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Sources et dynamique des méthanogènes chez l'Homme

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Affidavit

Je soussigné, Cheick Oumar GUINDO, déclare par la présente que le travail présenté dans ce manuscrit est mon propre travail, réalisé sous la direction scientifique du Professeur Michel DRANCOURT, 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.
Ce travail n'a pas été précédemment soumis en France ou à l'étranger dans une version identique ou similaire à un organisme examinateur.
Fait à Marseille, le 26 avril 2022.

Affidavit

I, undersigned, Cheick Oumar GUINDO, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific direction of Professor Michel DRANCOURT, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.
This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.
Place Marseille, April 26, 2022.

Liste de publications et participation aux conférences

1) Liste des publications réalisées dans le cadre du projet de Thèse :

1. **C.O. Guindo**, M. Drancourt, G. Grine. Digestive tract methanodrome: Physiological roles of human microbiota-associated methanogens. Microbial Pathogenesis. 2020;8. https://doi.org/10.1016/j.micpath.2020.104425.
2. **C.O. Guindo**, E. Terrer, E. Chabrière, G. Aboudharam, M. Drancourt, G. Grine. Culture of salivary methanogens assisted by chemically produced hydrogen. Anaerobe. 2020;6. https://doi.org /10.1016/j.anaerobe.2019.102128.
3. Y. Sereme*, **C.O. Guindo***, A. Filleron, P. Corbeau, T.A. Tran, M. Drancourt, J. Vitte, G. Grine. Meconial *Methanobrevibacter smithii* suggests intrauterine methanogen colonization in preterm neonates. Current Research in Microbial Sciences. 2021;100034. https://doi.org/10.1016/j.crmicr.2021.100034.
4. **C.O. Guindo**, B. Davoust, M. Drancourt, G. Grine. Diversity of Methanogens in Animals' Gut. Microorganisms. 2021;10. https://doi.org/10.3390/microorganisms9010013.
5. **C.O. Guindo**, M. Drancourt, G. Grine. Dairy products as sources of methanogens for humans, Microbiology, 2021. https://doi.org/10.1101/2021.11.16.468822.
6. **C.O. Guindo**, M. Morsli, S. Bellali, M. Drancourt, G. Grine. A *Tetragenococcus halophilus* human gut isolate. Current Research in Microbial Sciences. 2022; 100112. https://doi.org/10.1016/j.crmicr.2022.100112.
7. Y. Sereme, M. Michel, S. Mezouar, **C.O. Guindo**, L. Kaba, G. Grine, T. Mura, J.-L. Mège, T.A. Tran, P. Corbeau, A. Filleron, J. Vitte. A Non-Invasive Neonatal Signature Predicts Later Development of Atopic Diseases. Journal of Clinical Medicine. 2022;2749. https://doi.org/10.3390/jcm11102749.
8. **C.O. Guindo**, L. Amir, C. Couderc, M. Drancourt, G. Grine. Rapid identification of clinically interesting methanogens using an improved MALDI-TOF-MS assay. (In press, Access Microbiology).
9. **C.O. Guindo,** M. Morsli, S. Bellali, V. Pilliol, S. Belkacemi, M. Drancourt, G. Grine. Symbiotic Microbial Motility: the case for *Methanomassiliia massiliensis* gen. nov. sp. nov., and *Massiliisphaerochaeta oralis* gen. nov. sp. nov. (En préparation).

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

1. Tous les vendredis, depuis septembre 2019 : Participation aux séminaires IHU Méditerranée Infection à Marseille.
2. Tous les derniers mardis du mois, depuis octobre 2020 : Participation aux séances de WIP (Work In Progress) thématique IHU Méditerranée Infection à Marseille.

3. 24 février 2022 : Présentation de mes travaux de recherche au séminaire virtuel de MICAFRICA, Projet Européen associant quatre établissements de recherche en Tunisie, en Italie et en France, coordonné par la Professeure Leila KESKES, Université de Sfax, Tunisie.
4. 18-19 octobre 2021 : Participation en ligne (présentation du poster) au colloque du GDR Archaea.
5. 1er juillet 2021 : Participation à la journée Infectiopôle IHU Méditerranée Infection à Marseille.
6. Tous les derniers mardis du mois, de septembre 2019 – septembre 2020 : Participation aux séances de Bibliographie IHU Méditerranée Infection à Marseille.
7. 1er juillet 2020 : Participation à la journée Infectiopôle IHU Méditerranée Infection à Marseille.

Prix et distinctions

1. Février 2022 : Prix de la meilleure recherche « meilleur article publié » décerné par le comité scientifique lors des prix internationaux de recherche sur les nouvelles inventions scientifiques.
2. Juillet 2021 : Médaille de bronze pour mes travaux de recherche lors de la Journée Scientifique Infectiopôle Sud 2021.
3. Janvier 2021 : Médaille de reconnaissance pour ma participation aux efforts de lutte contre la Covid -19.

Vie associative

1. Juin 2022 : Observateur du jury 2 (Microbiologie – Génomique) du concours de l'Ecole Doctorale des Sciences de la Vie et de la Sante – ED62.
2. Février 2021 – Juin 2022 : Secrétaire général de l'association DynAMU - RÉSEAU DOCTORAL D'AIX-MARSEILLE UNIVERSITÉ.
3. Décembre 2019 – Juin 2022 : Représentant des doctorants au conseil de l'Ecole Doctorale des Sciences de la Vie et de la Sante – ED62.
4. Septembre 2019 – Juin 2022 : Représentant des étudiants/ doctorants au Conseil de laboratoire de l'UMR MEPHI au sein de l'IHU Méditerranée Infection (Institut Hospitalo-Universitaire) / AP-HM (Assistance des Hôpitaux Publics de Marseille).
5. Septembre 2019 – Juin 2022 : Responsable des étudiants/ doctorants de l'IHU Méditerranée Infection (Institut Hospitalo-Universitaire) / AP-HM (Assistance des Hôpitaux Publics de Marseille).

Avant-propos

Le format de présentation de cette Thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé, qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de Thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les Thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette Thèse.
En outre, la Thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale
Professeur Didier RAOULT.

Résumé

Les archaea méthanogènes (appelées méthanogènes dans ce document) sont les plus prévalentes parmi les archaea des microbiotes oral et digestif humains. Dans le microbiote digestif humain, les archaea méthanogènes ressortissent des genres *Methanobrevibacter* et *Methanomassiliicoccus*. Ces microorganismes consomment l'hydrogène issu des fermentations bactériennes anaérobies pour la production du méthane comme produit métabolique, d'où leur nom.

Les archaea méthanogènes sont présentes dans le microbiote humain dès la naissance avec une diversification au fil des années. Cependant, le répertoire complet, les sources et les dynamiques des méthanogènes restent méconnus chez l'Homme. Dans le cadre de cette Thèse, nous nous sommes intéressés à l'étude des sources et de la dynamique d'acquisition des méthanogènes chez l'Homme. Dans une première partie, nous avons revu la littérature décrivant l'ensemble des rôles physiologiques des méthanogènes associés au microbiote humain. Notre revue a mis en évidence l'énorme déficit de connaissances et des informations partiellement contradictoires concernant les méthanogènes humains, ainsi que la nécessité d'étudier la dynamique des populations de méthanogènes et leur rôle exact au sein du microbiote humain. Dans la seconde partie de nos travaux de Thèse, nous avons optimisé et développé de nouveaux protocoles pour l'identification et la culture en routine des méthanogènes d'intérêt clinique dans le laboratoire de microbiologie clinique. Nos travaux expérimentaux ont eu pour résultat de réduire le temps d'isolement par culture et de sous-culture de *Methanobrevibacter smithii* et de *Methanobrevibacter oralis*, d'isoler par culture une nouvelle espèce de méthanogène que nous avons nommée *Methanomassiliia massiliensis* à partir du fluide buccal et enfin, d'identifier les méthanogènes d'intérêt clinique par spectrométrie de masse MALDI-TOF-MS. Concernant la dynamique des méthanogènes chez l'Homme, nous avons montré par une approche polyphasique incluant la PCR-séquençage et les microscopies, que la période intra-utérine constitue le premier moment d'acquisition de *Methanobrevibacter smithii* avec la détection sans précédent de ce méthanogène dans le méconium de nouveau-nés prématurés. Également, nous avons ouvert la possibilité de transmission zoonotique des méthanogènes en montrant que les contacts étroits avec des animaux domestiques ainsi que la consommation du lait et des produits laitiers de certains animaux sont associés à la diversification des méthanogènes chez l'Homme.

Les résultats de notre Thèse constituent des avancées majeures dans la compréhension des sources et de la dynamique d'acquisition des méthanogènes du microbiote humain et invitent à développer un milieu biphasique standard pour l'isolement et la culture rapide des méthanogènes directement à partir des échantillons cliniques, permettant de poursuivre des investigations pour isoler par culture les méthanogènes détectés dans le méconium et dans les produits laitiers.

Mots-clés : Archaea, méthanogènes, Sources des méthanogènes, dynamique des méthanogènes, *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanomassiliia massiliensis*, microbiote humain, liquide buccal, méconium, produits laitiers, animaux domestiques, *Tetragenococcus halophilus*.

Abstract

Methanogenic archaea (referred to as methanogens in this document) are the most prevalent archaea in human oral and digestive microbiota. In the human digestive microbiota, the methanogenic archaea belong to the genera *Methanobrevibacter* and *Methanomassiliicoccus*. These microorganisms consume hydrogen from anaerobic bacterial fermentations to produce methane as a metabolic product, hence their name.
Methanogenic archaea are present in the human microbiota from birth with diversification over the years. However, the complete repertoire, sources and dynamics of methanogens remain unknown in humans. In the framework of this thesis, we are interested in the study of the sources and the dynamics of methanogen acquisition in humans. In the first part, we reviewed the literature describing the physiological roles of methanogens associated with the human microbiota. Our review highlighted the huge knowledge gap and partially conflicting information regarding human methanogens, as well as the need to study the population dynamics of methanogens and their exact role within the human microbiota. In the second part of our thesis work, we optimized and developed new protocols for the routine identification and culture of methanogens of clinical interest in the clinical microbiology laboratory. Our experimental work resulted in reducing the time of isolation by culture and subculture of *Methanobrevibacter smithii* and *Methanobrevibacter oralis*, isolation by culture a new species of methanogen that we have named *Methanomassiliia massiliensis* from oral fluid and finally, to identify methanogens of clinical interest by MALDI-TOF-MS mass spectrometry. Concerning the dynamics of methanogens in humans, we have shown by a polyphasic approach including PCR-sequencing and microscopies, that the intrauterine period constitutes the first moment of acquisition of *Methanobrevibacter smithii* with the unprecedented detection of this methanogen in the meconium of preterm newborns. Also, we opened the possibility of zoonotic transmission of methanogens by showing that close contact with domestic animals as well as the consumption of milk and dairy products from certain animals are associated with the diversification of methanogens in humans.
The results of our thesis constitute major advances in the understanding of the sources and the dynamics of methanogen acquisition in the human microbiota and invite the development of a standard biphasic medium for the isolation and rapid culture of methanogens directly from clinical samples, allowing further investigations to isolate by culture the methanogens detected in meconium and in dairy products.

Keywords: Archaea, methanogens, methanogen sources, methanogen dynamics, *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanomassiliia massiliensis*, human microbiota, oral fluid, meconium, dairy products, domestic animals, *Tetragenococcus halophilus*.

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Table des matières

Introduction

Les Archaea (nom féminin) sont des êtres vivants au même titre que les bactéries, les eucaryotes, et les virus [1]. Elles ont été datées d'environ trois milliards d'années dans les environnements extrêmes et ont été isolées pour la première fois chez l'Homme il y a plus de cinq décennies, comme constituants du microbiote digestif humain [2]. Les archaea présentes dans le microbiote humain sont divisées en trois phylum *Euryarchaeota*, *Crenarchaeota* et *Traumarchaeota*, les archaea du phylum *Euryarchaeota* sont les plus fréquemment isolées par culture ou par d'autres approches expérimentales, chez l'Homme [1,2] et comportent des archaea méthanogènes (qui seront simplement appelées méthanogènes dans ce mémoire de Thèse, et que nous qualifierons dans notre mémoire de Thèse comme nom masculin) et des archaea halophiles [1]. Les méthanogènes diffèrent des autres groupes d'Archaea par leur incapacité à assimiler l'oxygène qui est toxique pour eux et par la présence obligatoire d'hydrogène pour leur croissance [3,4]. Ils font partie du microbiote humain notamment du microbiote intestinal où ils représentent 10% des microorganismes anaérobies, du microbiote stomacal et du microbiote oral [1,2]. Ils ont aussi été retrouvés dans le sang [5], dans le colostrum et le lait maternel [6] , dans le vagin en cas de vaginose [7] ainsi que dans les voies urinaires en cas d'infection urinaire [8]. Dans le microbiote intestinal, les méthanogènes contribuent à la production du méthane comme sous-produit métabolique, d'où leur nom [1,9–11]. Ils sont divisés en trois groupes en fonction des substrats utilisés pour la production du méthane: les hydrogénotrophes utilisent le CO_2 ou le formate (*Methanobacteriales*, *Methanococcales*, *Methanocellales*); les méthylotrophes utilisent les composés méthylés (*Methanomassiliicoccales*); et les acétotrophes utilisent l'acétate (*Methanosarcinales*) [1,2]. Les méthanogènes sont des archaea d'intérêt clinique. Ils ont été associés à trois types de pathologies humaines: les abcès tels que abcès cérébral, abcès musculaire, sinusite réfractaire, gingivite, parodontite et péri implantite, phlegmon amygdalien, abcès péri-appendiculaires et infection de prothèse orthopédique; les dysbioses dont la vaginose et l'infection urinaire au cours desquelles les méthanogènes sont détectés ; et les malnutritions aiguës sévères de l'enfant au cours desquelles les méthanogènes ne sont pas détectés; et leur présence dans le sang appelée Archaeamie éventuellement associée à l'endocardite infectieuse [8,12–24].

Depuis la première description de l'isolement par culture d'un méthanogène chez l'Homme, huit espèces seulement ont été isolées par culture chez l'Homme (*Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, *Methanobrevibacter oralis*, *Methanomassiliicoccus luminyensis*, *Methanobrevibacter arboriphilus*, *Methanobrevibacter millerae*, *Candidatus* Methanomethylophilus alvus, C*andidatus* Methanomassiliicoccus intestinalis) [21,25–28]. *M. smithii* est détecté chez l'Homme dès les premiers instants de la vie et ensuite on observe une diversification des espèces de méthanogènes au fil des années. Cependant, il n'y a peu de connaissances sur les sources et la dynamique des méthanogènes chez l'Homme d'où notre travail de Thèse qui porte sur l'étude des sources

et la dynamique des méthanogènes chez l'Homme plus précisément sur la compréhension des modes d'acquisition des méthanogènes depuis le début de la vie jusqu'à l'âge adulte.
Pour mieux aborder cette Thèse, dès l'entame de nos travaux, nous avons fait une revue bibliographique sur les rôles physiologiques des méthanogènes associés au microbiote humain. Dans cette revue, nous avons fait le point sur l'ensemble des connaissances actuelles sur les méthanogènes associés au microbiote humain en abordant notamment les différentes méthodes d'étude des méthanogènes en microbiologie clinique, leur implication dans le métabolisme symbiotique avec d'autres microorganismes, leur incapacité de dégrader les hydrates de carbone, dans la libération de méthane et consommation de H_2, ainsi que dans la transformation des métaux lourds. Cette revue a aussi fait le point des connaissances sur l'utilisation éventuelle des méthanogènes comme probiotiques ainsi que leur rôle probable en pathologie humaine.
Dans un second temps, nous avons travaillé sur l'optimisation des méthodes d'identification et de culture des méthanogènes, avec pour but de mettre en place des méthodes beaucoup plus performantes pour l'identification et la culture rapide de ces microorganismes d'intérêt clinique, et ensuite d'isoler de nouvelles espèces de méthanogènes chez l'Homme. Nous avons donc mis en place une méthode chimique de production d'hydrogène permettant l'isolement et la culture rapides sur milieu solide de *M. smithii* et de *M. oralis* et, en parallèle nous avons optimisé le milieu SAB standard de culture des méthanogènes en ajoutant du rumen filtré ainsi que du méthanol pour isoler des méthanogènes méthylotrophes. Nous avons par la suite mis en place un protocole MALDI-TOF-MS permettant l'identification rapide des méthanogènes d'intérêt clinique.
Dans la dernière partie de notre Thèse, nous nous sommes intéressés sur les différents modes d'acquisition ainsi que la dynamique des méthanogènes chez l'Homme en explorant notamment le premier moment de leur acquisition chez le nouveau-né au cours de la période intra-utérine ainsi que les sources de leur diversification au cours de la croissance de l'enfant, en particulier la consommation des produits laitiers ainsi que la promiscuité avec les animaux domestiques. Nous avons démontré pour la toute première fois la présence de *M. smithii* dans le méconium collecté à partir de nouveau-nés prématurés et nous avons démontré au cours de ce travail, la transmission materno-fœtale durant la période intra-utérine. Nous avons par la suite démontré la part de la consommation des produits laitiers ainsi que le contact étroit avec les animaux domestiques dans la colonisation du tube digestif humain par des méthanogènes.

Chapitre I : Revue : Méthanodrome du tube digestif : Rôles physiologiques des méthanogènes associés au microbiote humain

Préambule

Dans cette première partie de notre Thèse, nous nous sommes intéressés à l'ensemble des rôles physiologiques des méthanogènes associés au microbiote humain. Nous avons notamment abordé les raisons pour lesquelles les méthanogènes restent toujours difficiles à étudier, notamment un examen microscopique qui n'est pas spécifique ; une culture nécessitant une atmosphère inhabituelle en microbiologie clinique, composée d'hydrogène et de dioxyde de carbone ainsi que des conditions anaérobies strictes ; une détection moléculaire nécessitant des amorces et des sondes spécifiques [29]. Nous avons démontré les relations syntrophiques entre les méthanogènes et certaines bactéries du microbiote digestif notamment via le transfert d'H_2 entre les genres *Christensenella* (bactérie) et *Methanobrevibacter* [30] ainsi que l'incapacité des méthanogènes à recycler les structures glucidiques qu'ils assemblent [31]. Cette revue a permis de démontrer qu'à travers leur production de méthane, les méthanogènes ont un impact positif sur le transit intestinal humain [32]. Nous avons aussi abordé le rôle possible des méthanogènes dans les maladies inflammatoires du tube digestif ainsi que dans certains abcès chez l'Homme [21–23,33–52], mais aussi la possibilité d'utiliser les *Methanomassiliicoccales* comme probiotiques pour prévenir les maladies cardiovasculaires liées à l'accumulation de triméthylamines dans le corps [53].

La rédaction de cette mini-revue nous a permis de comprendre les trous de connaissance relatifs aux rôles physiologiques des méthanogènes associés au microbiote humain. Il est donc nécessaire de développer de nouvelles méthodes pour l'identification et la culture des méthanogènes à partir d'échantillons cliniques afin d'isoler de nouvelles espèces et d'étudier la dynamique des populations de méthanogènes en précisant leur rôle exact au sein de la flore complexe associée au microbiote humain.

Article 1

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

C.O. Guindo, M. Drancourt, G. Grine.

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

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Methanogens

ABSTRACT

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

1. Introduction

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

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

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

Archaea are now recognized as members of human microbiota [1,6].

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However, the archaea stay as forgotten players in the human microbiota for three main reasons: microscopic examination is non-specific; the culturing of some archaea requires an unusual atmosphere consisting of hydrogen and carbon dioxide; and PCR-based detection requires specific primers and probes [7]. Though, several clinical microbiology teams have detected archaea including methanogens and halophilic archaea within the oral and gut microbiota with potential roles in some diseases [6,13].

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

2. Methods

2.1. .1Research strategy and article selection

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

2.2. .2Setting

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

3. Methods for studying human archaea in clinical microbiology

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

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

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

3.2. Molecular approach

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

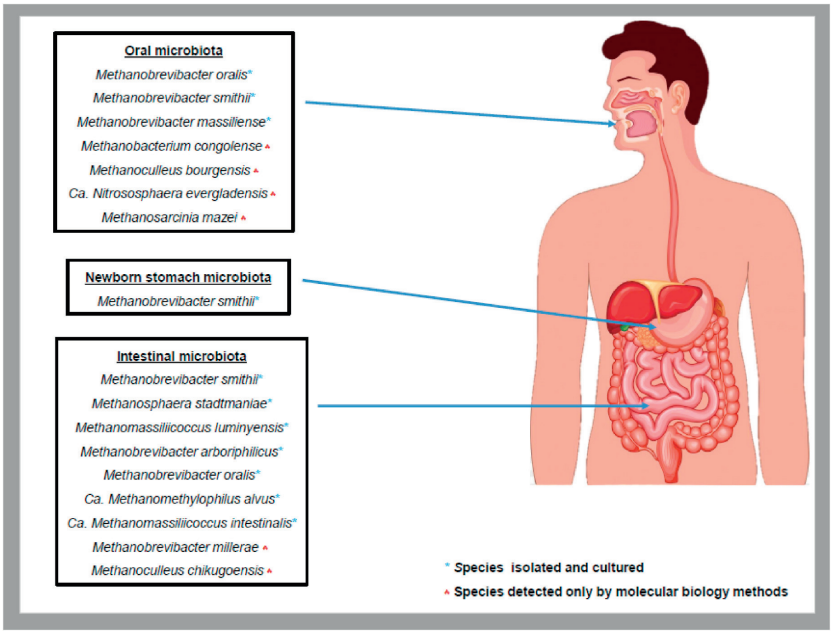

Fig. 1. Repertoire of methanogens in human digestive microbiota.

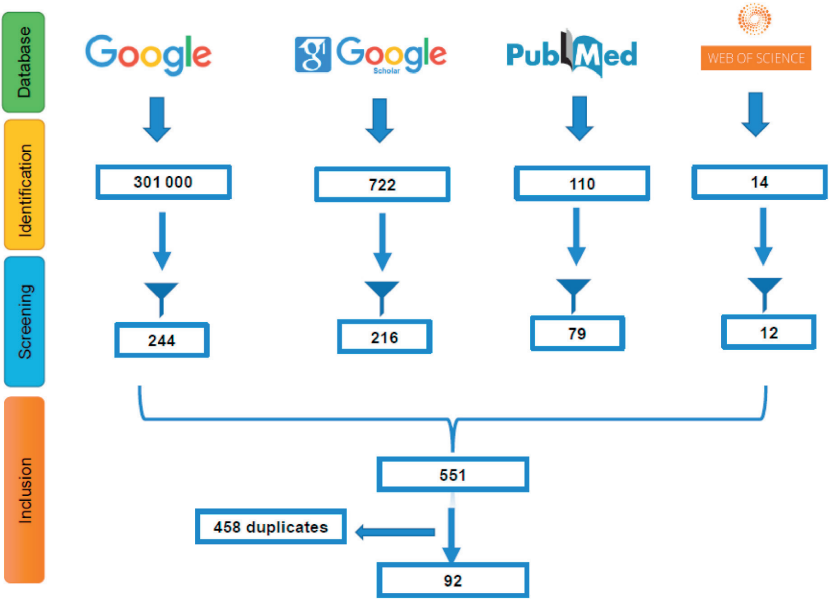

Fig. 2. Flow chart of selection of included articles in the review. Each box indicates the number of selected articles.

