligation de secret à laquelle vous étiez tenu, à l'égard des impératifs de la Défense Nationale.

L'instruction de votre dossier va se poursuivre en vue d'examiner sa conformité au regard des exigences des textes législatifs et règlementaires en vigueur.

J'attire votre attention sur le fait que conformément à la décision 2018-156 du Directeur Général de l'INPI, les démarches et échanges subséquents relatifs à votre demande s'effectuent en ligne, depuis le portail procédures https://procedures.inpi.fr

Veuillez agréer l'expression de ma considération distinguée.

Pour le Directeur général
de l'Institut national de la propriété industrielle
Joris REPPERT

Directeur du département administratif

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Page 1/1

Serment des Docteurs d'Université

« En présence de mes pairs. Parvenue à l'issue de mon doctorat en Maladies Infectieuses, et ayant ainsi pratiqué, dans ma quête du savoir, l'exercice d'une recherche scientifique exigeante, en cultivant la rigueur intellectuelle, la réflexivité éthique et dans le respect des principes de l'intégrité scientifique, je m'engage, pour ce qui dépendra de moi, dans la suite de ma carrière professionnelle quel qu'en soit le secteur ou le domaine d'activité, à maintenir une conduite intègre dans mon rapport au savoir, mes méthodes et mes résultats. »