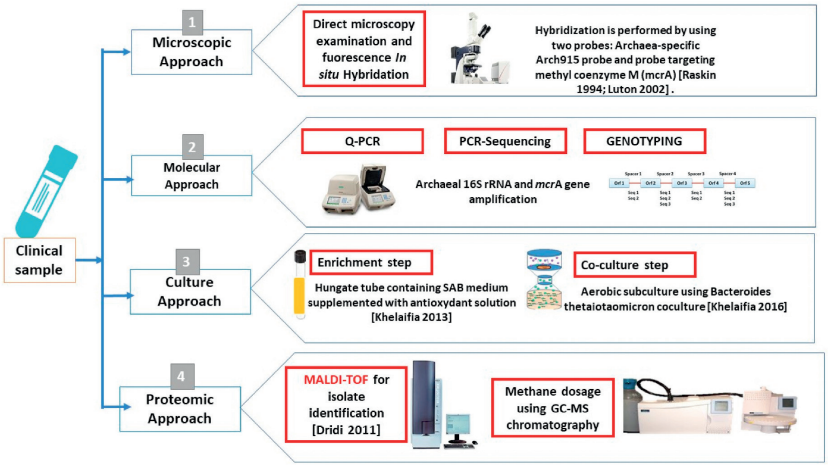

Fig. 3. Methods for studying human archaea in clinical microbiology.

used for the detection of *Methanobrevibacter smithii* (forward primer: Ms_rpoBF, 5′-AAGGGATTTGCACCCAACAC-3′reverse primer: Ms_rpoBR, 5′-GACCACAGTTAGGACCCTCTGG-3'; probe: Ms_rpoBVIC, 5′-ATTTGGTAAGATTTGTCCGAATG-3′) and *Methanosphaera stadtmanae* forward primer: Stadt_16SF, 5′-AGGAGCGACAGCAGAATGAT-3′ Reverse primer: Stadt_16SR, 5′-CAGGACGCTTCACAGTACGA-3'; probe: Stadt_16SFAM, 5′-TGAGAGGAGGTGCATGGCCG-3' [21].

3.3. Culture approach

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

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

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

3.5. In vitro susceptibility testing

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

4. Role of human-associated methanogens

4.1. Direct physical contact might support symbiotic metabolism

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

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

4.2. Methanogens and carbohydrates degradation

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

4.3. Methane release and H_2 consumption

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

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

4.4. Heavy metal transformation

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

4.5. Methanogens as probiotics

The possible use of methanogens as probiotics has received

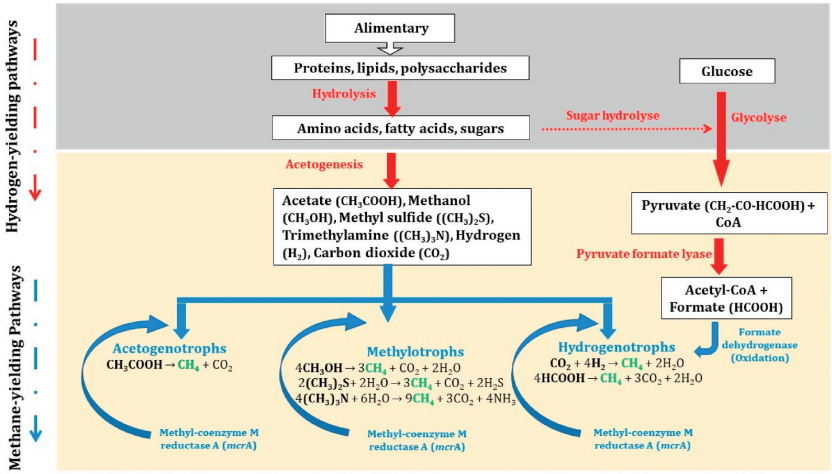

Fig. 4. Metabolisms supporting archaeal methanogenesis in aerobic (gray background) and anaerobic (yellow background): Bacterial enzymatic pathways in red characters and methanogens enzymatic pathways in blue characters.REVISED MANUSCRIPT. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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

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

5. Human associated methanogens and human diseases

5.1. Methanogens associated with dysbiosis

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

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

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

5.2. Methanogens associated with abscesses

There are only three studies carried out on the role of archaea in human abscesses and all these studies were done in our laboratory [87–89]. In a study conducted by Drancourt et al., *M. oralis* was isolated and cultured from a brain abscess [87]. In this study, the authors carried out a polyphasic approach (PCR-sequencing, metagenomics and culture) on brain abscess samples. They were able to detect 8/18 positive samples by qPCR, 28/32 positive samples by metagenomics and succeeded

in isolating by culture the *M. oralis* in a brain abscess sample taken from a 51-year-old woman with a history of dental avulsion of one month [87]. *M. smithii* was detected by PCR specific for 16 S RNA and *mcrA* genes in a paravertebral muscle abscess in a 41-year-old man suffering from lumbar swelling with night sweats and chronic fever [88]. In a study by Nkamga et al., *M. oralis* was detected by PCR-specific sequencing of the 16 S RNA and *mcrA* genes in a brain abscess sample from a 30-year-old woman suffering from persistent headaches for four days and a left temporal abscess [89].

6. Conclusions and perspectives

Archaea are an integral part of the human microbiota and are found in humans from the first day of birth [1,19]. There are three phyla found in humans including *Euryarchaeota* (the most important), *Crenarchaeota* and *Thaumarchaeota* [1] and methanogens are the most common archaea found in humans [1,15]. Since their first isolation in humans more than five decades ago, only ten species of archaea have been cultured in humans and all these species belong to the phylum *Euryarchaeota* (eight methanogens and two halophilic archaea) [1–3,6,23, 90]. This fact is linked to their fastidious culture which requires anaerobiosis control and the supply of hydrogen [23, 91, 92]. There is therefore a need to optimize current methods of isolating and culturing archaea in order to be able to isolate new species. It would also be very useful to conduct studies to better understand their role in human pathologies as well as their possible use as probiotics [52].

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

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

Declaration of competing interest

Guindo, C·O, Drancourt, M and Grine G declare that there is no conflict of interest.

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Chapitre II : Optimisation des méthodes d'identification et de culture des méthanogènes

Préambule

Les méthanogènes sont désormais reconnus comme des membres du microbiote humain, dont ils restent cependant des acteurs négligés [1,27]. En raison de la difficulté de leur mise en évidence, ils ne sont pas recherchés dans le diagnostic de routine malgré les données cumulatives indiquant leur rôle dans différentes pathologies. En particulier, il s'agit de microorganismes de culture fastidieuse dont l'isolement par culture dépend du contrôle de l'anaérobiose et de l'approvisionnement en H_2 [54,55]. Afin de faciliter l'isolement et la culture des méthanogènes, nous avons travaillé à optimiser et à mettre en place de nouveaux protocoles pour l'identification et la culture en routine des méthanogènes d'intérêt clinique dans le laboratoire de microbiologie clinique.

Nous avons développé une méthode alternative de production d'hydrogène utilisant de la limaille de fer et de l'acide acétique dans le but de cultiver les méthanogènes plus efficacement et plus rapidement. De façon intéressante, cette idée est venue de discussions avec le Prof. Eric CHABRIERE qui, dans l'Unité de recherche MEPHI dans laquelle nous avons réalisé nos travaux de Thèse, apporte une valence de biochimie et biophysique. Nous avons montré une apparition rapide des colonies de *M. smithii* et de *M. oralis* en utilisant cette méthode comparée à la méthode de référence utilisant *Bacteroides thetaiotaomicron* comme producteur d'hydrogène biotique [55,56]. Il s'agissait donc dans ce travail, de remplacer la production d'hydrogène biotique par une production chimique, éventuellement plus facile à maîtriser et à standardiser. Compte tenu de la simplicité, de la rapidité et de l'efficacité de cette méthode chimique de production d'hydrogène, son utilisation pourrait faciliter l'isolement et la culture de routine des méthanogènes dans les laboratoires de microbiologie.

Les méthanogènes méthylotrophes ont un intérêt très important sur la santé humaine étant donné leur capacité à utiliser la triméthylamine produite par les bactéries du microbiote intestinal et peuvent donc être proposés comme outils thérapeutiques pour prévenir la triméthylaminurie et les maladies cardiovasculaires [53]. Cependant, seulement trois espèces ont été isolées par culture dans le microbiote digestif humain. Nous avons donc mis au point un milieu de culture spécifique qui a permis l'isolement et la culture d'un nouveau genre de *Methanomassiliicoccales* à partir du fluide buccal humain. Cette étude a donc permis d'enrichir le répertoire des méthanogènes colonisant la cavité buccale avec l'isolement et la culture sans précédent de *Methanomassiliia massiliensis* à partir du fluide buccal humain.

Ensuite, nous avons travaillé à routiniser l'identification des méthanogènes ainsi plus facilement cultivés, par le développement de leur identification par spectrométrie de masse. Nous avons donc développé un nouvel outil de diagnostic fiable, rapide et

reproductible des méthanogènes d'intérêt clinique par spectrométrie de masse MALDI-TOF-MS. Nous avons créé une base de données comprenant 34 spectres de référence dérivés de 16 souches de référence de méthanogènes représentatives de huit espèces afin d'identifier des isolats cliniques de *M. smithii* et de *M. oralis* avec des scores > 2. Nous avons démontré que le MALDI-TOF-MS peut différencier certains génotypes de *M. smithii*. Ces résultats indiquent que la spectrométrie de masse MALDI-TOF-MS est une méthode de première ligne pour identifier les méthanogènes isolés au laboratoire.

Article 2

Culture of salivary methanogens assisted by chemically produced hydrogen.

C.O. Guindo, E. Terrer, E. Chabrière, G. Aboudharam, M. Drancourt, G. Grine.

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Culture of salivary methanogens assisted by chemically produced hydrogen

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ABSTRACT

Methanogen cultures require hydrogen produced by fermentative bacteria such as *Bacteroides thetaiotaomicron* (biological method). We developed an alternative method for hydrogen production using iron filings and acetic acid with the aim of cultivating methanogens more efficiently and more quickly (chemical method). We developed this new method with a reference strain of *Methanobrevibacter oralis*, compared the method to the biological reference method with a reference strain of *Methanobrevibacter smithii* and finally applied the method to 50 saliva samples. Methanogen colonies counted using ImageJ software were identified using epifluorescence optical microscopy, real-time PCR and PCR sequencing. For cultures containing pure strains of *M. oralis* and *M. smithii*, colonies appeared three days post-inoculation with the chemical method versus nine days with the biological method. The average number of *M. smithii* colonies was significantly higher with the chemical method than with the biological method. There was no difference in the delay of observation of the first colonies in the saliva samples between the two methods. However, the average number of colonies was significantly higher with the biological method than with the chemical method at six days and nine days postinoculation (Student's test, $p = 0.005$ and $p = 0.04$, respectively). The chemical method made it possible to isolate four strains of *M. oralis* and three strains of *M. smithii* from the 50 saliva samples. Establishing the chemical method will ease the routine isolation and culture of methanogens.

1. Introduction

Methanogenic archaea (referred to herein as methanogens) are acknowledged members of the digestive tract microbiota, and they have been detected by PCR-based methods and cultured from the oral cavity and the stools of apparently healthy individuals [1]. More particularly, *Methanobrevibacter oralis*, *Methanobrevibacter smithii* and *Methanobrevibacter massiliense* have been isolated from the oral cavity, whereas *M. smithii*, *Methanosphaera stadtmanae*, *Methanomassiliicoccus luminiyensis*, *Methanobrevibacter arboriphilicus*, *M. oralis*, *Ca.* Methanomethylophilus alvus and *Ca.* Methanomassiliicoccus intestinalis have been isolated from stools [1]. Accordingly, we previously showed that virtually all apparently healthy individuals would carry methanogens in the digestive tract microbiota [2] This observation indeed corroborated the pivotal role of methanogens, which detoxify hydrogen produced by bacterial fermentations into methane [3,4].

Moreover, methanogens are increasingly implicated in diseases; their presence or absence is associated with dysbioses such as those observed in the gut microbiota in cases of chronic constipation [5], obesity [6] and colonic diseases including ulcerative colitis, Crohn's disease and colorectal cancer [7–9], in the vaginal microbiota in cases of vaginosis [10,11]. In addition, the presence of methanogens is associated with anaerobic pus abscesses such as brain abscesses [12,13] and muscular abscesses [14].

As is usual in clinical microbiology, isolation and culture of methanogens is the gold standard to assess the detection of living methanogens in microbiota and in pathological clinical specimens collected by puncture or biopsy [1]. The routine application of methanogen culturing is hampered by the fact that methanogens

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are strictly aero-intolerant microbes and require hydrogen for culturing [15,16]. To facilitate the isolation and culture of methanogens from a routine perspective, we previously designed a new process in which methanogens are cultivated in the presence of hydrogen-producing *Bacteroides thetaiotaomicron* [17,18].

Here, we tested the conditions to replace the biological production of hydrogen with the chemical production of hydrogen and applied it to isolate methanogens from saliva as a proof-of-concept.

2. Materials and methods

2.1. Chemical production of hydrogen

The immediate chemical production of hydrogen resulting from the oxidation of iron by a weak acid has long been known [19]. We used acetic acid in our experiment because of its ability to oxidize iron, resulting in sufficient production of hydrogen [20]. We used two solutions for our experiments, namely, SAB culture broth [21] and distilled water (Fresenius, Bad Homburg voor Hoehe, Germany) with pH values of 7.3 and 7, respectively. We used 5 mL of each solution in our experiments. The experiments were performed in 10 Hungate tubes (Dominique Dutscher, Brumath, France) for each solution. Iron filings (Amazon, Clichy, France) were used at increasing quantities: 0.5 g for tube n°1, 1 g for tube n°2, and a 0.25 g increase from tube n°3 to tube n°10. The amount of acetic acid (VWR International, Pennsylvania, USA) used was 100 μL for tube n°1 to tube n°7 and 50 μL for tube n°8 to tube n°10. We first placed the iron filings in the tubes, followed by the solution (SAB broth or water) and then the acetic acid. We used three negative control Hungate tubes, one tube with iron filings in SAB culture medium, another tube with iron filings in distilled water and finally a third tube containing iron filings alone. Gas chromatography was then performed using a Clarus 580 FID chromatograph (PerkinElmer, Villebon-sur-Yvette, France) to measure hydrogen production in the different tubes, and the pH was monitored (Fisher Scientific, Illkirch, France).

2.2. Culture of methanogen strains using the chemical method for hydrogen production

M. oralis CSUR P9633, a human saliva isolate [22], was used for the development of the chemical method. We used the mini-double-chamber flask technique, which was derived from the previously described technique [21]. SAB agar plates (5-cm plates, Fisher Scientific) inoculated with 200 μL of a *M. oralis* suspension at 10^7 colony-forming units (CFUs) were placed in the upper compartment of a mini-double-chamber flask, which was sealed with parafilm to ensure an anaerobic atmosphere. We then placed 1 g of iron filings and 100 μL of acetic acid in 10 mL of distilled water in the lower compartment of the mini-double-chamber flask in a microbiological safety station. We used an SAB agar plate inoculated with 200 μL of sterile phosphate buffered saline (PBS) (Fisher Scientific) as a negative control. We then incubated the plates at 37 °C for nine days with visual inspection on day 3, day 6 and day 9 postinoculation.

Then, the growth of *M. smithii* CSUR P9632, a human stool isolate, using the chemical method was compared with that using the reference biological method using a double-chamber system as previously described for the aerobic culture of methanogens [17]. For the chemical method, we put 1.5 g of iron filings and 150 μL of acetic acid in 200 mL of distilled water in the lower compartment instead of *Bacteroides thetaiotaomicron*, which was used in the biological method. SAB agar plates inoculated with 200 μL of a *M. smithii* suspension at 10^6 CFU or 200 μL of PBS for the negative controls were placed in the upper compartment.

The pH of the SAB agar plates was measured at day 3, day 6 and day 9 postinoculation using pH indicator strips (Macherey-Nagel SARL, Hœrdt, France). We placed the pH indicator strip directly on the SAB agar plate for 2 min. We then took the value corresponding to the color obtained on the strip from the values indicated by the supplier. The pH and redox potential of the broth in the lower chamber were measured at day 3, day 6 and day 9 postinoculation using the Accumet™ AE150 apparatus (Fisher Scientific). The probe was rinsed thoroughly with distilled water and then immersed in the lower chamber until the device displayed the value on the reading screen. The rinsing step was performed after each use to avoid any error in reading the device. We used the pH and redox potential of distilled water alone and the SAB culture broth alone as controls. We also measured the redox potential of the upper compartment. For the two methods, we used one double-chamber system, in which we placed one Petri plate with 10 mL of distilled water and one Petri plate with 10 mL of the SAB culture broth. These double-chamber systems were then incubated at 37 °C, and the redox potential was measured in each of the Petri dishes at day 3, day 6 and day 9 postinoculation. The effectiveness of the two methods for the growth of methanogens was compared by observing the appearance of the first colonies as well as the average number of colonies at day 3, day 6 and day 9 postinoculation. Colonies were confirmed by autofluorescence optical microscopy as follows. Briefly, the colony deposited on a microscopy slide was observed at 63× using an epifluorescence microscope (Leica DMI 3000, Wetzlar, Germany) and at 100× magnification using another epifluorescence microscope (Leica DMI 6000). Colonies were enumerated at day 3, day 6 and day 9 postinoculation by focusing the image of the entire 5-cm plate on a black background using a portable camera without flash (Lenovo L38011, Beijing, China). Numbered images were analyzed and counted using ImageJ version 8 (https://imagej.nih.gov/ij/download.html) (Wayne Rasband, Java, National Institutes of Health, Bethesda, USA) as follows: each image was imported into ImageJ software and analyzed in a first step using the blue, diamidino phenylindole filter to normalized the image. In a second step, the normalized image was analyzed using the green fluorescein isothiocyanate filter and saved in the Joint Photographic Experts Group format. We standardized the images using the elliptical selection tabulation of the software. We then proceeded to manually count the enlarged colonies using the magnifying glass and the multipoint tabulation of the software.

2.3. Comparison of the two methods on saliva samples

The study was previously approved by the Ethics Committee of the IHU Méditerranée infection under n°2016–011. After having informed and collected the consent of the participants, we collected saliva samples from 50 people, including 25 tobacco smokers (15 men, 10 women, median age of 30) and 25 nonsmokers (12 men, 13 women, median age of 28). The collection and processing of samples were performed as previously described [22]. We used SAB agar plates inoculated with 200 μL of PBS as negative controls and then proceeded in the same manner as described above for the *M. smithii* strain.

2.4. Molecular analysis

PCR was performed to confirm the identity of the colonies. Colonies were suspended in 200 μL of ultrapure water (Fisher Scientific), and a sonication step was performed for 30 min. DNA was then extracted with the EZ1 Advanced XL Extraction Kit (QIAGEN, Hilden, Germany) using 200 μL as the sample volume and 200 μL as the elution volume. Gene amplification and PCR sequencing were performed as previously described [22–24]. Real-time PCR

analyses were performed as previously described [25].

3. Results and discussion

We report on the use of chemically produced hydrogen for the isolation and culture of methanogens as an alternative to the currently used biological method [17]. The method we report is based on the production of hydrogen resulting from the chemical reaction between iron filings and a weak acid such as acetic acid. All reported results were validated by negative controls that remained negative, and the colonies were identified by autofluorescence and PCR-based analyses.

We first determined an optimal balance between the concentration of iron filings and acetic acid to maintain the production of hydrogen over several days at a relative concentration compatible with the isolation and culture of methanogens. These preliminary experiments enabled the development of a safe and controlled production of hydrogen that was shown to be efficient in the culture of *M. oralis* and *M. smithii* reference strains.

In the first step, we measured the average redox potential and pH values of the various media investigated here. The pH and redox potential of the controls consisting of distilled water and SAB broth culture medium were 7 and +81.3 mV and 7.3 and −11.9 mV, respectively. After incubation at 37 °C for 3, 6 and 9 days, these values were 6.98, 6.92 and 6.87 units and +64.6 mV, +67.3 mV and +79.9 mV for distilled water; and 6.83, 6.78 and 6.27 units and +11.1 mV, +12.9 mV and +14.8 mV for the SAB broth culture medium, respectively (Table 1). These results agree with previous observations that most types of water, including tap water and bottled water, are oxidizing agents, and the value of their redox potential is positive, ≥ 20 mV [26]. The addition of antioxidants in the SAB medium makes this culture medium reductive. Then, the pH and redox potential of distilled water enriched with iron filings and acetic acid (chemical method) were measured at 5.23, 5.16 and 5.08 units and +109.77 mV, +108.6 mV and +112.7 mV, respectively (Table 2). These values were 5.8, 5.25, and 5.24 units and +113.8 mV, +113.8 mV and +114.5 mV at day 3, day 6 and day 9 for the SAB culture medium incubated with *B. thetaiotaomicron*, respectively (Table 2). These data indicated that the cultivation of *B. thetaiotaomicron* in the SAB medium releases fermenting compounds such as CO_2, hydrogen and acetate, which could oxidize the SAB medium [26]. Then, we measured these values in the Petri dishes placed in the upper compartment of the double-chamber system, which hosted the methanogen culture (Fig. 1). The redox potential values were −28.1 mV, −81.2 mV and −108.9 mV at day 3, day 6 and day 9 for distilled water, respectively, and +13.5 mV, −116.5 mV and −77.5 mV at day 3, day 6 and day 9 for the SAB culture broth in the chemical method, respectively. For the biological method, the redox potential values were −4.5 mV, −66 mV and −91.6 mV at day 3, day 6 and day 9 for distilled water, respectively, and +19.1 mV, −7.1 mV and −34.9 mV at day 3, day 6 and day 9 for the SAB culture broth in the biological method, respectively (Table 1). These data indicated that the hydrogen, being a reducer released into the bottom compartment, reaches the upper compartment and reduces the SAB medium or the water placed in the Petri dishes in the upper compartment [27].

Table 2
pH and redox potential of the lower compartment.

Methods	Redox potential (mV)			pH		
	D3	D6	D9	D3	D6	D9
Chemical method	+109.77	+108.6	+112.7	5.23	5.16	5.08
Biological method	+113.8	+113.8	+114.5	5.8	5.25	5.24

mV = millivolts; D: day.

In a second step, by culturing the reference methanogen strains in the presence of the negative and positive controls, we obtained hydrogen production in all tubes and observed a correlation in the hydrogen production, the amount of iron filings used and the acid acetic concentration. The hydrogen production using the chemical method was maintained until day 5, and the average pH values of the mixture were 4.09 ± 0.15 and 4.35 ± 0.23 in the SAB culture broth and distilled water, respectively. *M. oralis* colonies that were confirmed by PCR sequencing were visible on day 3 postinoculation using the chemical method (Supplementary Fig. 1). The negative control consisting of PBS inoculation instead of *M. oralis* remained negative.

To compare the chemical method with the biological method, *M. smithii* was inoculated in SAB agar plates using both methods. Using the chemical method, inoculation of SAB agar plates with *M. smithii* yielded colonies as early as day 3 postinoculation, whereas they appeared on day 9 using the biological method (Supplementary Fig. 1, Supplementary Fig. 2). All negative controls remained negative. The colonies were confirmed using PCR sequencing and real-time PCR. The average number of colonies of *M. smithii* was significantly higher with the chemical method than with the biological method, regardless of the day of follow-up (Student's test, $p < 0.0001$, $p < 0.001$ and $p = 0.0001$ for day 3, day 6 and day 9 postinoculation, respectively) (Table 3). Moreover, cultures of these two strains were obtained in three days using the chemical method rather than in nine days using the biological method [17]. In addition, the chemical method was easier to set up than the biological method because it incorporated distilled water instead of SAB culture broth, in contrast to the biological method in which only SAB culture broth can be used. Additionally, the biological method requires maintaining a *B. thetaiotaomicron* culture in the exponential growth phase to release efficient hydrogen production, which is not the case for the chemical method.

Finally, we compared the ability of the chemical method and the biological method to isolate methanogens from 50 saliva samples collected from 50 individuals after 9 days of incubation. The number of positive samples was 6/50 (12%) at day 3 post

Table 1
Controls of pH and redox potential.

Redox potential of the bottom compartment								
Controls	*Redox potential (mV)*				pH			
	D0	*D3*	*D6*	*D9*	*D0*	*D3*	*D6*	*D9*
Distilled water	+81.3	+64.6	+67.3	+79.9	7	6.98	6.92	6.87
SAB medium	−8.7	+11.1	+12.9	+14.8	7.3	6.83	6.78	6.27
Redox potential of the upper compartment								
Distilled water		−28.1	−81.2	−108.9		−4.5	−66	−91.6
SAB medium		+13.5	−116.5	−77.5		+19.1	−7.1	−34.9

mV = millivolts; D: day.

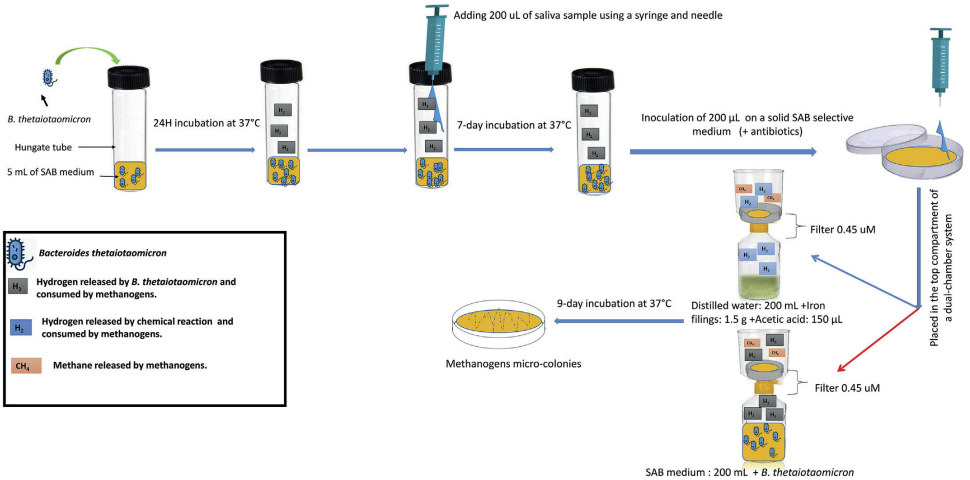

Fig. 1. Aerobic culture of methanogens in a double-chamber system on agar plates using either a microbiological method for hydrogen production (red arrow) or a chemical method for hydrogen production investigated here (blue arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Comparison of the two methods according to the average of the colonies counted by ImageJ.

Methods	Chemical method			Biological method			P-value		
	D3	D6	D9	D3	D6	D9	D3	D6	D9
M. smithii	377 ± 72	659 ± 168	1116 ± 193	0	0	436 ± 74	<0.0001	<0.001	0.0001
Saliva samples	190 ± 90	509 ± 133	700 ± 232	305 ± 139	722 ± 96	936 ± 136	0.09	0.005	0.04
Negative controls	0	0	0	0	0	0	NA	NA	NA

NA: Not adapted; D: Day.

inoculation using both methods, and one additional saliva sample was positive at day 6, so that a total of 7/50 (14%) were positive using both methods. No additional sample was positive at day 9 postinoculation, regardless of the culture method. In these 7 culture-positive saliva samples, the average number of colonies at day 6 and day 9 postinoculation was significantly higher using the biological method than using the chemical method (Student's test, $p = 0.005$ and $p = 0.04$, respectively), whereas there was no significant difference in the average number of colonies on day 3 (Student's test, $p = 0.09$) (Table 1). Cultures from 6/25 (24%) of the samples from tobacco smokers yielded colonies as early as day 3 in both methods versus 1/25 (4%) of the samples from nonsmokers at day 6 in both methods. Colonies yielded autofluorescence in both methods (Fig. 2 and Fig. 3), whereas no colony or autofluorescence was observed in the negative controls. PCR sequencing and real-time PCR identified three *M. oralis*-positive and three *M. smithii*-positive samples in the tobacco smoker samples and one *M. oralis*-positive sample in one nonsmoker sample (Supplementary Fig. 2). Applying the chemical method for the isolation of *M. oralis* from saliva samples yielded significantly more colonies more rapidly than the biological method used in parallel. We hypothesized that the chemical method allowed the kinetics of hydrogen production to be mastered in such a way to optimize the atmosphere in the double-chamber system used in our experiments. These results show that the chemical method is more effective than the biological method for the rapid culture of methanogens. Accordingly, the observation that almost a quarter of the saliva samples contained *M. oralis* and *M. smithii* is in agreement with a previously published report [22]. Interestingly, we confirmed that methanogens are more prevalent in the saliva from tobacco smokers than in the saliva collected from nonsmokers. These results are consistent with those found previously [22], in which there was also a predominance of *M. oralis* over *M. smithii* in saliva samples. These observations also confirm that *M. oralis* is the most prevalent methanogen in the oral cavity. We measured the redox potential in the culture using the chemical method to monitor whether this chemical reaction led to anaerobiosis. Our results indicate that this chemical reaction is reducing and confirm that acetic acid is an efficient oxidizer of iron filings [20]. The pH of the agar plates remained neutral throughout the inoculation period both in the chemical method and the biological method; this observation suggests that the acidity produced in the lower compartment of the double-chamber device in the chemical method does not reach the upper compartment.

The present study highlighted some advantages of the chemical method over the biological method. The chemical method is simple to set up, the hydrogen production is controlled so that the first colonies appear quickly due to the immediate hydrogen production, and this chemical method can be performed with both distilled water and SAB culture broth. Given the simplicity, speed and efficiency of this chemical method of hydrogen production, it could replace the biological method

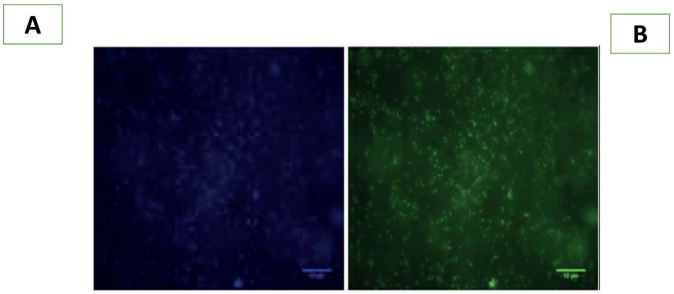

Fig. 2. (A) Fluorescence emitted by *M. smithii* by applying the blue filter set. (B) Fluorescence emitted by *M. smithii* by applying the green filter set. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

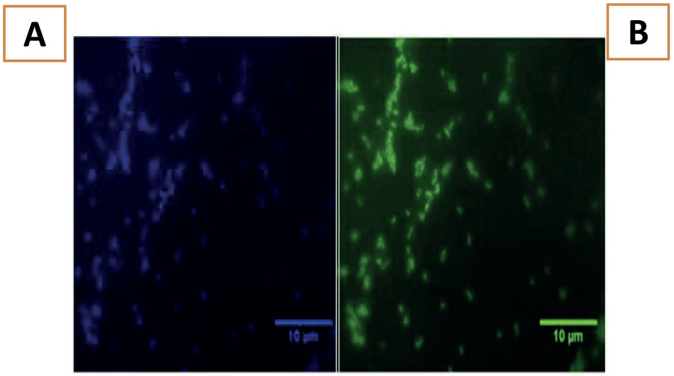

Fig. 3. (A) Fluorescence emitted by *M. oralis* by applying the blue filter set. (B) Fluorescence emitted by *M. oralis* by applying the green filter set. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

incorporating *B. thetaiotaomicron* in the isolation and culture of methanogens. Using the chemical method will improve the routine isolation and culture of methanogens in microbiology laboratories to ease and speed the isolation and culture of these opportunistic pathogens [1] and rarely encountered *Methanomassiliicoccales*, of medical interest as potential archaeobiotics [28–30].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2019.102128.

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Article 3

Rapid identification of clinically interesting methanogens using an improved MALDI-TOF-MS assay.

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To the Editor

Marseille November 29, 2021

Dear Editor,

Please find enclosed the original manuscript entitled "**Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identification of methanogens of clinical interest"** that I would like to submit to Access Microbiology journal.

The aim of this study is to establish a reliable, rapid, and reproducible diagnostic protocol to identify methanogens of clinical interest by matrix-assisted laser desorption time-of-flight mass spectrometry for use in routine diagnostic.

Thank you in advance for your consideration of this manuscript.

Sincerely Yours,

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REVISED VERSION

Rapid identification of clinically interesting methanogens using an improved MALDI-TOF-MS assay.

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ABSTRACT

Methanogens, the archaea uniquely detoxifying fermentative hydrogen into methane in the digestive tract, are increasingly detected in pathology situations, rendering their rapid identification mandatory. We improved the experimental protocol to identify broth-cultured methanogens by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS). A database incorporating 34 reference spectra derived from 16 methanogen reference strains representative of eight species, supported further identification of 21 *Methanobrevibacter smithii* and 14 *Methanobrevibacter oralis* isolates broth-cultured from human stool and oral fluid, respectively, with scores > 2. In addition, MALDI-TOF-MS differentiated five *Methanobrevibacter smithii* genotypes incorporated in the study. Data here reported found MALDI-TOF-MS as a first line identification method for methanogens recovered from microbiota and clinical samples.

Keywords: Methanogens, culture, identification, multispacer sequence typing, MALDI-TOF-MS, clinical microbiology.

1. INTRODUCTION

Methanogens are acknowledged members of the digestive tract microbiota where these strictly aero intolerant archaea are detoxifying molecular hydrogen issued from anaerobic bacterial fermentation, into methane [1,2]. They are the only sources of methane [2]. Accordingly, the oral and gut cavities of virtually all humans harbor methanogens, with respect to this indispensable physiological role , and eight different species of methanogens have been detected and cultured from the oral and intestinal microbiota [3,4]. More specifically, *Methanobrevibacter smithii* (*M. smithii*), *Methanobrevibacter oralis* (*M. oralis*) and *Methanobrevibacter massiliense* have been isolated from the oral cavity, whereas *M. smithii, M. oralis*, *Methanosphaera stadtmanae* (*M. stadtmanae*)*, Methanomassiliicoccus luminyensis* (*M. luminyensis*), *Methanobrevibacter arboriphilicus* (*M. arboriphilicus*)*,* "*Candidatus*" Methanomethylophilus alvus and "*Candidatus*" Methanomassiliicoccus intestinalis have been isolated from stools [3,5,6]. Other methanogens including *Methanosarcina mazei* (*M. mazei*), *Methanoculleus chikugoensis*, *Methanoculleus bourgensis*, *Methanobacterium congolense* have been PCR-detected in the gut microbiota but not cultured yet from clinical specimens [4].

Methanogens are emerging pathogens increasingly detected in various situations of pathology, such as dysbiosis, abscesses [2]; and more recently archaeamia [6]. In all these pathological situations, methanogens have been always detected in the presence of bacteria, including Enterobacteriaceae, Staphylococci and Streptococci.

Owing to this increasing role in diverse pathological processes supported by the isolation and culture of methanogens in the clinical microbiology laboratory, there is a demand for a routine and specific method of identification of these still unusual microorganisms in clinical microbiology. Variable microscopic morphological aspects ranging from single coccobacillary

for *M. smithii*, pairs or short chains oval rods for *M. oralis*, single cell cocci for *M. luminyensis* and pairs or tetrads cocci for *M. stadtmanae*, may not be specific enough for the accurate identification of methanogens [5]. Fluorescent *in situ* hybridization (FISH) added to specificity of the microscopic examination of methanogens in clinical specimens but is yet too laborious for a routine usage [7]. Accordingly, the firm identification of cultured methanogens still relies upon the detection of species-species DNA sequences [7,8]. However, DNA-based identification methods delay identification as current archaeal DNA extraction protocols add 3-24 hours to one-hour real-time PCR protocol or 3-hour PCR-sequencing protocol when looking for methanogen species [9]. Potential carry-over of DNA resulting in false-positive results as well as the cost of PCR-based protocols may also limit their use for the routine identification of methanogen colonies so that a more appropriate method is desired for this purpose.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is currently the first-line method for identification of bacteria and yeasts cultured in clinical microbiology as this method precisely overcomes the potential limitations of PCR-based methods as reported above [10]. In addition, the benefit of MALDI-TOF over sequencing is that the downstream is quicker, which would be important for a screening/clinical point of view[10]. The rapid and reproducible identification of some environmental archaea by MALDI- TOF-MS has been reported by some authors [11,12]. In addition, in another study, the authors succeeded in identifying by MALDI- TOF-MS some strains of methanogens of clinical interest for humans at a time when isolation and culture of methanogens was still in its infancy in clinical microbiology laboratories [13]. However, the authors of this study used a tedious protein extraction protocol including the use of glass beads during the lysis phase [13] making it very

difficult to use this method for routine identification of methanogens of clinical interest clinical microbiology laboratories.

Here, following improvements in experimental and informatic protocols, we are reporting on the MALDI-TOF-MS identification of broth-cultured methanogens, renewing interest in MALDI-TOF-MS-based identification of methanogens of clinical interest.

2. MATERIALS AND METHODS

2.1 Methanogen clinical isolates and strains.

Sixteen reference strains of methanogens available in the Collection de Souches de l'Unité des Rickettsies (CSUR, Marseille, France) were used to create a MALDI-TOF-MS reference database (Table 1). These sixteen reference strains of methanogens were subcultured in broth using a culture protocol previously established in our laboratory [14] and their identification was firmly confirmed by PCR-sequencing of the 16S rRNA gene, as previously described [15]. Further clinical isolates were made from mucosa-associated specimens as previously described [16,17]. In total, 35 clinical isolates included 21 stool isolates and 14 oral swab isolates, identified by PCR-sequencing targeting 16S rRNA Archaea and cultured in broth for nine days before being assessed by MALDI-TOF-MS against the MALDI-TOF reference database. Furthermore, we performed multispacer sequence typing (MST) of *M. smithii* 21 clinical isolates. The multispacer sequence typing technique and analysis was performed on each of the *M. smithii* clinical isolates according to the previously described protocol [9,18–20]. This part of the study was previously approved by the Ethics Committee of the University-Hospital Institute (IHU) Méditerranée Infection under n° 2016-020 and participants were given all information about the process of the study and gave informed consent before participation in this study.

2.2 Methanogens MALDI-TOF-MS reference database.

We used a protocol of the manufacturer MALDI Biotyper® (Bruker Daltonics, Wissembourg, France), adding two additional washing steps to remove the culture medium as well as extending the centrifugation time after the ethanol addition step. Briefly, one milliliter of archaeal suspension corresponding to 108 UFC/mL transferred into a sterile Eppendorf tube (Fisher Scientific, Illkirch, France) was centrifuged at 17,000 g for 30 minutes. The pellet was suspended into 500 µL of High Purity Liquid Chromatography water or HPLC water (VWR International, Strasbourg, France), vortexed, and centrifuged at 17,000 g for 10 minutes and this washing step was repeated twice. The pellet was suspended in 300 µL of HPLC water (VWR International), homogenized by pipetting, and 900 µL of ethanol absolute for HPLC Chromanorm (VWR International, Fontenay-sous-Bois, France) were added and homogenized by pipetting. After 5-min centrifugation at 17,000 g, the pellet was suspended in 50 µL of 70% formic acid and 50 µL of acetonitrile and vortexed for ten seconds. After a final 2-min centrifugation at 17000g, 1.5 µL of supernatant was deposited in one of a MALDI-TOF 96 MSP target polished steel BC ref number: 8280800 (Bruker Daltonik GmbH, Bremen, Germany) and 10 spots were deposited for each methanogen species. After drying, each spot was coated with 1.5 µL of a matrix solution consisting of saturated α-cyano-4-hydroxycynnamic acid or HCCA (C2020 Sigma, Lyon, France), 50% acetonitrile (CarboErba for HPLC) 2,5% trifluoroacetic acid or TFA (Uvasol for spectrometry, Sigma-Aldrich, Dorset, UK) and 47.5% HPLC water (VWR International). After drying in ambient air, the target plate was introduced into the Microflex LT® MALDI-TOF-MS device (Bruker Daltonics). Each spot was then analyzed with the help of FlexControl version 3.4 acquisition software and MALDI Biotyper® Compass version 4.1.80 (MBT Compass) analysis software. Positive control consisted of a protein extract Bacterial Test Standard Bruker ref:

8255343. Non-inoculated media and matrix solutions were used as negative controls. Also, to prevent any cross-contamination, every MALDI-TOF plate (Bruker Daltonics) used in the study, was thoroughly cleaned to remove any previous deposits of other microorganisms. The MALDI-TOF plate was first soaked in 70% ethanol (Absolute for HPLC Chromanorm) for 15 min then rinsed with HPLC water (VWR International). We subsequently stripped the MALDI-TOF plate with 500 µL of 80% TFA (Sigma-Aldrich) while gently scrubbing and then rinsed with HPLC water (VWR International), to thoroughly clean the plate under a chemical hood.

2.3 Reference MALDI-TOF profiling reproducibility.

For each of the sixteen methanogen reference strains tested, ten deposits were made on MALDI-TOF-MS plate and all experiments were performed on day 9 of culture corresponding to the optimal growth period approved by Ct score of 18 using Real-time PCR [14,17,19].

2.4 Blind MALDI-TOF identification and clustering of methanogen clinical isolates.

MALDI Biotyper® version 4.1.80 software (Bruker Daltonics) was used to create reference spectra for blind identification.

Spectral references (MSP) are created using Biotyper® MSP Creation Standard Method. Five raw spectra were used for creating an MSP for each species. We performed the experiments in triplicate and a total of 34 MSP were found to be of very good quality and therefore used as a reference database. We compared the 35 clinical isolates to this database.

3. RESULTS AND DISCUSSION

We improved the MALDI-TOF-MS protocol for identifying cultured methanogens after a specific database was created, allowing for the rapid and routine identification of colonies issued from clinical specimens collected from mucosa-associated specimens and pathological

specimens. The data here reported were validated by the negativity of the negative controls introduced in every one of the experimental steps and the reproducibility of data which were correlated with PCR-sequencing molecular identification used as the gold standard.

The database was created after three independent runs, each incorporating 10 spots for each one of 16 methanogen reference strains. We obtained a total of 270 spectra and 135 spectra (50%) analyzed of good quality, were incorporated in the database. We therefore set up a database with 34 reference spectra. We then blindly tested the identification of these 16 reference strains using the updated local database by analyzing five spots for each of them. We obtained a correct identification of these different spots with scores > 2 for each of the reference strains. We observed a unique protein profile for each one of methanogen reference strains studied (Fig.1 and Fig.2). We did not observe any spectra on the negative controls (Fig.3). There are fifteen different MST genotypes of *M. smithii* among *M. smithii* strains known in humans according to a previous study [18]. The multispacer sequence typing is used as the first-line method for genotyping *M. smithii* strains found in humans. It allows the differentiation of *M. smithii* strains isolated from humans [18]. We observed a difference in the number and intensity of spectra between *M. smithii* strains from different MST genotypes (Fig.2B). These observations proved the reproducibility and the inter-species specificity of the method. These data confirmed the ability of MALDI-TOF-MS to identify archaea cultured in broth, as previously reported in a proof-of-concept study using a difficult protein extraction protocol [13]. We further tested our database against all the MALDI-TOF-MS databases available in our laboratory, incorporating 16,908 spectra representative of 12,290 different microbial species i.e., 4,335 bacteria, 5,989 parasites and 1,966 fungi, and no identification was observed which proved the specificity of our spectra and an absence of contaminating spectra. Genotyping the 21 clinical isolates of *M.*

smithii revealed five different genotypes. Genotype 1 was found in 10/21 (47.76%); genotype 2 in 5/21 (23.80%); genotype 3 in 3/21 (14.28%); genotypes 5 in 2 /21 (9.52%) and genotype 6 in 1/21 (4.76%). These results confirm the predominance of the MST1 genotype in clinical isolates of *M. smithii* found in the human digestive tract [9,19].

We then used the reference database for the identification of clinical isolates of *M. smithii* and *M. oralis* obtained by broth culture of human stool and oral fluid, respectively. We obtained a correct identification of all the clinical isolates tested with scores >2 i.e., 21/21 (100%) for *M. smithii* and 14/14 (100%) for *M. oralis,* respectively. We noticed that for a good identification by MALDI-TOF-MS of the *M. smithii* species, it is necessary to incorporate a larger set of reference spectra of *M. smithii* of different MST genotype in the reference database due to the diversity observed within this species [9,18,19], which results in different spectral profiles depending on each MST genotype as obtained in our study. To confirm this hypothesis, we repeated the blind test with only two reference strains of *M. smithii* with different MST genotype i.e., *M. smithii* CSURP5816 of MST genotype 3, and *M. smithii* CSURP5922 of MST genotype 2. We observed that with these two reference strains, we obtained only 23% (5/21) of correct identification with scores> 2 and 19% (4/21) with scores> 1.7. Our results demonstrate the need for rapid expansion of the MALDI-TOF-MS database to incorporate isolates of clinical interest.

The results here confirmed the proof-of-concept study carried out in our laboratory in which MALDI-TOF-MS was able to perfectly identify the methanogens and environmental archaea [13]. We further observed that MALDI-TOF-MS could differentiate between different genotypes of *M. smithii*, opening the possibility to use MALDI-TOF-MS as a first line typing method for this species of prime clinical interest. We are implanting the experimental protocol and MALDI-TOF-MS methanogen database in the workflow of our clinical microbiology

196 laboratory, for the routine, rapid identification of methanogens of clinical interest. The MALDI-
197 TOF MS database created in this study are available on the website of the University-Hospital
198 Institute (IHU) Méditerranée Infection on the link https://www.mediterranee-
199 infection.com/acces-ressources/base-de-donnees/urms-data-base/. This database is accessible to
200 the entire scientific community.

ETHICS APPROVAL

Before using mucosa-associated specimens for culture, we have obtained the approval from the Ethics Committee of the University-Hospital Institute (IHU) Méditerranée Infection under n° 2016-020 and participants were given all information about the process of the study and gave informed consent before participation in this study.

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AUTHOR CONTRIBUTIONS

COG Collection of samples, study design, manipulations, data information, results interpretation, draft writing; LA Study design, data information; CC Results interpretation, draft writing; MD Supervision, study design, draft writing; GG Supervision, study design, draft writing.

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COG benefits from a PhD training course grant from Fondation Méditerranée Infection, Marseille, France.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare for this work. In particular, the Brucker Daltonics society did not interfere at all with any decision regarding the experimental work, nor the decision to publish data.

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290 **TABLE**

291 **Table 1.** Methanogen reference strains used in this study.

292

SPECIES	GENOTYPES	SOURCES	CSUR NUMBERS	ORIGINS
Methanomassiliicoccus luminyensis	NA	IHU [21]	CSURP9636	Human stool
Methanosphaera stadtmanae	NA	DSMZ	CSURP9634	Human stool
Methanobrevibacter arboriphilicus	NA	DSMZ	CSURP9635	Human stool
Methanobrevibacter smithii	Genotype 3	IHU [16,17]	CSURP5816	Human stool
Methanobrevibacter smithii	Genotype 2	IHU [16,17]	CSURP5922	Human stool
Methanobrevibacter smithii	Genotype 5	IHU [16,17]	CSURQ5493	Human stool
Methanobrevibacter smithii	Genotype 1	IHU [16,17]	CSURQ5497	Human stool
Methanobrevibacter smithii	Genotype 6	IHU [16,17]	CSURQ5501	Human stool
Methanobrevibacter oralis	NA	IHU [16,17]	CSURP5701	Oral fluid
Methanobrevibacter oralis	NA	IHU [16,17]	CSURQ5479	Oral fluid
Methanobrevibacter oralis	NA	IHU [16,17]	CSURQ5481	Oral fluid
Methanobrevibacter oralis	NA	IHU [16,17]	CSURQ5483	Oral fluid
Methanobrevibacter oralis	NA	IHU [16,17]	CSURQ5485	Oral fluid
Methanosarcina mazei	NA	DSMZ	CSURP9637	Environment
Methanosarcina barkeri	NA	DSMZ	CSURP9601	Environment
Methanobacterium beijingense	NA	DSMZ	CSURP9638	Environment

293 NA: Not applicable.

294 IHU: University-Hospital Institute.

295 DSMZ: German Collection of Microorganisms and Cell Cultures.

296

297 **FIGURES**

298 **Figure 1.** Comparison of the sixteen reference methanogen strains MALDI-TOF- MS spectra
299 using MALDI Biotyper® version 4.1.80 software (Bruker Daltonics). Normalized gel view
300 representation of MS profile intensity of the sixteen reference methanogenic strains used in this
301 study.

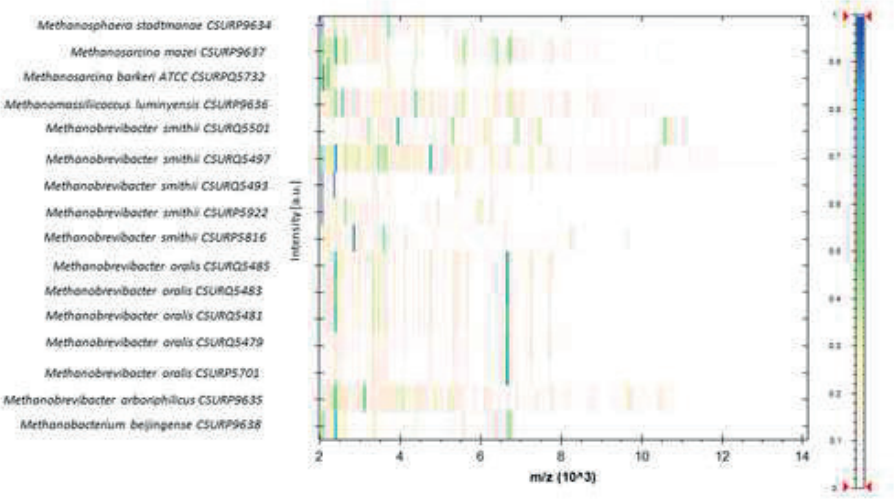
302 a.u., arbitrary units; m/z, mass-to-charge ratio.

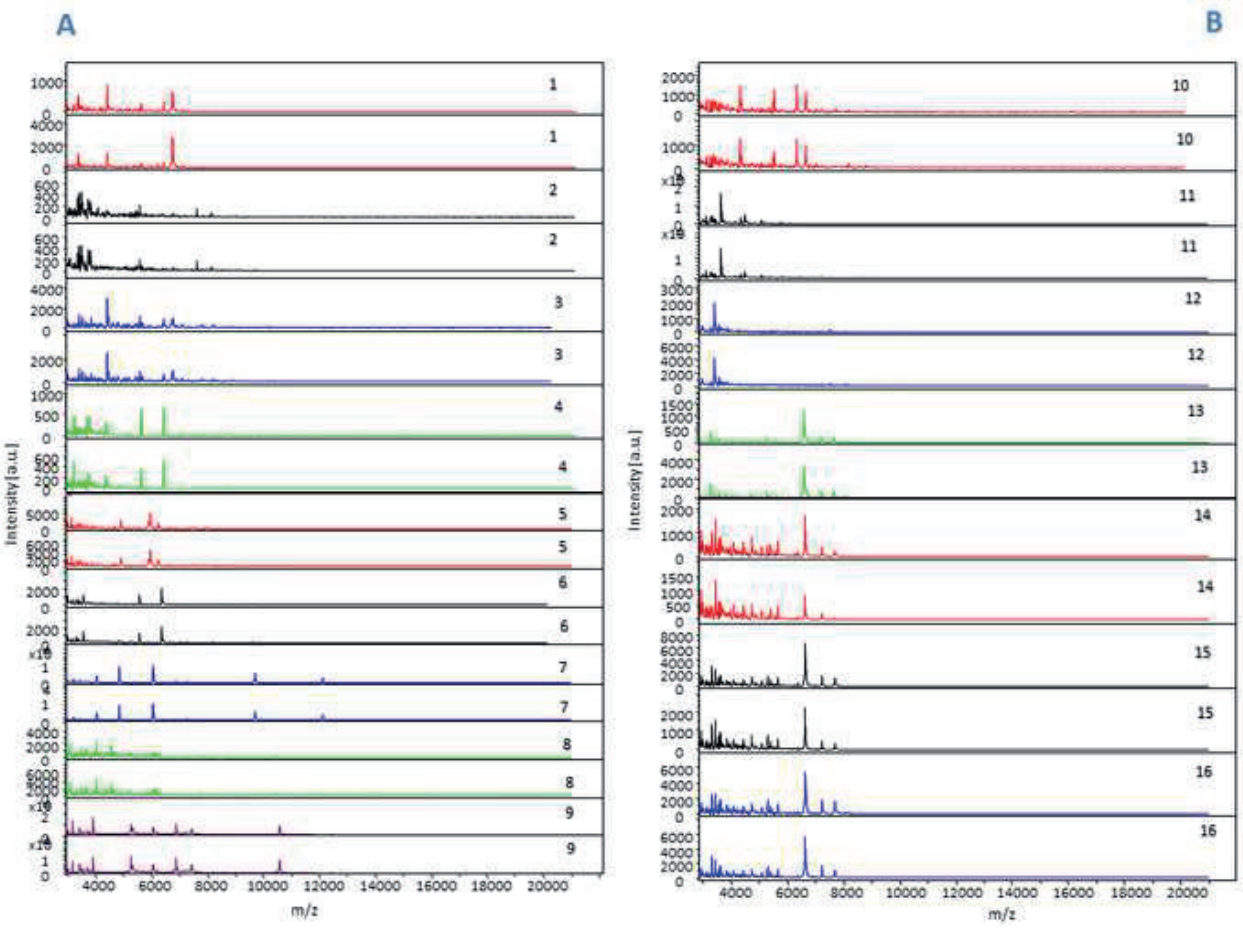
303 **Figure 2.** Representative MS profiles of the sixteen methanogen reference strains studied using
304 FlexAnalysis software. **(1).** MS profiles of *M. beijingense* CSURP9638. **(2).** MS profiles of *M.*
305 *arboriphilicus* CSURP9635. **(3).** MS profiles of *M. luminyensis* CSURP9636. **(4).** MS profiles of
306 *M. barkeri* CSURQ5732. **(5).** MS profiles of *M. smithii* CSURP5922. **(6).** MS profiles of *M.*
307 *smithii* CSURP5816. **(7).** MS profiles of *M. smithii* CSURQ5493. **(8).** MS profiles of *M. smithii*
308 CSURQ5497. **(9).** MS profiles of *M. smithii* CSURQ5501. **(10).** MS profiles of *M. mazei*
309 CSURP9637. **(11).** MS profiles of *M. stadtmanae* CSURP9634. **(12).** MS profiles of *M. oralis*
310 CSURP5701. **(13).** MS profiles of *M. oralis* CSURQ5479. **(14).** MS profiles of *M. oralis*
311 CSURQ5481. **(15).** MS profiles of *M. oralis* CSURQ5483. **(16).** MS profiles of *M. oralis*
312 CSURQ5485. The sixteen methanogen reference strains are presented in duplicate to
313 demonstrate reproducibility of MS profiles.

314 a.u., arbitrary units; m/z, mass-to-charge ratio.

315 **Figure 3.** Negative controls used in the MALDI-TOF-MS experiment. Non-inoculated media in
316 blue and matrix solution in red.
317 a.u., arbitrary units; m/z, mass-to-charge ratio.

318





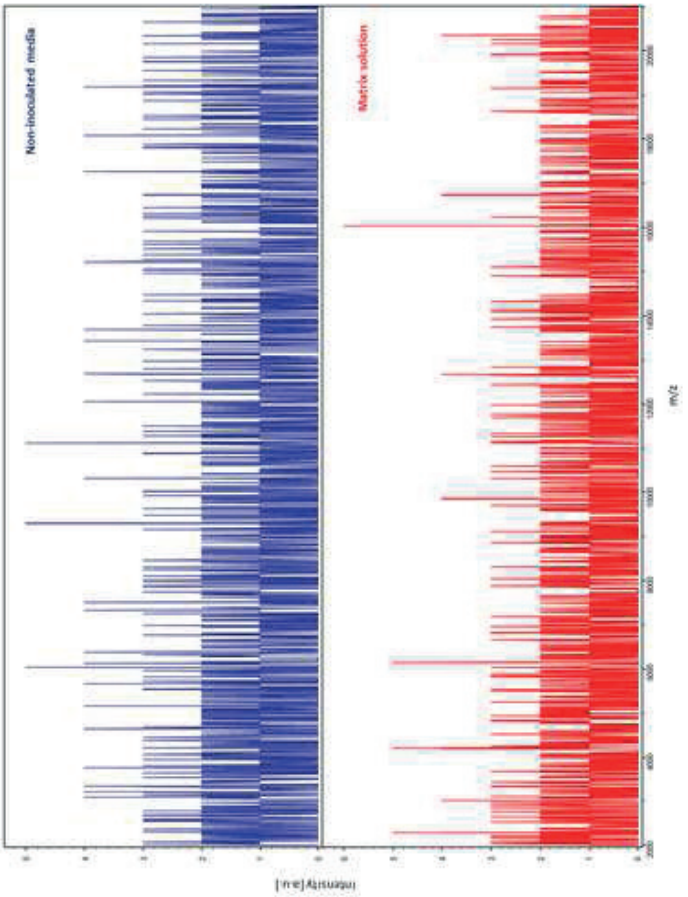


Figure 3

Chapitre III : Sources et la dynamique d'acquisition des méthanogènes chez l'Homme

Préambule

La présence de méthanogènes dans le microbiote digestif d'un nouveau-né serait liée en partie au contact avec la maman et à l'allaitement maternel [6]. Une transmission materno-fœtale au cours de la grossesse pourrait aussi être évoquée en raison de la présence de *M. smithii* dans le suc gastrique des nouveau-nés de moins de 24h en absence de toute vaginose [7]. De plus, des études antérieures ont prouvé la présence de bactéries dans le liquide amniotique, le sang du cordon ombilical, le méconium, les membranes placentaires et fœtales en absence de toute infection materno-fœtale [57–73]. Nous avons donc utilisé une approche polyphasique incluant la microscopie et la PCR-séquençage pour investiguer la présence des méthanogènes dans le méconium chez des nouveau-nés prématurés. Nous avons démontré pour la première fois la présence de *M. smithii* dans le méconium montrant ainsi que c'est réellement la période intra-utérine qui constitue le premier moment d'acquisition de ce méthanogène chez l'Homme.

Les méthanogènes font aussi partie du microbiote des mammifères non humains, notamment la vache [74–89]. Une étude récente a démontré une association entre l'acquisition de *M. smithii* chez les enfants et la consommation de produits laitiers [90]. Nous avons caractérisé par biologie moléculaire, les méthanogènes du microbiote digestif des animaux domestiqués par l'Homme ainsi que ceux des produits laitiers pour vérifier si le contact avec les animaux et/ou la consommation du lait/produits laitiers de certains animaux, en particulier la vache pouvaient être considérés comme l'une des sources possibles d'acquisition des méthanogènes chez l'Homme. Nous avons détecté dans le microbiote digestif de ces animaux domestiques et dans les produits laitiers, des méthanogènes présents dans le microbiote digestif humain. Ces résultats ont permis de confirmer que le contact étroit avec des animaux domestiques ainsi que la consommation du lait/ produits laitiers de certains animaux est l'une des sources probables de diversification des méthanogènes chez l'Homme.

Article 4

Meconial *Methanobrevibacter smithii* suggests intrauterine methanogen colonization in preterm neonates.

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Meconial *Methanobrevibacter smithii* suggests intrauterine methanogen colonization in preterm neonates

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ABSTRACT

To understand the dynamics of methanogens in the human intestinal microbiota, we investigated the presence of methanogens in meconium using a polyphasic approach including microscopy and PCR-sequencing in 33 meconium samples collected from 33 pre-term neonates, in accordance with current ethics regulation. In the presence of negative controls, 90.9% samples were real-time PCR-positive for methanogens and 69.7 % were PCR-sequencing positive, identified as *Methanobrevibacter (M.) smithii*. Further, auto-fluorescent analysis detected methanogens in the two meconium samples analyzed, with a morphology suggesting *M. smithii*. Multispacer Sequence Typing found *M. smithii* genotypes ST1 and ST2, previously described as intestinal microbiota inhabitants. C-section delivery and non-use of peripartum antibiotics significantly correlated with PCR-detection of methanogens in meconium. These data position *M. smithii* among the early inhabitants of the human gut, detectable immediately after birth and suggest the contribution of methanogens to the perinatal development of intestinal microbiota and physiology.

Introduction

Meconium, the first stool of the newborn, lines the intestinal tract of the fetus during pregnancy (Gosalbes et al., 2013). Its composition varies during fetal development and comprises 72 to 80% water, intestinal secretions, cellular desquamations, bile pigments, inflammatory proteins, and blood (Gosalbes et al., 2013). Meconium, found in the fetal digestive system at the end of the first trimester of pregnancy, results from the ingestion of amniotic fluid during pregnancy (Gosalbes et al., 2013; Hu et al., 2013; Moles et al., 2013). Physiologically, the emission of the first meconium occurs 24 to 48 hours after birth, being most often expelled by the newborn at the time of birth reflex and usually abundantly (Gosalbes et al., 2013; Hu et al., 2013; Moles et al., 2013). In some studies, bacteria have been isolated and/or detected by Polymerase Chain Reaction (PCR) in amniotic fluid (Bearfield et al., 2002; Hitti et al., 1997; Oh et al., 2010), umbilical cord blood (Jiménez et al., 2005), meconium (Ardissone et al., 2014; Dominguez-Bello et al., 2010; Gosalbes et al., 2013; Hu et al., 2013; Jiménez et al., 2008; Madan et al., 2012; Moles et al., 2013; Mshvildadze et al., 2010), placenta (Aagaard et al., 2014; Satokari et al., 2009; Stout et al., 2013), and fetal membranes (Rautava et al., 2012; Steel et al., 2005) without any clinical or histological evidence of infection or inflammation in the mother or the newborn. However, intestinal methanogens have recently gained attention as players of immune-mediated diseases (Sereme et al., 2019).

Methanogens are strict aero-intolerant archaea that produce methane in the presence of H_2, CO_2 and other substrates (Guindo, 2020; Nkamga et al., 2017; Sereme et al., 2019; Sogodogo et al., 2019). They are divided into three groups according to the substrates used in the production of methane: hydrogenotroph methanogens use CO_2 and formate as substrates; methylotroph methanogens use methyl com-

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Table 1
Clinical data for 33 premature newborns here investigated for the presence of meconial methanogens.

Code	Peripartum maternal antibiotic therapy	Mode of delivery	Gestational age	Weight	Size
1	Yes	Cesarean section	26	925	35
2	No	Cesarean section	32	1260	39
3	No	Vaginal delivery	30	1480	41
4	No	Vaginal delivery	30	1460	38
5	No	Cesarean section	29	1565	42
6	No	Cesarean section	29	880	35
7	Yes	Vaginal delivery	30	1670	43
8	No	Cesarean section	31	1120	38
9	No	Cesarean section	24	530	31
10	No	Cesarean section	28	890	33
11	No	Cesarean section	26	925	35
12	No	Cesarean section	26	565	31
13	Yes	Cesarean section	27	925	34
14	Yes	Cesarean section	27	680	31
15	Yes	Cesarean section	30	1335	39
16	Yes	Cesarean section	30	1355	47
17	No	Cesarean section	30	1480	39
18	No	Cesarean section	31	1570	43
19	No	Cesarean section	32	1155	39
20	No	Cesarean section	29	1050	39
21	No	Cesarean section	29	1190	38
22	No	Cesarean section	32	1930	44
23	No	Cesarean section	32	1575	44
24	No	Cesarean section	25	870	34
25	No	Cesarean section	30	1750	43
26	No	Cesarean section	30	1360	39
27	No	Cesarean section	27	600	29
28	No	Vaginal delivery	25	750	32
29	No	Cesarean section	31	980	36
30	No	Cesarean section	31	1410	39
31	No	Cesarean section	30	770	33
32	Yes	Vaginal delivery	32	1568	41
33	No	Cesarean section	30	1680	39

pounds as substrates; and acetogenotroph methanogens use acetate as substrate (Guindo, 2020; Nkamga et al., 2017; Sereme et al., 2019; Sogodogo et al., 2019). Methanogens are part of the human microbiota, in particular the intestinal representing 10 % of the anerobic microorganisms in the human digestive tract and oral microbiota (Guindo, 2020; Nkamga et al., 2017; Sereme et al., 2019; Sogodogo et al., 2019). They have even been found in colostrum and breast milk (Togo et al., 2019), in blood during infectious endocarditis (Drancourt et al., 2020), in vagina only in case of vaginosis (Grine et al., 2019a) and in urinary tract during urinary tract infections (Grine et al., 2019b). They are present in humans from birth (Grine et al., 2017).

Detection of methanogens requiring specific laboratory methods is not routinely developed in clinical microbiology. Accordingly, the detection of methanogens in the meconium has not been reported. Here, as part of a clinical research protocol on the development of the intestinal microbiota and immune status in a cohort of premature infants, we have used such specific methods we are mastering, to explore the presence of methanogens in the meconium of premature newborns.

Results

Clinical data

Thirty-three meconium samples collected from 33 pre-term neonates were investigated in this study. The mean birth weight was 1.190 g (range 440–2.130 g), mean gestational age of 30 weeks (range 25–32 weeks) and mean height of 39 cm (range 32–45 cm) (Table 1). Of these 33 subjects, 5/33 (15.15%) were moderate preterm premature, 19/33 (57.57%) were very preterm, 9/33 (27.27%) were extremely preterm, 28/33 (84.84%) were from C-section delivery, 5/33 (15.15%) were from vaginal delivery, and 7/33 (21.21%) were from mothers with a history of peripartum antibiotic therapy (Table 2).

Methanobrevibacter smithii *is very frequently detected in meconium by PCR*

DNA extraction yielded 22.04 ± 4.96 ng/μL and incorporating 16S rRNA archaeal gene PCR primers recently designed in our laboratory into real-time PCR, we detected the presence of methanogen DNA in 30 (90.9%) of meconium samples here investigated and none of the negative controls. PCR-sequencing yielded *M. smithii* in 23 cases (69.69%) and sequences exhibited a 99.5% similarity with the reference 16S rRNA gene sequence of *M. smithii* ATCC 35061 (accession NCBI: NR_074235). The phylogenetic tree showed that all these 23 sequences clustered with *M. smithii* previously detected in the human digestive tract (Fig. 1). MST genotyping revealed the presence of genotype ST1 in 22/23 (95.65%) meconium sample, and the genotype ST2 in one meconium sample (4.34%) (Table 3).

Microscopic observation is compatible with the presence of M. smithii *in meconium*

By using confocal microscopy, we were able to observe fluorescent microorganisms exhibiting a green fluorescent and diplococcus morphology characteristic of *M. smithii* supporting the presence of methanogens in two meconium specimens (Fig. 2)

The presence of methanogens in meconium is correlated with c-section delivery

Further exploitation of the five available clinical variables (peripartum maternal antibiotic therapy, mode of delivery, gestational age, sex and weight) indicated no significant correlation between gestational age ($p$value = 0.318), weight at birth ($p$value = 0.229), sex ($p$value = 0.476) and the detection of methanogens in meconium specimens. However, we observed a significant correlation between the detection of methanogens

Table 2
Distribution of newborns according to gestational age, mode of delivery and maternal antibiotic intake.

Moderate preterm	Very preterm	Extremely preterm	Cesarean section	Vaginal delivery	Peripartum maternal antibiotic therapy
5/33(15.15%)	19/33(57.57%)	9/33(27.27%)	28/33(84.84%)	5/33(15.15%)	7/33(21.21%)

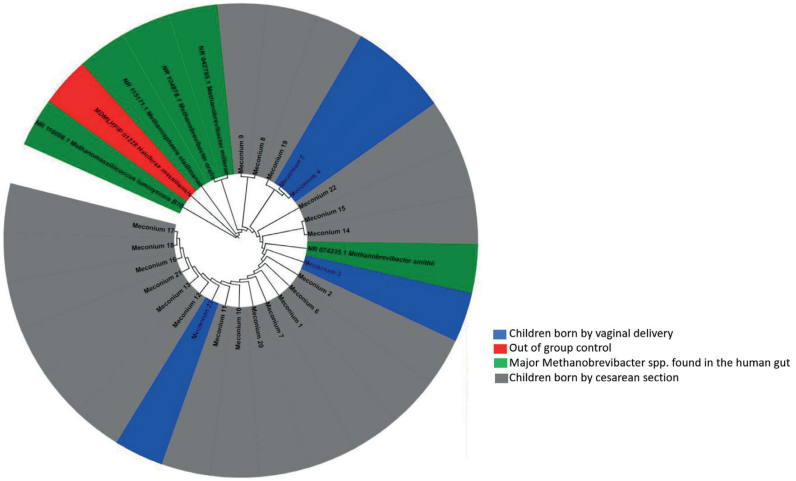

Fig. 1. Molecular phylogenetic analysis, based on 16S rRNA partial gene, showed the position of *Methanobrevibacter smithii* sequences detected in meconium samples. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1716.60) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 473 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.

Table 3
Multispacer sequence typing of 23 *Methanobrevibacter smithii* detected in 23 meconium preterm babies.

Samples	Peripartum maternal antibiotic therapy	Mode of delivery	Gestational age	Genotype				
				Spacer1	Spacer2	Spacer 3	Spacer 4	Spacer type*
1	Yes	Cesarean	26	X	X	X	X	1
2	No	Cesarean	32	X	X	X	X	1
3	No	Vaginal delivery	30	X	X	X	X	1
4	No	Vaginal delivery	30	X	X	X	X	1
5	Yes	Vaginal delivery	30	X	X	X	X	1
6	No	Cesarean	28	X	X		X	2
7	Yes	Cesarean	27	X	X	X	X	1
8	Yes	Cesarean	30	X	X	X	X	1
9	No	Cesarean	30	X	X	X	X	1
10	No	Cesarean	31	X	X	X	X	1
11	No	Cesarean	32	X	X	X	X	1
12	No	Cesarean	29	X	X	X	X	1
13	No	Cesarean	29	X	X	X	X	1
14	No	Cesarean	32	X	X	X	X	1
15	No	Cesarean	32	X	X	X	X	1
16	No	Cesarean	25	X	X	X	X	1
17	No	Cesarean	30	X	X	X	X	1
18	No	Cesarean	30	X	X	X	X	1
19	No	Cesarean	27	X	X	X	X	1
20	No	Vaginal delivery	25	X	X	X	X	1
21	No	Cesarean	31	X	X	X	X	1
22	No	Cesarean	31	X	X	X	X	1
23	Yes	Vaginal delivery	32	X	X	X	X	1

*Spacer type was determined according to references (Grine et al., 2017; Guindo et al., 2021; Nkamga et al., 2015).

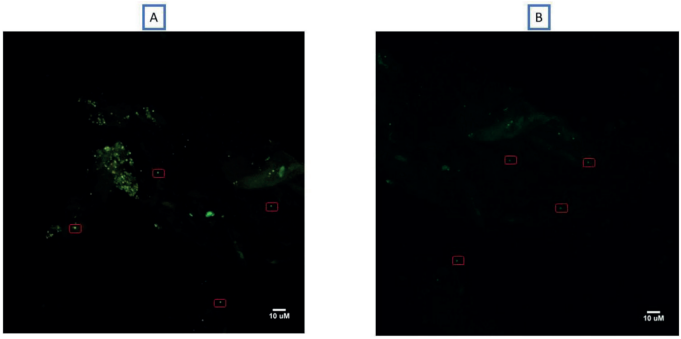

Fig. 2. Fluorescent microorganisms exhibiting a green fluorescent and diplococcus morphology characteristic of *M. smithii* from two meconium samples (A and B) using a confocal microscope at 63X magnification.

Table 4
Results of Principal Component Analysis (PCA) (Results of PCA. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1).

	Estimate	Standard error	zvalue	Statistical significance
Intercept	-4.0665	1.1784	-3.451	0.0005***
Peripartum maternal antibiotic therapy (No)	0.8014	1.3026	0.615	0.0381*
Peripartum maternal antibiotic therapy (Yes)	-0.3257	1.104	-0.295	0.7679
Cesarean section delivery	2.4192	1.233	1.962	0.0049**
Vaginal delivery	-0.3673	1.3532	0.271	0.7769
Gestational age	1.4219	1.4268	0.997	0.3189
Weight	-0.3642	1.3234	-0.292	0.2292
Sex	0.671	1.822	0.152	0.4766

in meconium specimens and c-section delivery (*p*value = 0.004) and with non-use of antibiotic peripartum (*p*value = 0.038) (Table 4).

Discussion

We are reporting the first ever detection in the meconium of the methanogen *M. smithii*, firmly identified by a polyphasic approach including microscopy and PCR-sequencing. All data here reported were ascertained by the negativity of negative controls and the fact that concordant results were obtained by different techniques.

The data here reported are in line with previous detection of *M. smithii* in 50/50 (100%) of gastric juice samples collected from one-day-old newborns (Grine et al., 2017), pushing back the colonization of the newborn digestive tract most probably during the *in-utero* period of life; questioning the sources of such a colonization. In this perspective, herein genotyping meconial *M. smithii* using the sequence-based MST method yielded ST1 previously found in one-day newborns' gastric juice and in adult gut microbiota (Grine et al., 2017; Nkamga et al., 2015); and ST2 also part of the adult gut microbiota (Nkamga et al., 2015). Viable *M. smithii* and *Methanobrevibacter oralis* (*M. oralis*) colonize the colostrum and the mother milk (Togo et al., 2019) but *M. oralis* was not detected in meconium in the present study, in line with its lack of detection in one-day newborns' gastric juice (Grine et al., 2017). This observation and the fact that, here, meconium samples have been collected prior to any feeding, plea against the hypothesis that mother milk was the source of *M. smithii* in preterm newborns. Likewise, *M. smithii* has been detected in the vaginal fluid only in the case of vaginosis, making the vaginal fluid an unlikely source of *M. smithii* in this study (Grine et al., 2019a). Accordingly, meconial *M. smithii* was here significantly associated with cesarean section, further ruling-out the vaginal fluid as a source of *M. smithii* (Figure 3). It has been suggested that, during pregnancy, gut microorganisms translocating through the intestinal epithelium move to the placenta via the bloodstream (Rodríguez et al., 2015). *M. smithii* could also be found in the blood, after we recently reported a series of *M. smithii* archaemia in febrile adult patients, including three cases of infectious endocarditis (Drancourt et al., 2020). Indeed, dendritic cells present in the intestinal barrier, do recover bacteria as well as the intestinal methanogens *Methanosphaera stadtmanae* and *M. smithii*, eventually transported to lymphoid organs (Rodríguez et al., 2015). There, methanogens activate the adaptive immune response, as illustrated in rabbit and mouse models of immunization (Macario et al., 1984, 1983) (Figure 3).

In conclusion, this study demonstrating the presence of *M. smithii* as an *in-utero* member of the gut microbiota. This finding, together with previous literature showing the absence of *M. smithii* in infant life-threatening kwashiorkor (Million et al., 2016), suggests that *M. smithii* is an early and crucial player of gut microbiota and immunity in infants.

Methods

Patients and sampling

Premature newborns were included in the "Influence of Intestinal Microbiota Implantation in Preterm Infants on Microbiota and Immune Orientation at 3 Years" (NCT02738411, principal investigator AF) cohort after written informed parental consent was obtained for each preterm. This research project was approved by the Ethics Committee on Clinical Research of Nîmes and Montpellier University Hospitals. To be eligible for enrolment, preterm neonates must have been born at a gestational age ≤ 32 weeks. First spontaneously evacuated meconium was collected by the medical staff at the Nîmes, and Montpellier University Hospitals noticed peripartum maternal antibiotic therapy, mode of delivery, gestational age, and weight of premature newborns (Table 1). Collections were done between May and September 2018. Before sampling, the pediatrician washed his or her hands with alcoholic solution and then put gloves on before manipulating meconium samples and

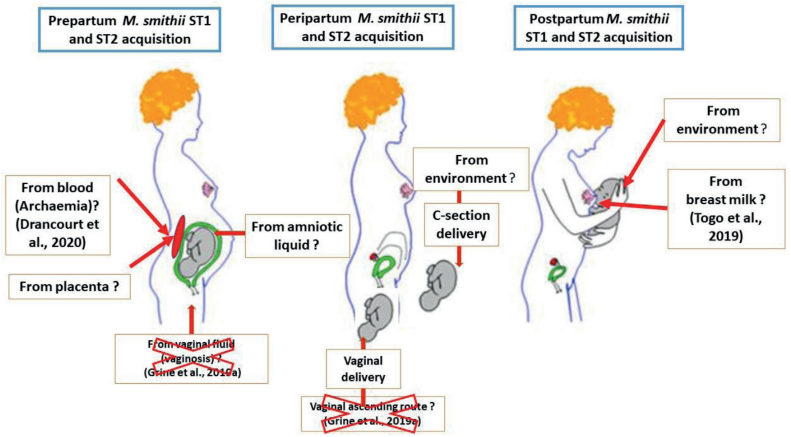

Fig. 3. Peripartum hypothetical routes of transmission of *M. smithii* in preterm infants. In this study, *M. smithii* were genotyped using multi-spacer typing, indicating spacer type 1(ST1) and spacer type 2 (ST2). Hypothetical routes of transmission of *M. smithii* prior, during and after delivery of preterm infants were derived from data gathered from this study and up-to-date literature (references are shown in brackets). Unlikely routes of transmission are indicated by red crosses.

each meconium sample was transferred from a diaper to one sterile Falcon tube (Sigma-Aldrich, Saint Quentin Fallavier, France) using a sterile tongue depressor by the medical staff and stored at -80°C until analysis.

Molecular analysis

DNA extraction was performed as described previously (Dridi et al., 2009). Briefly, 0.2 g of each meconium sample has been mixed with 500 μL of G2 buffer (QIAGEN, Hilden, Germany), then, shaked with 0.3 g of acid-washed beads $\leq$ 106 μm (Sigma-Aldrich, Saint-Quentin Fallavier, France) in a FastPrep BIO 101 device (MP Biomedicals, Illkirch, France) for 45 s. 20 µL of proteinase K (QIAGEN) was added to a volume of 180-µL mixture, then incubated 56 °C overnight. Total DNA was finally extracted with the EZ1 Advanced XL extraction kit (QIAGEN) and 50 μL eluted volume. In each DNA extraction run, we used sterile PBS as a negative control.

Once the DNA Extracted, a real-time PCR targeting a 156 pb 16S rRNA regions (691 and 843) was performed using Metha_16S_2_MBF: 5′-CGAACCGGATTAGATACCCG -3′ and Metha_16S_2_MBR: 5′-CCCGCCAATTCCTTTAAGTT-3′ primers and the FAM_Metha_16S_2_MBP 6FAM- CCTGGGAAGTACGGTCGCAAG probe targeting the 16S DNA gene of methanogens (Eurogentec, Angers, France) designed according the following steps, the 16S rRNA gene of *Methanobrevibacter smithii* ATCC 35061 (GenBank accession number CP000678), *Methanosphaera stadtmanae* DSM 3091 (GenBank accession number NC 007681), *Methanobrevibacter oralis* M2 CSUR P5920 (GenBank accession number GCA_900289035.1), *Methanobrevibacter arboriphilus* ANOR1 (GenBank accession number GCA_000513315.1), *Methanomassiliicoccus luminiyensis* B10 (GenBank accession number GCA_000308215.1) was targeted using MEGA7 software (https://www.megasoftware.net/). Using the online Primer 3 program(http://biotools.umassmed.edu/bioapps/primer3_www.cgi), we found that all these published genomes exhibit only one copy of the 16S rRNA gene. The specificity of the PCR primers and probes have been verified by testing experimentally the DNA extracted from 30 bacterial species representative of common gut inhabitants and *in silico* using the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

The real-time PCR amplification reaction was performed as previously described (Guindo et al., 2021) following this program: 50 °C for 2 min, followed by 39 cycles of 95°C for 45 s, 95 °C for 5 s and finally 60 °C for 30 s. The amplifications were carried out in CFX96 thermocycler (BioRad, Marnes-la-Coquette, France). We considered as positive all meconium samples which PCR exhibited a CT<40. Gene amplification and PCR sequencing were performed as previously described (Grine et al., 2018, 2017; Guindo et al., 2020; Nkamga et al., 2015).

Multispacer sequence typing

The multispacer sequence typing (MST) technique was performed on meconium as previously described (Grine et al., 2017; Guindo et al., 2021; Nkamga et al., 2015). Briefly, all positive PCR products were sequenced in both directions using the same primers as used for PCRs in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) with an initial 1-minute denaturation step at 96°C, followed by 25 cycles denaturation for 10-second each at 96°C, a 20-second annealing step at 50°C and a 4-minute extension step at 60°C. Sequencing products were purified using the MultiScreen 96-well plates Millipore (Merck, Molsheim, France) containing 5 % of Sephadex G-50 (Sigma-Aldrich) and sequences were analyzed on an ABI PRISM 31309 Genetic Analyzer (Applied Biosystem) and edited using the ChromasPro software (version 1.42; Technelysium Pty Ltd). MST genotypes were defined as a unique combination of the four spacer sequences (Grine et al., 2017; Guindo et al., 2021; Nkamga et al., 2015).

Phylogenetic analyses

Sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Molecular phylogenetic and evolutionary analyses were conducted in MEGA7 as previously described (Kumar et al., 2016). We used sequences of the major methanogens present in the human digestive tract (*Methanobrevibacter smithii, Methanosphaera stadtmanae, Methanobrevibacter oralis, Methanobrevibacter millerae* and *Methanomassiliicoccus luminiyensis*) in the construction of the phylogenetic trees. The non-methanogen archaea species *Haloferax massiliensis* was used as an out-of-group control.

Direct microscopic examination

Based on factor 420 carried by methanogens (Dridi, 2012), the presence of methanogens in meconium has been investigated by confocal microscopy on the only two fresh meconium samples as follows. Briefly, meconium suspension was prepared with distilled water. A drop of the prepared meconium suspension deposited on a microscopy slide was observed at 63X magnification using a confocal microscope (LSM800 Airyscan Zeiss, Oberkochen, Germany).

Statistical analyses

All statistical processes were done using the open-source statistical language R (R Development Core Team, 2010). The threshold of 0.05 was the maximal p-value for each statistical conclusion. The model hypothesis was that the presence of methanogens could be associated with the peripartum maternal antibiotic therapy, mode of delivery, gestational age, sex and weight. We tested this hypothesis using a Principal Component Analysis (PCA) (Groth et al., 2013) with the functions of the FactoMineR (https://cran.rproject.org/web/packages/FactoMineR/index.html) and factoextra (https:/cran.r-project.org/web/packages/factoextra/index.html).

Authors contributions

YS and COG conducted the experiments, analyzed the data and wrote the paper; AF and TA contributed to the collection of samples; JV, PC and MD designed the project, participated in the writing of the paper and provided great support carrying out the experiments; GG designed the project, helped conduct the experiments and participated in the writing of the paper.

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Declaration of Competing Interest

The authors declare no competing interests in relation to this study. Outside this study, JV reports speaker and consultancy fees in the past 5 years from Meda Pharma (Mylan), Novartis, Sanofi, Thermo Fisher Scientific, outside the submitted work.

We confirm that the manuscript has been read and approved by all named authors.

We confirm that the order of authors listed in the manuscript has been approved by all named authors.

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Article 5

Diversity of methanogens in animals' gut.

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Diversity of Methanogens in Animals' Gut

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Abstract: Methanogens are members of anaerobe microbiota of the digestive tract of mammals, including humans. However, the sources, modes of acquisition, and dynamics of digestive tract methanogens remain poorly investigated. In this study, we aimed to expand the spectrum of animals that could be sources of methanogens for humans by exploring methanogen carriage in animals. We used real-time PCR, PCR-sequencing, and multispacer sequence typing to investigate the presence of methanogens in 407 fecal specimens collected from nine different mammalian species investigated here. While all the negative controls remained negative, we obtained by PCR-sequencing seven different species of methanogens, of which three (*Methanobrevibacter smithii*, *Methanobrevibacter millerae* and *Methanomassiliicoccus luminyensis*) are known to be part of the methanogens present in the human digestive tract. *M. smithii* was found in 24 cases, including 12/24 (50%) in pigs, 6/24 (25%) in dogs, 4/24 (16.66%) in cats, and 1/24 (4.16%) in both sheep and horses. Genotyping these 24 *M. smithii* revealed five different genotypes, all known in humans. Our results are fairly representative of the methanogen community present in the digestive tract of certain animals domesticated by humans, and other future studies must be done to try to cultivate methanogens here detected by molecular biology to better understand the dynamics of methanogens in animals and also the likely acquisition of methanogens in humans through direct contact with these animals or through consumption of the meat and/or milk of certain animals, in particular cows.

Keywords: mammals' digestive tract; dynamics of methanogens; sources of methanogens and zoonotic methanogens

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1. Introduction

Methanogens are archaea characterized by their unique capability in producing methane from byproducts of bacterial anaerobe fermentations, being members of anaerobe microbiota of the digestive tract microbiota of several mammals [1]. Methanogens as strict anaerobes are classified to be limited to anoxic habitats. However, some studies have shown that some methanogens are able to produce methane in soils rich in oxygen [2] and even in human microbiota [3]. Methanogens were classified into three biochemical groups based on the substrates used for hydrogen production: hydrogenotrophic, aceticlastic, and methylotrophic [2,4,5]. The group most described in human microbiota is hydrogenotrophic methanogens, which oxidize H_2, formate or a few simple alcohols, and reduce CO_2 to CH_4.

Accordingly, methanogens gained interest in the clinical microbiology over the past years after they were detected by PCR-based methods and cultured from the gut microbiota [6,7]; their translocation in milk and urines has been further observed [8]. Moreover, methanogens have been associated with dysbiosis such as in the case of vaginosis [9], urinary tract infections [10], and anaerobe abscesses of the brain [11,12], the muscle [13], the oral cavity in the case of periodontitis, and periimplantitis [14,15] in the case of refractory sinusitis [16]. Recently, we observed blood-borne methanogens associated with endocarditis [17]. In all these situations, anaerobe bacteria were associated in the methanogen-disease process, and this observation was probably reflecting methanogen specificities,

including the absolute oxygen intolerance and the necessity of bacterial fermentative products to produce methane [4,18].

Currently, 16 different methanogens have been cultured from digestive-tract microbiota of animals [19–22], and PCR-based methods of detecting species-specific sequences traced an additional 4 species [21] (Table 1).

Table 1. Methanogens found in digestive tract microbiota of animals.

Methanogens Species Obtained by Culture	Additional Species Detected by PCR-Based Methods But Not by Culture
Methanosarcina sp.	
Methanobacterium formicicum	
Methanomicrobium mobile	
Methanosarcina barkeri	
Methanobacterium bryantii	
Methanobrevibacter, ruminantium	
Methanobrevibacter millerae	*Methanobrevibacter smithii*
Methanobrevibacter olleyae	*Methanimicrococcus* spp.
Methanoculleus olentangyi	*Methanosphaera* spp.
Methanobrevibacter woesei	*Methanobacterium* spp.
Methanobrevibacter gottschalkii	
Methanobrevibacter thaueri	
Methanobrevibacter wolinii	
Methanobrevibacter cuticularis	
Methanobrevibacter curvatus	
Methanobrevibacter filiformi	

The sources, modes of acquisition, and dynamics of digestive-tract methanogens remain poorly investigated. We previously reported that one-day newborns exhibited culturable *Methanobrevibacter smithii* (*M. smithii*) in the gastric fluid [23], suggesting a perinatal source of acquisition. Accordingly, we reported that mother milk did contain culturable *M. smithii* and culturable *Methanobrevibacter oralis* (*M. oralis*) [8]. Yet, it is unclear whether these one-day methanogens do persist along with the digestive tract of the newborns or whether this is just one of several waves of acquisition of methanogens along the first months of life [23–27]. Therefore, the search for methanogens sources other than mother milk is of interest.

Certain mammals (cow, sheep, donkey, horse, cat, pig, rabbit, rat, rhinoceros, baboon, monkey, and hippopotamus); birds (goose, turkey, and chicken) and insects (termites) are acknowledged to harbor digestive tract methanogens, and *M. smithii* in particular has already been detected from bovine and also from Wistar rats [21,22,28–43]. In this study, we aimed to expand the spectrum of animals that could be sources of methanogens for humans, by exploring methanogen carriage in animals.

2. Materials and Methods

2.1. Feces Samples

After the obtention of verbal consent from animals' owners, feces samples were collected from nine different animal species, namely cat, dog, horse, sheep, rabbit, cow, pig, goat, and donkey from animals living in metropolitan France, more precisely in the Marseille metropolitan area, Southeastern France (Table 2). Dogs and cats were fed industrial dry-kibble feed; horses were fed hay + straw + pellets; sheep and goats were fed pasture (grass) and dry supplementary feed; rabbits were fed dehydrated alfalfa + hay + pellets (other vegetables, cereals, mineral salts, and vitamins); cows were fed hay + straw + pasture (grass) and whole plant maize silage; pigs were fed straw + dry pelleted feed (formula consisting mainly of maize, wheat, oats, peas, soybeans, cereals, oilseeds, and minerals), and donkeys were fed grass and hay. Feces samples were stored at +4 °C for five weeks before being processed for DNA extraction as reported below.

Table 2. Details of 407 feces samples here investigated for the presence of methanogens.

Origin of Samples	Species	Collected Number per Sample	Collection Sites
Cat	*Felis silvestris catus*	105	Marseille
Dog	*Canis lupus*	52	Marseille
Horse	*Equus caballus*	89	Marseille and Carnoux
Sheep	*Ovis aries*	29	Bourganeuf
Rabbit	*Oryctolagus cuniculus*	2	Allauch
Cow	*Bos taurus*	57	Bourganeuf and Allauch
Pig	*Sus scrofa domesticus*	64	Avignon
Goat	*Capra aegagrus hircus*	5	Allauch
Donkey	*Equus asinus*	4	Allauch

2.2. DNA Extraction and PCR Assays

DNA extraction was performed by mixing 0.2 g of each feces sample with 500 µL of G2 buffer (QIAGEN, Hilden, Germany) in an Eppendorf tube (Fisher Scientific, Illkirch, France). Then, 0.3 g of acid-washed beads ≤106 µm (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added in each tube and shaken in a FastPrep BIO 101 device (MP Biomedicals, Illkirch, France) for 45 s for mechanical lysis before 10-min incubation at 100 °C. A 180 µL volume of the mixture was then incubated with 20 µL of proteinase K (QIAGEN) at 56 °C overnight before a second mechanical lysis was performed. Total DNA was finally extracted with the EZ1 Advanced XL extraction kit (QIAGEN) and 200 µL eluted volume. Sterile phosphate-buffered saline (PBS) was used as a negative control in each DNA extraction run. Extracted DNA was incorporated into real-time PCR performed using Metha_16S_2_MBF: 5′-CGAACCGGATTAGATACCCG -3′ and Metha_16S_2_MBR: 5′- CCCGCCAATTCCTTTAAGTT-3′ primers (Eurogentec, Angers, France) and a FAM_Metha_16S_2_MBP 6FAM- CCTGGGAAGTACGGTCGCAAG probe targeting the 16S DNA gene of methanogens, designed in our laboratory (Eurogentec). PCR amplification was done in a 20 µL volume including 15 µL of mix and 5 µL of extracted DNA. Five µL of ultrapure water (Fisher Scientific) was used instead of DNA in the negative controls. The amplification reaction was performed in a CFX96 thermocycler (BioRad, Marnes-la-Coquette, France) incorporating a protocol with a cycle of 50 °C for 2-min, followed by 39 cycles of 95 °C for 5-min, 95 °C for 5 s and finally 60 °C for 30 s. The PCR-sequencing was done in a 20 µL volume, including 15 µL of mix and 5 µL of extracted DNA. Five µL of ultrapure water (Fisher Scientific, Illkirch, France) was used instead of DNA in the negative controls. The amplification reaction was performed in a CFX96 thermocycler (BioRad, Marnes-la-Coquette, France) incorporating a protocol with a cycle of 50 °C for 2-min, followed by 39 cycles of 95 °C for 5-min, 95 °C for 5 s and finally 60 °C for 30 s. Amplification of the archaeal 16S rRNA gene (primers used: SDArch0333aS15, 5-TCCAGGCCCTACGGG-3 and SDArch0958aA19, 5-YCCGGCGTTGAMTCCAATT-3) was performed as previously described [8,9,33,34]. Sequencing reactions (Sangers' method) were carried out using the BigDye Terminator, version 1.1, cycle sequencing kit DNA according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). Nucleotide sequences were assembled using 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 (http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi). We considered the sequences as belonging to the same species if the percentage of identity was >98.7%; as different species if between 95–98.7%, and different genera if this threshold was < 95% with respect to the first hit obtained by BLAST [44].

2.3. Multispacer Sequence Typing

We carried out a multispacer sequence typing (MST) technique on all fecal specimens positive by PCR-sequencing as previously described in our laboratory [23,45]. PCRs were realized in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) and followed all the steps described for standard PCR used for the molecular analysis of fecal specimens. Negative controls consisting of PCR mixture without DNA template were included in each PCR run. All PCR products were sequenced in both directions using the same primers as used for PCRs in a 2720 Thermal Cycler (Applied Biosystems) with an initial 1-min denaturation step at 96 °C, followed by 25 cycles denaturation for 10 s each at 96 °C, a 20 s annealing step at 50 °C, and a 4-min extension step at 60 °C. Sequencing products were purified using the MultiScreen 96-well plates Millipore (Merck, Molsheim, France), containing 5% of Sephadex G-50 (Sigma-Aldrich), and sequences were analyzed on an ABI PRISM 31309 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and edited using the ChromasPro software (version 1.42; Technelysium Pty Ltd., Tewantin, Australia). For each intergenic spacer, a spacer type (ST) was defined as a sequence exhibiting unique genetic polymorphism (SNPs and indels). MST genotypes were defined as a unique combination of the four spacer sequences [23,45].

2.4. Phylogenetic Analyses

Sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Molecular phylogenetic and evolutionary analyses were conducted in MEGA7 as previously described [46].

2.5. Statistical Analyses

We used R software for data analysis (https://www.r-project.org/). The Chi 2 test was used to compare the prevalence between the different animal species with a threshold $\alpha = 0.05$.

3. Results

In this study, a total of 407 fecal specimens collected from nine different mammalian species were investigated by real-time PCR and PCR-sequencing for the presence of methanogens using primers targeting the broad-range archaeal 16S rRNA gene.

Firstly, incorporating the 16S rRNA archaeal gene PCR primers newly designed in our laboratory into real-time PCR, we detected the presence of methanogen DNA in all animals here investigated and none of the negative controls. We found that 100.0% of cat feces specimens were positive with Ct values of 33.51 ± 1.28; 78.8% of dog feces specimens were positive with Ct values of 27.71 ± 0.94; 84.4% of horse feces specimens were positive with Ct values of 25 ± 2.95; 96.6% of sheep feces specimens were positive with Ct values of 27.19 ± 3.11; 100% of rabbit feces specimens were positive with Ct values of 27.1 ± 1.36; 100% of cow feces specimens were positive with Ct values of 24.11 ± 1.94; 100% of pig feces specimens were positive with Ct values of 22.15 ± 2.75; 80% of goat feces specimens were positive with Ct values of 19.18 ± 2.46; and 100% of donkey feces specimens were positive with Ct values of 18.82 ± 1.44 (Table 3).

Secondly, sequencing the standard PCR products was used for the precise identification of methanogens at the genus and species levels in each sample. In cats, 50/105 successfully sequenced samples yielded 20 *Methanocorpusculum aggregans* (*M. aggregans*), 13 *Methanocorpusculum labreanum (M. labreanum)*, 09 *Methanobrevibacter millerae (M. millerae)*, 04 *M. smithii*, 02 *Methanobrevibacter thaueri (M. thaueri)*, and 02 *Methanobrevibacter olleyae (M. olleyae)*. In dogs, 30/52 successfully sequenced samples yielded 13 *M. labreanum*, 06 *M. smithii*, 05 *M. aggregans*, 03 *M. thaueri*, 02 *M. millerae*, and 01 *M. olleyae*. In horses, 24/89 successfully sequenced samples yielded 11 *M. aggregans*, 10 *M. olleyae*, 01 *M. smithii*, 01 *M. millerae*, and 01 *M. labreanum*. In sheep, 28/29 successfully sequenced samples yielded 23 *M. labreanum*, 03 *M. millerae*, 01 *M. smithii*, and 01 *M. aggregans*. In rabbits, 2/2 successfully sequenced samples yielded 02 *M. thaueri*. In cows, 44/57 successfully sequenced samples

yielded 22 *M. aggregans*, 11 *M. millerae*, 06 *M. labreanum*, and 05 *M. thaueri*. In pigs, 25/64 successfully sequenced samples yielded 12 *M. smithii*, 09 *M. millerae*, 03 *Methanomassiliicoccus luminiyensis* (*M. luminiyensis)*, and 01 *M. olleyae*. In goats, 4/5 successfully sequenced samples yielded 03 *M. labreanum* and 01 *M. aggregans*. Finally, in donkeys, 4/4 successfully sequenced samples yielded 04 *M. aggregans* (Table 4).

Table 3. Comparison of prevalence based on real-time PCR between animal species.

Animal Species	Number of Samples Analyzed	Number of Positive Samples by RT-PCR	Prevalence [IC 95%]	*p*-Value
Cat	105	105	100.0 [96.5–100.0]	9.9×10^{-8}
Dog	52	41	78.8 [65.3–88.9]	
Horse	89	75	84.4 [75.0–91.1]	
Sheep	29	28	96.6 [82.2–99.9]	
Rabbit	2	2	100.0 [15.8–100.0]	
Cow	57	57	100.0 [93.7–100.0]	
Pig	64	64	100.0 [94.4–100.0]	
Goat	5	4	80.0 [28.4–99.5]	
Donkey	4	4	100.0 [39.8–100.0]	

Table 4. Comparison of prevalence based on PCR-sequencing between animal species.

Animal Species	Number of Samples Analyzed	Number of Positive Samples by PCR-Sequencing	Prevalence [IC 95%]	*p*-Value
Cat	105	50	47.6 [37.8–57.6]	1.4×10^{-12}
Dog	52	30	57.7 [43.2–71.3]	
Horse	89	24	27.0 [18.1–37.4]	
Sheep	29	28	96.6 [82.2–99.9]	
Rabbit	2	2	100.0 [15.8–100.0]	
Cow	57	44	77.2 [64.2–87.3]	
Pig	64	25	39.1 [27.1–52.1]	
Goat	5	4	80.0 [28.4–99.5]	
Donkey	4	4	100.0 [39.8–100.0]	

We obtained a total of seven different species of methanogens in our study as illustrated with Venn diagrams (Figure 1).). The Venn diagram shows which species of methanogens are found in common in humans and in animal samples analyzed in this study, and which species of methanogens are found exclusively in animals and exclusively in humans. Indeed, three methanogens species (*M. smithii, M. millerae,* and *M. luminyensis*) are known to be part of the methanogens present in the human digestive tract. The remaining four (*M. thaueri*, *M. olleyae, M. labreanum* and *M. aggregans*) are not known to date in humans. However, we did not find in our study the other 10 species of methanogens present in the human digestive tract, including *Methanobrevibacter arboriphilicus*, *M. oralis*, *Methanosphaera stadtmanae* (*M. stadtmanae)*, *Candidatus* Methanomethylophilus alvus (*Ca.* Methanomethylophilus alvus), *Candidatus* Methanomassiliicoccus intestinalis (*Ca*. Methanomassiliicoccus intestinalis), *Methanoculleus chikugoensis* (*M. chikugoensis*), *Methanobacterium congolense* (*M. congolense*), *Methanoculleus bourgensis* (*M. bourgensis*), *Candidatus* Nitrososphaera evergladensis (*Ca.* Nitrososphaera evergladensis), and *Methanosarcinia mazei* (*M. mazei*).

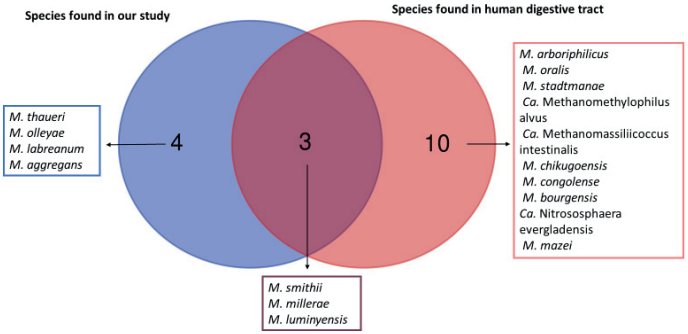

Figure 1. Venn diagram between the methanogens found in our study and those known from the human digestive tract. This Venn diagram shows which species of methanogens are found in common in humans and in animal samples analyzed in this study and which species of methanogens are found exclusively in animals and exclusively in humans.

Among the 211 sequences obtained, 153 (72.51%) of them have an identity percentage greater than 99%, 43 (20.37%) have an identity percentage lower than 98.7%, and 15 (7.10%) have an identity percentage lower than 95% (Table 5). The phylogenetic trees of sequences obtained with a percentage identity lower than 98.7% and sequences with a percentage identity lower than 95% indicated new species and new genera, respectively (Figure 2 and Supplementary Figures). We obtained 24 *M. smithii* by PCR-sequencing including 12/24 (50%) in pigs, 6/24 (25%) in dogs, 4/24 (16.66%) in cats, and 1/24 (4.16%) in both sheep and horses. Genotyping the 24 *M. smithii* revealed five different genotypes. Genotype 1 was found in 8/24 (33.33%); genotype 2 in 10/24 (41.66%); genotype 3 in 4/24 (16.66%); and genotypes 4 and 5 in 1/24 (4.16%) each (Table 6).

Table 5. Percentage of identity among the sequences obtained.

Animal Species	Percentage > 99%	Percentage < 98.7%	Percentage < 95%
Cat	50	0	0
Dog	30	0	0
Horse	7	15	2
Sheep	18	7	3
Rabbit	2	0	0
Cow	29	12	3
Pig	16	4	5
Goat	0	2	2
Donkey	1	3	0

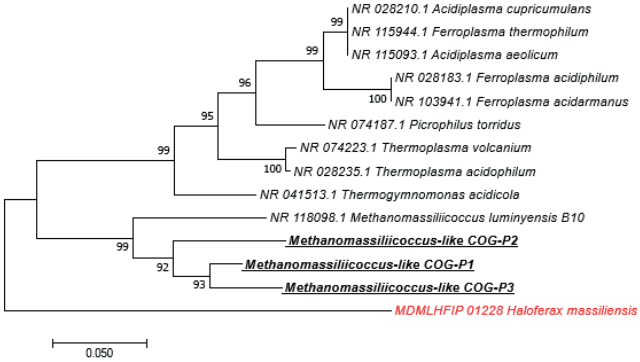

Figure 2. Molecular phylogenetic analysis, based on 16S rRNA partial gene, showed the position of *Methanomassiliicoccus-like* sequences detected in feces of pigs. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.82722721 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1.000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 415 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Bootstrap values $\geq$ 95% are indicated at nodes. In red: out of group. Species highlighted: methanogens species detected in this study.

Table 6. Summary of the results of multispacer sequence typing.

			Genotypes				
Samples	**Origin**	**Collection Sites**	**Spacer 1**	**Spacer 2**	**Spacer 3**	**Spacer 4**	**Spacer Type ***
1	Sheep	Bouganeuf			×		1
2	Horse	Marseille			×		1
3	Pig	Avignon	×	×	×	×	2
4	Pig	Avignon	×	×	×	×	2
5	Pig	Avignon	×	×	×	×	2
6	Pig	Avignon	×	×	×	×	2
7	Pig	Avignon	×	×	×		3
8	Pig	Avignon	×	×			4
9	Pig	Avignon		×	×		5
10	Pig	Avignon			×		1
11	Pig	Avignon	×	×	×	×	2
12	Pig	Avignon	×	×	×		3
13	Pig	Avignon	×	×	×	×	2
14	Pig	Avignon	×	×	×	×	2
15	Dog	Marseille	×	×	×	×	2
16	Dog	Marseille	×	×	×	×	2
17	Dog	Marseille	×	×	×		3
18	Dog	Marseille	×	×	×		3
19	Dog	Marseille	×	×	×	×	2
20	Dog	Marseille			×		1
21	Cat	Marseille			×		1
22	Cat	Marseille			×		1
23	Cat	Marseille			×		1
24	Cat	Marseille			×		1

Genotyping these *M. smithii* strains revealed the presence of five different genotypes. * Spacer type was determined according to reference [23,45].

4. Discussion

It is known and published that methanogens colonize the gastrointestinal tract of certain mammals, particularly herbivorous ones [35]. Most methanogens identified in mammals belong to the phylum Euryarchaeota, with a high percentage of the species *M. smithii* [36], a species being the most prevalent one in humans [47]. Our report is the largest one showing the presence of methanogens in nine mammals in the same study. Our results confirmed the published data on the presence of methanogens in the digestive tract of cats, dogs, horses, cows, sheep, rabbits, goats, pigs, and donkeys [21,22,28–34,36–38]. In addition, all methanogens found in this study belong to the phylum Euryarchaeota, which is in accordance with the results obtained in studies conducted on the human digestive tract [6,48]. Our results give an insight on the concentration of methanogens present in the intestinal microbiota of each animal species analyzed and on the prevalence of methanogens in domestic animals by humans.

The results of the analysis of the 16S RNA sequences obtained from our samples show that there is a real diversity of methanogenic archaea genera (*Methanosphaera*, *Methanocorpusculum*, *Methanocalculus*, *Methanoculleus*, *Methanogenium*, *Methanoplanus*, *Methanolacinia*, *Methanobacterium*, *Methanomicrobium*, *Methanomassiliicoccus* and *Methanobrevibacter*) in the digestive tract of animals (cats, dogs, horses, sheep, cows, rabbits, goats, pigs, and donkeys) as in humans [6,48]. All sequences with a percentage lower than 98.7% have been deposited in the GenBank database (accession no MT587812 to MT587864) and EBI database (accession no MT793590; MT819603; MT822292; MT822293; and MT822482).

Methanomassiliicoccus luminiyensis was known to colonize the human digestive tract, and it has never been detected in animals' digestive tracts [46]. For the first time, this study demonstrated the presence of the species *M. luminiyensis* in pigs and not in the other animals investigated here. These results could be explained by the fact that the pig is an omnivore, which means that its diet is close to that of humans compared to other animals. In addition, 50% of *M. smithii* in our study was found in pigs, indicating that *M. smithii* was the most prevalent methanogen in the digestive tract of pigs, consistent with work carried out in humans where the high prevalence of *M. smithii* in the digestive tract has been demonstrated [45,49].

These results are representative of the methanogen community present in the digestive tract of certain animals domesticated by humans, and other future studies must be done to try to cultivate methanogens detected here by molecular biology to better understand the dynamics of methanogens in animals. The possible ways of methanogens' acquisition in humans could be contact with animals and/or through consumption of milk/dairy products of certain animals, in particular cows, since a recent study demonstrated an association between the acquisition of *M. smithii* in children and the consumption of dairy products [50].

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/1/13/s1.

Author Contributions: C.O.G.: Samples collection, study design, manipulations, data information, results interpretation, draft writing. B.D.: samples collection, data information. M.D.: Supervision, study design, results interpretation, draft writing. G.G.: Grant acquisition, supervision, study design, samples collection, results interpretation, draft writing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: All the authors declare that there is no conflict of interest.

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Article 6

Dairy products as sources of methanogens for humans.

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Dairy products as sources of methanogens for humans.

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ABSTRACT

Methanogens are detected in human gut from the first moments of life and there is a diversification of methanogens during infancy. However, the sources of acquisition of methanogens are not well elucidated. We therefore investigated 56 dairy products as potential sources of methanogens by applying molecular biology. In the presence of negative controls, we obtained an overall prevalence of methanogens in 85.7% (48/56) of samples by real-time PCR. Further PCR-sequencing identified 73.2% (41/56) of *Methanobrevibacter smithii*. We also found for the first time in dairy products 1.8% (1/56) of *Methanobrevibacter oralis*, 7.1% (4/56) of *Methanobrevibacter millerae*, 1.8% (1/56) of *Methanobrevibacter ruminantium*, 1.8% (1/56) of *Methanocorpusculum* sp. We observed a significant presence (p-value=0.001) of methanogens in fermented dairy products compared to unfermented dairy products. This study gives credit to the fact that dairy products could be considered as a source of methanogens for humans, especially for children.

Keywords: Diary products, sources, methanogens, humans.

1. Introduction

Methanogens represent the archaea most present in the mammalian microbiota, especially in the human digestive microbiota where they account for 10% digestive tract of the anaerobic microorganisms (Dridi et al., 2011; Guindo, 2020; Nkamga et al., 2017). Methanogens are detected in humans from birth (Grine et al., 2017) and there is a diversification of methanogens in humans over the years: while only *Methanobrevibacter smithii* (*M. smithii*) has been detected and cultured in the neonates (Grine et al., 2017; Koenig et al., 2011; Mihajlovski et al., 2010; Odamaki et al., 2016; Palmer et al., 2007; Sereme et al., 2021), *M. smithii, Methanosphaera stadtmanae* (*M. stadtmanae*)*, Methanobrevibacter millerae* (*M. millerae*)*, Methanomassiliicoccus luminiyensis, Methanobrevibacter arboriphilicus, Methanobrevibacter oralis* (*M. oralis*)*, Candidatus* Methanomethylophilus alvus *and Candidatus* Methanomassiliicoccus intestinalis have been isolated from adult stools (Dridi et al., 2012; Nkamga et al., 2017; Sogodogo, Drancourt, et al., 2019). The various sources for each one of these different species remain unknown. Accordingly, a recent study demonstrated that the presence of methanogens, especially *M. smithii* in children stools is linked to the consumption of dairy products (van de Pol et al., 2017); but this study targeted only two of the methanogens strains present in the human digestive tract, namely *M. smithii* and *M. stadtmanae*. Therefore, we further explored the presence of methanogens in dairy products using molecular biology to target all methanogens currently known in the human digestive tract.

2. Materials and methods

2.1 Sampling of dairy products

We have investigated the presence of methanogens in different types of dairy products including unfermented dairy products (formula milk, fresh milk, and fresh cheese) and fermented dairy products (yogurt and fermented milk) (Table 1). All these dairy products were purchased in randomly selected supermarkets in Marseille, France in November 2019.

2.2 DNA extraction and PCR assays

DNA extraction was performed as previously described (Guindo et al., 2020). Briefly, for cheeses, yogurt and formula milk, 0.2 g was suspended in 200 µL of ultrapure water (Fisher Scientific, Illkirch, France), and a sonication step was performed for 30 minutes. DNA was then extracted with the EZ1 Advanced XL Extraction Kit (QIAGEN, Hilden, Germany) using 200 µL as sample volume and 50 µL as the elution volume. For fresh milk and fermented milk, 200 µL were taken and a sonication step was performed for 30 minutes as above. DNA was then extracted with the EZ1 Advanced XL Extraction Kit (QIAGEN) using 200 µL as the sample volume and 50 µL as the elution volume. The PCR assays targeting the 16S rRNA gene of methanogens, including real-time PCR and PCR-sequencing were performed to investigate the presence of methanogens in dairy products using primer pairs and PCR conditions described previously (Guindo et al., 2021). Sterile phosphate buffered saline (PBS) (Fisher Scientific) was used as a negative control in each DNA amplifications steps.

2.3 Phylogenetic analyses

Sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) as previously used (Guindo et al., 2021; Sereme et al., 2021; Sogodogo, Doumbo, et al., 2019; Sogodogo, Fellag, et al., 2019). Molecular phylogenetic and

evolutionary analyses were conducted in MEGA7 as previously described (Kumar et al., 2016).

2.4 Statistical analyses

Data were analyzed with RStudio (https://www.R-project.org/) by Fisher. test (** $p < 0.01$, * $p < 0.05$, ns: non-significant). We used the former to compare the proportion of methanogen detection by real-time PCR in fermented dairy products compared to unfermented dairy products.

3. Results and discussion

We investigated the presence of methanogens in 56 dairy products including 30 unfermented dairy products, and 26 fermented dairy products, of which 48 were positive (Table 1). The overall prevalence of methanogens in dairy products was 85.7% (48/56) by real-time PCR. The prevalence of methanogens in fermented dairy products was 32.1% (18/56) versus 53.6% (30/56) in unfermented dairy products (p-value=0.001). The results here reported were authentified by the fact that negative controls introduced in all experiments, remained negative. PCR-sequencing yielded *M. smithii* in 41 cases, i.e., 73.2% (41/56) exhibiting a 100% sequence similarity with the reference 16S rRNA gene sequence of *M. smithii* ATCC 35061 (accession NCBI: NR_074235) isolated from human stool. *M. smithii* was therefore the most frequent species in dairy products samples (Fig.1). These results are consistent with the literature where *M. smithii* was the only species identified in dairy products (van de Pol et al., 2017). The high prevalence of *M. smithii* in the human digestive tract (Dridi et al., 2009) would therefore be related to the consumption of dairy products (van de Pol et al., 2017). However, we also found for the first time *M. oralis* in one formula milk and this sequence exhibits 99.82% similarity with the sequence of the reference 16S rRNA gene of *M. oralis*

CSUR P5920 (NCBI accession: LR590665.1) isolated from Breast milk of healthy breast-feeding mother; *M. millerae* in three fermented milk and in one fresh milk and these sequences have more than 99% similarity with the sequence of the reference 16S rRNA gene of *M. millerae* strain CY31 (NCBI accession: MH443315.1). These two sequences have already been found in human microbiota (Nkamga et al., 2017; Togo et al., 2019). We also found *Methanobrevibacter ruminantium* (*M. ruminantium*) in one fermented milk exhibiting 99.65% similarity with the sequence of the reference 16S rRNA gene *M. ruminantium* strain Yak M20 (NCBI accession: KP123415.1), but also *Methanocorpusculum* sp. T07 in one fermented milk which has 92.24% similarity with the sequence of the reference 16S rRNA gene of *Methanocorpusculum* sp. T07 (NCBI accession: AB288279.1). These two species are only known from the digestive tract of animals especially in the feces of domestic animals (Guindo et al., 2021). In addition, statistical analysis has shown that there is significant correlation between the presence of methanogens in fermented dairy products compared to unfermented dairy products (p-value=0.001), suggesting that fermentation processes might have impact on the DNA of methanogens present in dairy products. This study gives an insight on the concentration of methanogens present in dairy products with the demonstration for the first time ever of the presence of *M. oralis*, *M. millerae*, *M. ruminantium* and *Methanocorpusculum* sp. in dairy products. We therefore detected many more species of methanogens compared to the previous study (van de Pol et al., 2017). The presence of *M. smithii*, *M. oralis* and *M. millerae* in dairy products suggests another mode of acquisition of these methanogens in children through artificial breastfeeding. In addition, a previous study demonstrated that *M. smithii* and *M. oralis* can be acquired in children through breastfeeding (Togo et al., 2019), which strengthens our hypothesis that methanogens present in the digestive tract can be found in milk through mechanisms still unknown. These data add to previous reports of the detection of methanogens in dairy products (van de Pol et al., 2017)

133 and thus giving credit that dairy products could be considered as a source of methanogens for
134 humans, especially for children.

135

136 **4. Conclusions**

137 This study demonstrates, for the first time, the presence of *M. oralis*, *M. millerae*, *M.*
138 *ruminantium* and *Methanocorpusculum* sp. in dairy products and suggests that dairy products
139 may be essential to seed the infant's microbiota with these neglected critical commensals
140 from the first hours of life.

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AUTHORS CONTRIBUTIONS

COG contributed to the collection of samples, conducted the experiments, analyzed the data, and wrote the paper; MD designed the project, participated in the writing of the paper, and provided great support carrying out the experiments; GG designed the project, helped conduct the experiments and participated in the writing of the paper.

CONFLICTS OF INTEREST

All the authors declare that there is no conflict of interest.

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222 **Table 1.** Distribution of dairy products according to the number and results of real-time PCR and PCR-sequencing.

	Dairy products	Samples analyzed	Real-time PCR	PCR-sequencing	Methanobrevibacter species	p-value
Unfermented dairy products	**Fresh cheese**	5	5	5	*M. smithii*	0.001**
	Fresh milk	9	9	9	*M. smithii* and *M. millerae*	
	Formula milk	16	16	16	*M. smithii* and *M. oralis*	
Fermented dairy products	**Yogurt**	10	7	7	*M. smithii*	
	Fermented milk	16	11	11	*M. smithii, M. millerae, M. labreanum* and *M. ruminantium*	

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223 **: High significant

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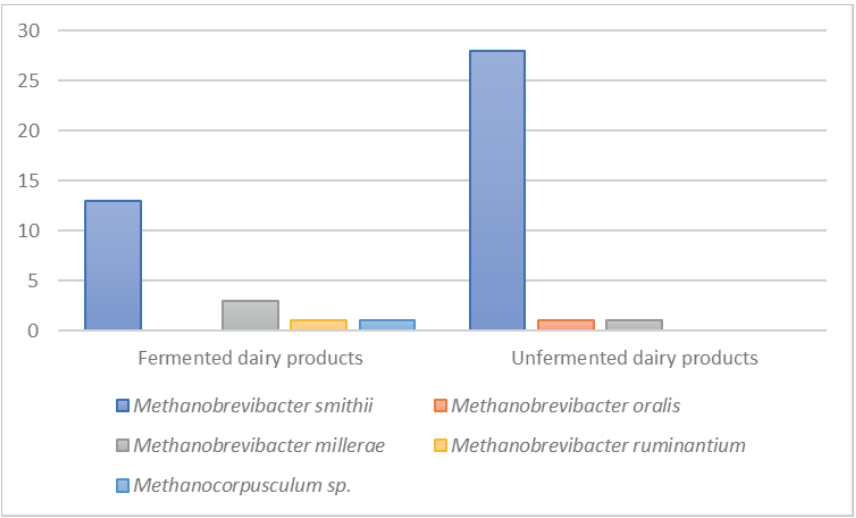

225

226 **Figure 1.** Methanogen species detected in dairy products.

Conclusions et perspectives

Jusqu'à la réalisation de nos travaux de Thèse, les seuls méthanogènes connus de l'intestin humain appartenaient aux genres *Methanobrevibacter* et *Methanomassiliicoccus* [1,91]. Au cours de cette Thèse, nous nous sommes intéressés aux sources et à la dynamique d'acquisition des méthanogènes chez l'Homme depuis la période néonatale jusqu'à l'âge adulte. Notre travail de recherche bibliographique sous forme d'une revue sur les rôles physiologiques des méthanogènes associés au microbiote humain nous a montré un déficit de connaissances et des informations partiellement contradictoires concernant les méthanogènes humains et la nécessité de développer une méthodologie et des procédures opérationnelles standard permettant la détection, la quantification et la caractérisation des méthanogènes dans les échantillons cliniques ainsi que l'étude de la dynamique d'acquisition des populations de méthanogènes chez l'Homme.

À la suite de ce travail de revue bibliographique, nous nous sommes intéressés à l'optimisation et au développement de nouveaux protocoles pour l'identification et la culture en routine des méthanogènes d'intérêt clinique dans le laboratoire de microbiologie clinique. Nous avons inventé une méthode chimique de production d'hydrogène combinant la limaille de fer et l'acide acétique, permettant l'isolement et la culture rapide sur gélose à partir des échantillons cliniques, des deux méthanogènes les plus prévalents dans le microbiote digestif, *Methanobrevibacter smithii* et dans le microbiote buccal, *Methanobrevibacter oralis*. Cette méthode évite toute source externe d'hydrogène un petit peu difficile de mise en œuvre et, comme perspective, pourrait être développée pour faciliter l'isolement par culture des méthanogènes à partir des échantillons cliniques. Enfin, nous avons mis au point un milieu de culture spécifique permettant la découverte par isolement et culture d'un nouveau genre de *Methanomassiliicoccales* que nous avons nommé *Methanomassiliia massiliensis* à partir du fluide buccal humain. En parallèle à ces travaux de développement de nouvelles conditions d'isolement par culture des méthanogènes associés aux microbiotes humains, nous avons développé une méthode fiable, rapide et reproductible pour l'identification des méthanogènes ainsi cultivés et d'intérêt clinique, par spectrométrie de masse MALDI-TOF-MS qui permet également de différencier les génotypes différents de *Methanobrevibacter smithii*. La perspective d'utilisation en routine de ces différentes techniques développées dans cette Thèse dans les laboratoires de microbiologie clinique, permettra non seulement le diagnostic de routine des méthanogènes d'intérêt clinique mais également d'enrichir le répertoire des méthanogènes du microbiote humain.

Quant aux sources et la dynamique d'acquisition des méthanogènes chez l'Homme, nous avons montré dans le courant de notre Thèse que la période intra-utérine constitue le premier moment d'acquisition de *Methanobrevibacter smithii* avec la détection sans précédent de ce méthanogène dans le méconium des nouveau-nés prématurés; confirmant l'hypothèse d'une étude précédente dans laquelle *Methanobrevibacter smithii* avait été isolé par culture dans le suc gastrique des nouveau-nés [92]. Nous avons aussi

démontré que le contact étroit avec des animaux domestiques ainsi que la consommation du lait et produits laitiers de certains animaux est l'une des sources de la diversification des méthanogènes chez l'Homme. Ces données sont conformes avec la littérature indiquant que la détection des méthanogènes dans le colostrum et le lait maternel [6] et l'association significative entre acquisition de *Methanobrevibacter smithii* et consommation de produits laitiers, chez les enfants [90].

A la lumière des résultats de notre Thèse et des données de la littérature, nous concluons qu'il existe quatre modes probables d'acquisition des méthanogènes chez les enfants : une acquisition intra-utérine à travers les échanges materno-fœtaux durant la grossesse, et après la naissance une acquisition à travers la consommation du lait maternel, de produits laitiers, et enfin une transmission à partir de l'environnement notamment le contact avec les parents mais aussi avec les animaux. Il serait très utile de mener des investigations futures pour isoler par culture les méthanogènes détectés par biologie moléculaire dans le méconium ainsi que dans les produits laitiers pour mieux les caractériser phénotypiquement et explorer leur génome par séquençage pour mieux préciser leur rôle exact au sein du microbiote humain. Il serait aussi très utile d'étudier les mécanismes de la transmission materno-fœtale en explorant la présence des méthanogène dans le liquide amniotique, le sang du cordon ombilical et le placenta par une approche polyphasique incluant la microscopie, la culture et le séquençage génomique. Ces données nouvelles permettraient de mieux comprendre la dynamique d'acquisition des méthanogènes chez les nouveau-nés, notamment dans la perspective de la pathologie de la malnutrition aiguë sévère et mortelle au cours de laquelle les méthanogènes sont constamment absents [93].

Le développement d'un milieu biphasique standard pour l'isolement et la culture rapide des méthanogènes à partir des échantillons cliniques est aussi nécessaire. La mise au point d'un tel milieu permettrait l'isolement et la culture en routine des méthanogènes directement à partir des échantillons cliniques et par la même occasion la culture des méthanogènes déjà détectés chez l'Homme par biologie moléculaire ainsi que des espèces non encore connues dans le microbiote humain.

ANNEXES

Annexe 1 : Un isolat intestinal humain de *Tetragenococcus halophilus*

Préambule

Tetragenococcus halophilus est une bactérie à Gram positif, non mobile, non sporulante, anaérobie facultative, en forme de Cocci, productrice d'acide lactique [94,95] . Elle a été isolée par culture dans divers aliments salés et elle produit des acides organiques, des acides aminés et des composés aromatiques pendant la fermentation de ces aliments salés [94–101]. Elle aurait aussi des effets bénéfiques dans la santé humaine, en particulier un effet immunomodulateur surtout dans l'amélioration des maladies atopiques [102,103]. Cependant, cette espèce n'a jamais été détectée dans un échantillon clinique. Nous avons donc investigué par une approche polyphasique incluant la culture, la microscopie électronique à balayage et le séquençage génomique la présence de *Tetragenococcus halophilus* dans les échantillons de matières fécales prélevés sur des individus apparemment en bonne santé exposés à différentes régions d'Europe et d'Afrique. Cette étude a révélé la présence sans précédent de *Tetragenococcus halophilus* dans des échantillons de matières fécales humaines.

Article 7

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A *Tetragenococcus halophilus* human gut isolate

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ABSTRACT

Tetragenococcus halophilus (*T. halophilus*) is a facultative anaerobic, coccus-shaped halophilic lactic acid-producing bacterium previously detected and cultured in various salty foods and credited for beneficial effects on human health. In this study, we investigated the presence of *T. halophilus* in human samples using a polyphasic approach including scanning electron microscopy, molecular biology methods and microbial culture. This unique investigation yielded the unprecedented presence of *T. halophilus* in human feces samples, thus enriching the repertoire of halophilic microorganisms colonizing the human gastrointestinal tract with the isolation and culture of *T. halophilus* for the first time in humans. Using the E-test strips, the MIC was assessed for T. *halophilus* strain CSURQ6002: rifampicin (MIC at 0.002 µg/mL), benzylpenicillin (MIC at 0.094 µg/mL), amoxicillin (MIC at 0.5 µg/mL), erythromycin (MIC at 2 µg/mL), clindamycin (MIC at 4 µg/mL), and vancomycin (MIC at 8 µg/mL). However, this strain showed a MIC up to 256 µg/mL for ciprofloxacin, fosfomycin, doxycyclin, imipenem, and colistin. *In-silico* profiling derived from whole genome sequencing (NCBI accession number: PRJNA780809), was confirmed. This discovery suggested that *T. halophilus* was part of the human digestive microbiota and that its potential role on human health should be considered.

1. Introduction

Tetragenococcus halophilus (*T. halophilus*) is a gram-positive, non-motile, non-sporulating, facultative anaerobic, coccus-shaped lactic acid-producing bacterium (Chun et al., 2019, Justé et al., 2012). This halophilic microorganism has been initially isolated from miso (Kumazawa et al., 2018) and further detected in various salty foods (Chun et al., 2019, Justé et al., 2012, Guan et al., 2011, Lee et al., 2015, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016), being incorporated into soy sauce, miso, anchovy brines and fermented and smoked fish eggs (Kim et al., 2018, Hanagata et al., 2003, Roling and van Verseveld, 1996, Udomsil et al., 2011, Tanasupawat et al., 2002). Indeed, *T. halophilus* has been shown to produce organic acids, amino acids and flavoring compounds during the fermentation of salty foods (Udomsil et al., 2010, Udomsil et al., 2017, Lee et al., 2018). Moreover, *T. halophilus* has been credited from beneficial effects on human health, in particular an immunomodulatory effect leading to improvement of atopic diseases (Masuda et al., 2008, Ohata et al., 2011). Despite the widespread presence of *T. halophilus* in salty foods, this species has never been described in humans in the many studies which have proven the presence of halophilic bacteria in the human microbiota (Oxley et al., 2010, Seck et al., 2018, Khelaifia et al., 2017, Khelaifia and Raoult, 2016). We herein questioned whether *T. halophilus* could also be detected in the human gut, investigating feces samples collected from apparently healthy individuals exposed to different regions in Europe and Africa. This unique investigation yielded the unprecedented presence of *T. halophilus* in human feces samples, and this finding was confirmed by the sequencing of the first genome of this clinical isolate from the cultivated colonies.

2. Materials and methods

2.1. Sample collection

A series of 184 human anonymized leftover fecal specimens were retrospectively investigated for the prevalence of *T. halophilus*. In France, Mali, and Senegal, where stools samples have been anonymously collected, informed consent was obtained from individuals before

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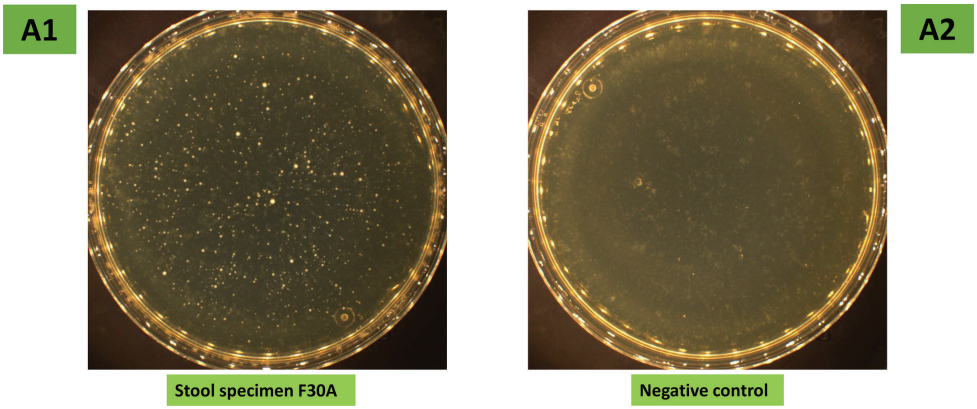

Fig. A. (A1) Spherical white colonies of *T. halophilus* CSURQ6002 growing on agar. **(A2)** Negative control.

participation in this study in agreement with French, Malian and Senegalese laws.

2.2. DNA extraction and PCR assays

Total DNA extracted as previously described (Guindo et al., 2020), was incorporated into PCR for the amplification of the 16S rRNA gene from *T. halophilus* using the primers *T. halophilus* F, 5-ACAGGGGTAGGAGTGAAATCTTG-3, and *T. halophilus* R, 5- TGCTGGCAACAGTGG GCAC-3 (Eurogentec, Angers, France), designed in our laboratory and using a specific program for the amplification reactions (**Appendix A**). Sequencing reactions (Sangers' method) were carried-out as previously described (Guindo et al., 2020).

2.3. Isolation and culture of T. halophilus

We selected 20 human anonymized leftover stool specimens proved to harbor *T. halophilus* following specific DNA sequencing of which five have been collected in five individuals living in Senegal, 13 have been collected in 13 individuals living in Mali and two have been collected in two individuals living in France. Salinity was measured in these 20 stool specimens according to a previously established protocol (Seck et al., 2019). The total mineral salt content of all 20 stool specimens was measured by a salinity refractometer (Thermo Scientific). One gram of each stool specimen was diluted in 10 mL of distilled water (Thermo Scientific, Villebon sur Yvette, France) and vortexed briefly. Then, 100 µL of supernatant was deposited in the refractometer and the result was directly displayed on the screen. One measure was performed per sample.

For stool culturing, we used a specific culture medium containing 100 g of NaCL (**Appendix B**). We established a four-week culture protocol with weekly follow-up. We poured 25 mL of culture medium in bottle serum (Sigma-Aldrich, Saint-Quentin Fallavier, France) and then degassed with 2-bar nitrogen for ten minutes. Then, one gram of each stool specimen was diluted in 10 mL of 10X PBS (Fisher Scientific, Illkirch, France) under anaerobic conditions and subsequently 2 mL of each dilution were injected into the degassed bottle serum and incubated at 37°C during a four-week follow-up. Then, each stool specimen was subcultured in an anaerobic chamber (Don Whitley Scientific, Bingley BD16 2NH, UK) on a solid medium comprising the same components as the liquid medium used and placed in an anaerobic bag (Becton Dickinson, Pont-de-Claix, France) with a CO_2 generator (Becton Dickinson) and incubated at 37°C for ten days.

2.4. Scanning electron microscopy investigations

Morphological features of *T. halophilus* were further assessed using a SU5000 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). Pure cultures were obtained from solid and liquid cultures fixed for three hours with 2.5% glutaraldehyde. Fixed bacteria were cyto-centrifuged on glass slides and platinum sputtered coated (5 µm thick platinum layer) using ion sputter MC1000 (Hitachi). Micrographs were acquired at magnifications ranging from x10,000 to x120,000 with a 10-kV voltage and a spot intensity of 30 using the backscatter electron detector under a high vacuum mode.

2.5. Whole genome sequencing

For whole genome sequencing, *T. halophilus* DNA was extracted flowing an in-house adapted procedure used EZ1 protocol (Qiagen Tissue, Hilden, Qiagen, Germany). Briefly, 200 µL of *T. halophilus* suspension mixed with 200 µL G2 buffer in presence of 20 µL proteinase K (Qiagen) was incubated over night at 56°C. After proteinase K treatment, glass powder was added to the mix and incubated 20 minutes at 100°C and immediately vortexed 90S at 6.5 m/S with FastPrep (MP Biomedical Europe, Illkirch, France). After five-minute centrifugation at 17,000 g, DNA was extracted from 200 µL supernatant and eluted in 50 µL final volume. Extracted DNA was normalized using Qubit dsDNA High Sensitivity Assay Kit (Life Technology, Villebon-sur-Yvette, France). Genome sequencing was performed using 1 ng of normalized DNA following Illumina Nextera-XT paired-end protocol and sequenced on Illumina iSeq 100 and MiSeq instruments (Illumina, San Diego, USA)

Table A
Salinity assessment.

ID	C028	C068	F30A	C036	C014	M34A	C040	C112	C137	C037	C054	C020	C153	C169	C141	S1	S2	S3	S4	S5
Salinity(%)	1.7	2.1	2.2	1.3	0.6	0.6	0.8	0.4	0.6	0.3	0.5	0.46	0.47	0.47	0.47	0.26	0.31	0.21	0.22	0.2

after library preparation, as previously described (Morsli et al., 2021, Morsli et al., 2021, Diop et al., 2016). In parallel, 1 μg of *T. halophilus* DNA was used for Oxford Nanopore library preparation as previously described (Morsli et al., 2021), then sequenced using MinION sequencer (Oxford Nanopore technologies, Oxford, UK). NGS-generated data were analyzed using CLC Genomics Workbench, version 7.5.0 (Qiagen). Phylogenetic tree based on whole genome sequence was generated using Orthologous Average Nucleotide Identity Tool (OAT) software version (0.93.10) (Lee et al., 2016) and pangenome analysis was performed using Roary command after genome annotation on galaxy Europe online software (https://usegalaxy.eu/). Antibiotic resistance and bacteria virulence were analyzed using on-line CGE-database bio-tools (CGE Server "dtu.dk"), and Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi).

3. Results and Discussion

We are reporting on the first isolation and culture of *T. halophilus* from human stools. The results here reported were authentified by the fact that negative controls introduced in all experiments remained negative; in agreement with the fact that *T. halophilus* has never been previously worked in our laboratory and the fact that concordant results were obtained by polyphasic approaches, including molecular methods, culture, and microscopy. The fact that this observation occurred twelve years after the initial detection of food-borne *T. halophilus* in Asia (Kumazawa et al., 2018, Guan et al., 2011, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016), testifies that the search for halophiles is still in its infancy, in clinical microbiology.

In the first step, we searched for *T. halophilus* by PCR-sequencing in stools specimens collected in individuals living in France, Senegal, and Mali. By incorporating 16S rRNA *T. halophilus* gene PCR primers recently designed in our laboratory into PCR-sequencing, we detected the presence of *T. halophilus* DNA in 28/184 (15%) of stools samples here investigated but not in the negative controls (**Appendix C**). All sequences obtained exhibited a 100% similarity and 100% coverage with the sequence of the complete genome of *T. halophilus* strain YJ1 (NCBI accession number: CP046246.1). These data indicated an average prevalence of *T. halophilus* in humans compared to the high prevalence in salty foods (Chun et al., 2019, Justé et al., 2012, Guan et al., 2011, Lee et al., 2015, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016). This average prevalence could be explained by the generally very low salt diet in the Caucasian population, which represented 67.39% (124/184) of the stool samples here investigated. *A contrario*, the *T. halophilus* strain here reported had been recovered from stool sample collected in an individual exposed to high salt content food.

In a second step, we initiated the isolation by culture of *T. halophilus*, by using a specific salt medium. We used a very rich medium because *T. halophilus* has complex nutritional needs (Collins et al., 1990). After ten days of culture, we observed spherical white colonies suggestive of *T. halophilus* growing on agar (**Fig. A**), as previously described (Vos et al., 2011). This observation was made on 5% (1/20) of the samples cultured after ten-day follow-up. These results suggested the low prevalence of *T. halophilus* in humans regardless of the population studied, but also demonstrated that *T. halophilus* was a slow growing bacterium. The culture-positive sample had a salinity of 2.2% (**Table A**), compatible with the presence of *T. halophilus* in salty stool as defined by a salinity > 1.5% (Seck et al., 2019). However, the mean salinity of here cultured samples was 0.7%, hence the low prevalence of *T. halophilus* in the cultured samples.

To characterize this strain of *T. halophilus*, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Wissembourg, France) on the colonies obtained on agar as previously described (Seng et al., 2009, Seng et al., 2013) and also performed an antibiogram with E-tests (bio-Mérieux, Craponne, France) using 11 of the most prescribed antibiotics in human pathologies (amoxicillin, vancomycin, erythromycin,

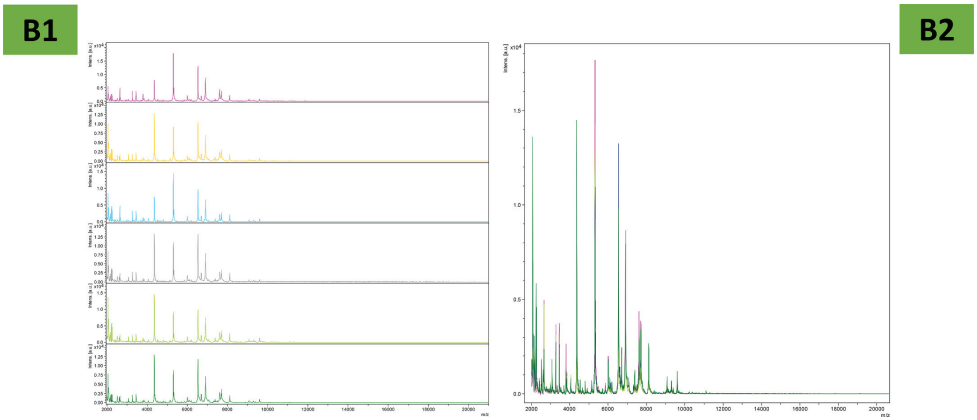

Fig. B. Spectral profiles of *T. halophilus* CSURQ6002. **(B1)** Spectra from six different deposits of *T. halophilus* CSURQ6002. **(B2)** Spectral superposition of *T. halophilus* CSURQ6002.

clindamycin, fosfomycin, ciprofloxacin, benzylpenicillin, rifampicin, doxycyclin, imipenem, and colistin) according to a previously established protocol (Konate et al., 2021). Specifically, to perform E-tests, fresh *T. halophilus* colonies were resuspended in 0.9% NaCl to a 3 McFarland density. We obtained reproducible spectra with intensities at 10^4 (**Fig. B**). These spectra were added to our MALDI-TOF MS database and are available on the website of the University-Hospital Institute (IHU) Méditerranée Infection on the link https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/urms-data-base/.

Using the E-test strips, the MIC was assessed for *T. halophilus* strain CSURQ6002: rifampicin (MIC at 0.002µg/mL), benzylpenicillin (MIC at 0.094 µg/mL), amoxicillin (MIC at 0.5 µg/mL), erythromycin (MIC at 2 µg/mL), clindamycin (MIC at 4 µg/mL), and vancomycin (MIC at 8 µg/mL). However, this strain showed a MIC up to 256 µg/mL for ciprofloxacin, fosfomycin, doxycyclin, imipenem, and colistin (**Appendix D**).

Using SEM, *T. halophilus* cells appeared as cocci with average diameter of 450 µm, shaped and grape-like clusters (**Fig. C**). At high magnification the surface of cells was not smooth.

Blast nucleotide on NCBI Blast database of the NGS data after assembly using spades on galaxy Europe online software, showed *T. halophilus* strain YJ1 (NCBI accession number: CP046246) at 99.98% sequence similarity, strain isolated from Salty fermented fish sauce in south Korea in 2018 (unpublished data). The *T. halophilus* strain YJ1 complete genome (NCBI accession number: CP046246.1), was used as reference sequence for mapping of total reads by CLC Genomics Workbench software. Mapping MiSeq, iSeq and MinION-generated reads yielded 2,863,767 reads (59%) were matched to the reference genome, generating 2,511,991 nucleotides with 97-read depth. Annotation of identified *T. halophilus* CSURQ6002 (NCBI accession number: PRJNA780809) genome yielded 36.2% GC nucleotides, 0.82 gap ratio and 78% coding ratio including 2,694 encoding gene (CDSs), 15 rRNA genes, 62 tRNA genes and two genes encoding for CRISPRs. Whole genome phylogenetic analysis of the identified *T. halophilus* CSURQ6002 (NCBI accession number: PRJNA780809) with the six hit-blast strains genome recovered from GenBank NCBI database using OrthoANI Tool version (0.93.1), including *Methanohalophilus halophilus* strain Z-7982 (NCBI accession number: NZ_CP017921) as an out of the group, showed that the identified *T. halophilus* genome belonged to *T. halophilus*, sharing 97.37% whole genome sequence similarity with *T. halophilus* strain MJ4, isolated form myeolchi-jeotgal, Korean traditional fermented anchovy (NCBI accession number: NZ_CP012047.1) (Kim et al., 2018), 97.19% whole genome sequence similarity with *T. halophilus* strain KUD23 isolated from Doenjang, fermented soybeans (NCBI accession number: CP020017), and only 97.07% sequence similarity with the first hit-blast *T. halophilus* strain YJ1 (**Fig. D**). This was confirmed by pangenome analysis after annotation of the encoding genome fraction of the identified *T. halophilus* CSURQ6002 (accession number: PRJNA780809) and the first six-blast genomes recovered from GenBank database, yielded that the isolated bacteria strain belonged to the *T. halophilus* species genetically near to *T. halophilus* strain YJ1, first blast-result obtained by blast against GenBank database (**Appendix E**). Based on genomic data analysis, the isolated bacteria probably translocated to the digestive tracts by alimentation, illustrating the role of nutrition in the diversity of the human gut microbiota. Also, *in silico* analysis did not reveal any gene known to encode for virulence and antibiotic resistance, a result somewhat discrepant with E-test results as above, potentially due to the fact that online databases used for such investigations, do not contain halophilic genomes, therefore lacking references for antibiotic resistance.

The *T. halophilus* strain herein isolated has been deposited the Collection de Souches de l'Unité des Rickettsies (CSUR) as *T. halophilus* CSURQ6002.

In conclusion, the present study demonstrated for the first time, isolation by culture of *T. halophilus* from the human digestive tract and enabled the isolation and cultivation of the first strain of *T. halophilus* in humans. However, we observed a low prevalence of *T. halophilus* in humans, and its detection was associated with high stool salinity. This discovery therefore suggested that *T. halophilus* was part of the human

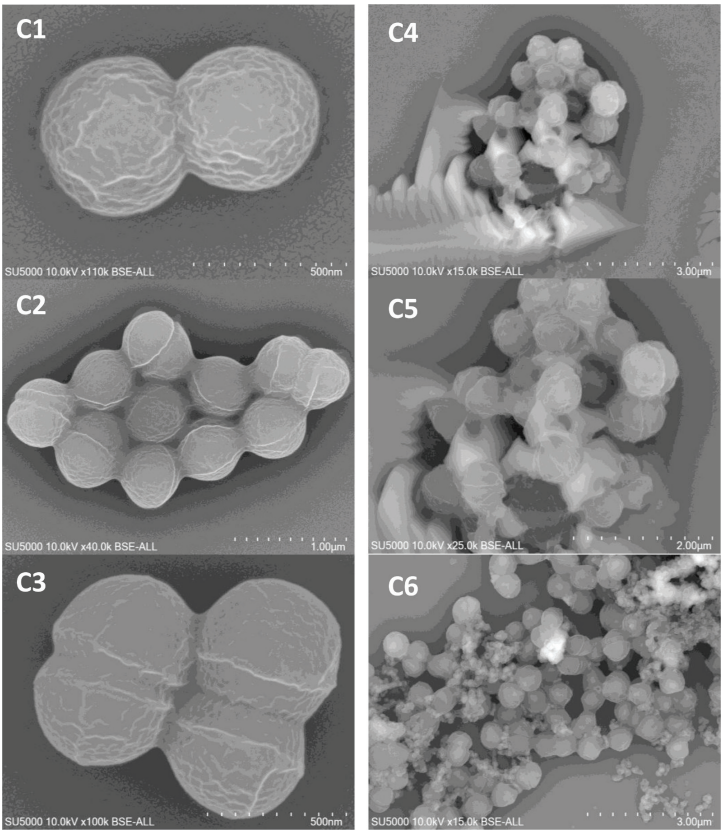

Fig. C. SEM micrographs of *T. halophilus* CSURQ6002. Fresh cultures were obtained from agar plates **(C1, C2, C3)**, as well as from liquid medium **(C4, C5, C6)**.

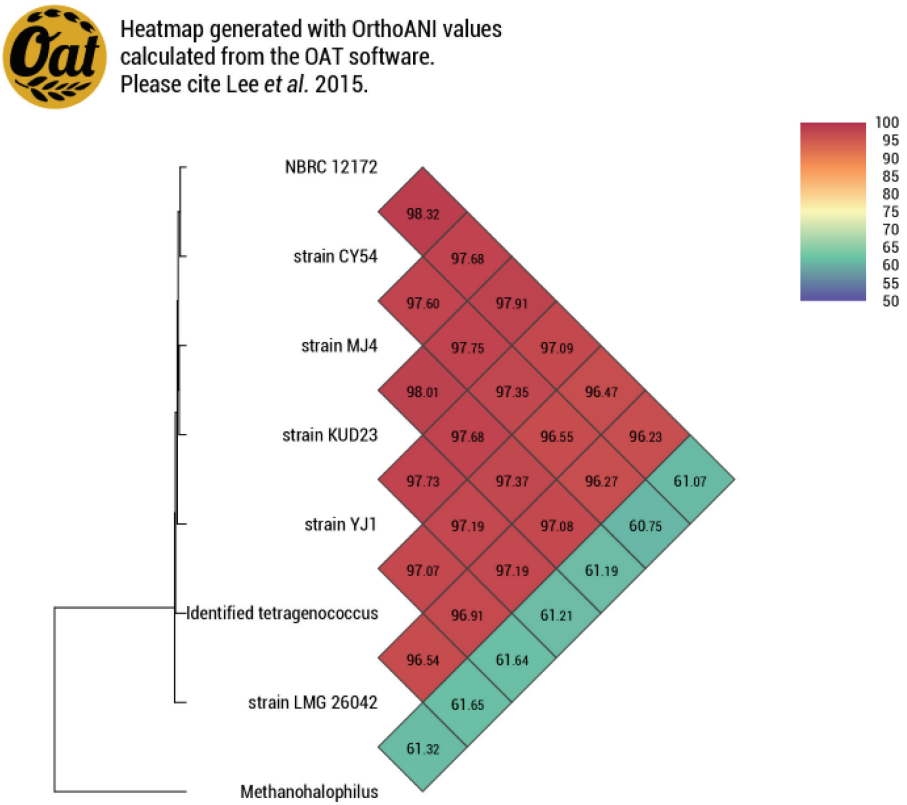

Fig. D. Phylogenetic analysis based on complete genome sequences of the identified *T. halophilus* and the six hit-blast strains recovered from NBCI GenBank nucleotide sequence database (https://www.ncbi.nlm.nih.gov/nucleotide/, accessed on 24 October 2021). The identified *T. halophilus* CSURQ6002 had 97.37% genome identity with *T. halophilus* strain MJ4, 97.19 with *T. halophilus* strain KUD23 and only 97.07% genome identity with the first hit-blast *T. halophilus* strain YJ1. The evolutionary history was inferred in Orthologous Average Nucleotide Identity Tool software used OrthoANI to measure overall similarity between two genome sequences. Unlike the original ANI algorithm, OrthoANI produces identical reciprocal similarities. It has been shown by a large comparison study, values generated by the original ANI and OrthoANI are comparable. The proposed cut-off for species demarcation is 95~96% for both OrthoANI and the original ANI.

digestive microbiota in some individuals, in whom its role on human health should be further considered (Masuda et al., 2008, Ohata et al., 2011).

Ethics approval

The study was previously approved by the Ethics Committee of the University-Hospital Institute (IHU) Méditerranée Infection under n° 2016-011, the Ethics Committee of the Faculty of Medicine and Odonto-Stomatology of Bamako, Mali, under n° 2014/46/CE/FMPOS, and the National Health Research Ethics Committee of Senegal under n°SEN16/45.

Credit author statement

COG contributed to the collection of samples, conducted the experiments, analyzed the data and wrote the paper; MM contributed to the whole genome sequencing and *in silico* data analysis and participated in the writing of the paper; SB contributed to the electron microscopy analysis and participated in the writing of the paper; MD designed the project, participated in the writing of the paper and provided great support carrying out the experiments; GG designed the project, helped conduct the experiments and participated in the writing of the paper. All authors have read and approved the manuscript.

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Declaration of Competing Interest

All the authors declare that there is no conflict of interest.

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Appendix

Appendix A. Program for T. halophilus DNA amplification reactions

An initial 15min denaturation step at 95°C was followed by 40 cycles of denaturation of 30s each at 95°C, a 45s annealing step at the appropriate Tm 55°C and a 60s extension at 72°C. Holding at 72°C for 5min allowed the complete extension of the PCR products.

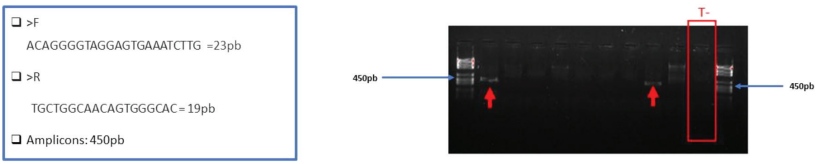
Appendix B. T. halophilus CSURQ6002 culture medium

NaCl	100.0 g
$MgSO_4 \bullet 7H_2O$	3.45 g
$MgCl_2 \bullet 6H_2O$	2.75 g
Sodium acetate	1.0 g
KCl	0.335 g
NH_4Cl	0.25 g
$CaCl_2 \bullet 2H_2O$	0.14 g
$K_2HPO_4 \bullet 3H_2O$	0.14 g
Resazurin	1.0 mg
$NaHCO_3$ solution	80.0 mL
Trimethylamine•HCl solution	20.0 mL
Na_2CO_3 solution	10.0 mL
Trace elements solution	10.0 mL
Vitamin solution	10.0 mL
L-Cysteine•HCl solution	10.0 mL
$Na_2S \bullet 9H_2O$ solution	10.0 mL
Distilled water	860.0 mL

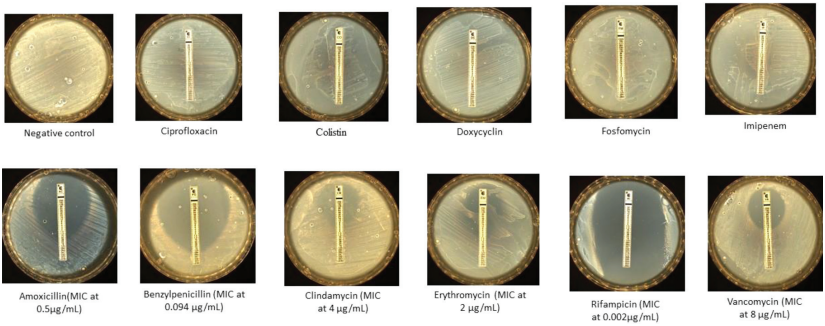
pH 6.9 ± 0.2 at 25°C

Preparation of Medium: Add components, except $NaHCO_3$ solution, trimethylamine•HCl solution, Na_2CO_3 solution, vitamin solution, L-cysteine•HCl solution, and $Na_2S \bullet 9H_2O$ solution, to distilled/deionized water and bring volume to 860.0mL. Mix thoroughly. Sparge with 100% N_2 for 20 min. Then sparge with 80% N_2 + 20% CO_2 for 10 min. Anaerobically distribute into tubes or bottles. Autoclave for 15 min at 15 psi pressure–121°C. Aseptically and anaerobically add 80.0mL of sterile $NaHCO_3$ solution, 20.0mL of sterile trimethylamine•HCl solution, 10.0mL of sterile Na_2CO_3 solution, 10.0mL of sterile vitamin solution, 10.0mL of sterile L-cysteine•HCl solution, and 10.0mL of sterile $Na_2S \bullet 9H_2O$ solution. Mix thoroughly.

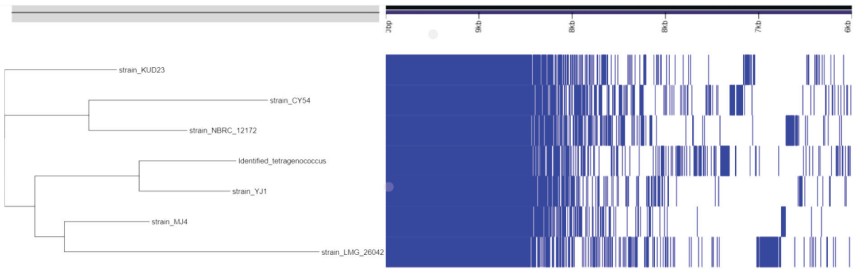
Appendix C. PCR electrophoresis picture of T. halophilus CSURQ6002



Appendix D. Antibiotic susceptibility of T. halophilus CSURQ6002



Appendix E. Pangenome analysis of the identified-Tetragenococcus (T. halophilus CSURQ6002) and the first six hit-blast genomes recovered from GenBank database



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Annexe 2 : Une signature néonatale non invasive permet de prédire le développement ultérieur des maladies atopiques

Préambule

La prématurité est une cause majeure de morbidité et de mortalité chez les nourrissons et les enfants [104]. Les méthanogènes font partie du microbiote humain néonatal et contribuent à former l'empreinte immunitaire précoce [105,106]. Cependant, les méthodes non invasives de dépistage du statut immunitaire néonatal font défaut. Dans cette étude, nous avons analysé la relation entre les biomarqueurs immunologiques, l'abondance des méthanogènes et les maladies atopiques à l'âge d'un an. Nous avons rapporté dans cette étude, une méthode non invasive pour montrer des preuves d'une corrélation entre la charge méconiale et néonatale de méthanogènes et certains biomarqueurs fécaux, et l'apparition ultérieure de conditions atopiques chez les enfants prématurés.

Article 8

A Non-Invasive Neonatal Signature Predicts Later Development of Atopic Diseases.

Y. Sereme, M. Michel, S. Mezouar, **C.O. Guindo**, L. Kaba, G. Grine, T. Mura, J.L. Mège, T.A. Tran, P. Corbeau, A. Filleron, J. Vitte.

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Article

A Non-Invasive Neonatal Signature Predicts Later Development of Atopic Diseases

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Abstract: Background: Preterm birth is a major cause of morbidity and mortality in infants and children. Non-invasive methods for screening the neonatal immune status are lacking. Archaea, a prokaryotic life domain, comprise methanogenic species that are part of the neonatal human microbiota and contribute to early immune imprinting. However, they have not yet been characterized in preterm neonates. **Objective:** To characterize the gut immunological and methanogenic Archaeal (MA) signature in preterm neonates, using the presence or absence of atopic conditions at the age of one year as a clinical endpoint. **Methods:** Meconium and stool were collected from preterm neonates and used to develop a standardized stool preparation method for the assessment of mediators and cytokines and characterize the qPCR kinetics of gut MA. Analysis addressed the relationship between immunological biomarkers, Archaea abundance, and atopic disease at age one. **Results:** Immunoglobulin E, tryptase, calprotectin, EDN, cytokines, and MA were detectable in the meconium and later samples. Atopic conditions at age of one year were positively associated with neonatal EDN, IL-1β, IL-10, IL-6, and MA abundance. The latter was negatively associated with neonatal EDN, IL-1β, and IL-6. **Conclusions:** We report a non-invasive method for establishing a gut immunological and Archaeal signature in preterm neonates, predictive of atopic diseases at the age of one year.

Keywords: preterm birth; fecal mediator and cytokine; methanogenic Archaea; allergy; atopy

1. Introduction

Preterm birth, defined as delivery at fewer than 37 completed weeks of gestation, is the leading cause of neonatal mortality and morbidity and has long-term adverse health consequences [1]. The global incidence of preterm births was estimated at 10.6 in 2014, and 9.8 in 2000 [2]. The etiology of preterm birth is multifactorial and not yet fully understood [3]. However, factors related to preterm birth include maternal or fetal medical conditions, genetic and epigenetic [4] influences, environmental exposures, infertility treatments, behavioral and socioeconomic elements [3,5]. Preterm infants experience abnormal immune and metabolic programming, which might exert a lasting influence on the risk of future disease [6,7]. Preterm-born children have been shown to have immune mediator dysregulation [8], impaired innate immunity and adaptive responses characterized by reduced levels of immunoglobulin (Ig) G, opsonization and phagocytosis, and increased activation of Th1 cells compared to that of Th2 cells [9]. Cohort studies show that preterm-born children are at increased risk for preschool wheezing and school-age asthma [10], but not for food allergy [11,12] or atopic dermatitis (AD) [11,13].

During fetal life, maternal microbiota produces compounds that are transferred to the fetus and enhance the generation of innate immune cells [14]. This process is halted prematurely in preterm infants, leaving them vulnerable to disease [9]. Preterm infants have an inflammatory and hypoxic state, which has a negative impact on lung maturation, the risk of respiratory infections, and susceptibility to subsequent exposures [9,14,15]. As early as the neonatal period, the gut microbiota imprints a persistent effect on the immune system through multiple mechanisms, including the modulation of epithelial functions, the production of cytokines, and the recruitment and training of immune cells [16–18].

Archaea, considered a separate domain of life from Eukarya, giant bacteria and viruses, are part of the human microbiota [19–21]. Gut methanogenic Archaea consume hydrogen produced by bacterial fermentation, releasing methane and short chain fatty acids (SCFA) and thus taking part in the energy supply to the host [19]. They interact with the host immune system, triggering innate and adaptive immune responses, generation of specific T and B cells, and hypersensitivity responses in animals and humans [22–24]. We have shown that the neonatal gut is colonized by methanogenic Archaea from the first postnatal hours, possibly starting in utero [21,22]. Gut microbiome establishment is altered in preterm and low- birth-weight infants [25,26].

Clinical investigations and research studies in neonates, including those born before term, are usually performed with peripheral blood. However, the search for non-invasive alternatives has gained momentum in recent years [27,28].

We hypothesize the existence of an association between intestinal methanogenic Archaea and the intestinal immunological signature, understood as a pattern of immune biomarkers including cell-specific products, e.g., mast cell tryptase and eosinophil-derived neurotoxin (EDN) and major pro- and anti-inflammatory cytokines. The validation of this hypothesis would open the prospect of a predictive score for the later occurrence of immune disorders, including atopic diseases.

We addressed this question through the development of a non-invasive standardized method for the assessment of the neonatal gut immune and microbial status, implemented in a cohort of preterm infants. The aims of the present study were: (1) establish a non-invasive method adequate for the investigation of preterm neonates, (2) characterize the gut immune and Archaeal components longitudinally from birth to six weeks in the study cohort, and (3) correlate the results of gut immune and Archaeal investigations at birth and up to six weeks to the later occurrence of allergic or atopic conditions.

2. Methods

2.1. Patients and Sampling

Stool samples from 43 preterm neonates were collected without the use of preservatives at the Nimes and Montpellier University Hospitals and stored at −80 °C. Samples

were collected as meconium (n = 33) and later stool samples at 2 (n = 33), 4 (n = 29), and 6 (n = 24) weeks.

2.2. Ethics Statement

2.2.1. A-Immunological Analysis

Preparation of Fecal Samples

One gram of feces was solubilized in two milliliters of an in-house extraction buffer consisting of phosphate buffered saline supplemented with 4 mM 4-(2-aminoethyl)-benzensulphonyl fluoride, 0.26 mM bestatin, 28 μM E-64, 2 μM leupeptin and 0.6 μM aprotinin, pH 7.4, and a protease inhibitor (Sigma-Aldrich, St. Louis, MN, USA [29]. The stool–buffer mixture was incubated for 20 min at room temperature, prior to centrifugation at 2000 rpm for 15 min at 4 °C. The supernatant liquids were freeze-dried for 24 h, resolubilized in one milliliter of extraction buffer, and used for mediator and cytokine determination (Figure 1).

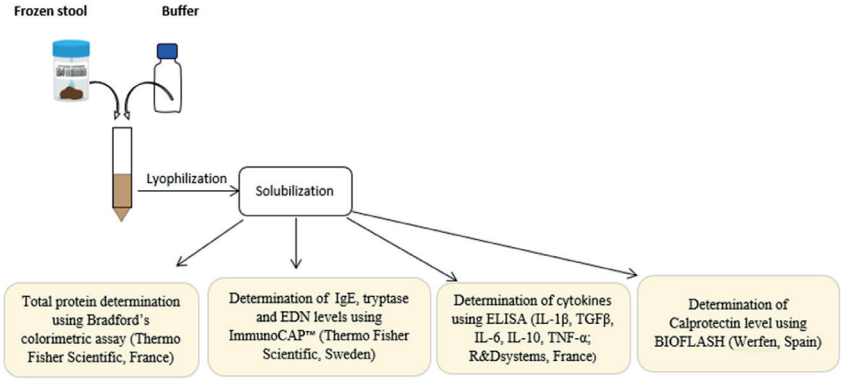


Figure 1. Fecal extraction protocol.

Total IgE, Tryptase, and EDN Determination

The concentration of total IgE, tryptase, and EDN were measured using an automated fluoro-enzymo-immunoassay with the ImmunoCAP™ 250 platform (Thermo Fisher Scientific, Uppsala, Sweden), according to ISO 15,189 standards [30]. The measurement range was 2–5000 kIU/L (4.8–12,000 μg/L) for total IgE, 1–200 μg/L for tryptase, and 2–200 μg/L for EDN.

Calprotectin and Total Protein Determination

Fecal calprotectin was measured using the BIOFLASH (Werfen, Barcelona, Spain) chemo-luminescent analyzer platform according to ISO 15,189 standards. Assay sensitivity was greater than 20 μg/mL. The total protein concentration of the samples was measured by the colorimetric method (BCA Protein assay, Thermo Fisher Scientific).

Immunoassays

Cytokines (IL-6, IL-10, IL-1β, TGF-β, and TNF-α) were measured by ELISA using specific immunoassay kits according to the manufacturer's protocols (R&D systems, Minneapolis, MN, USA). The sensitivity of the assays was 1.0 pg/mL.

2.2.2. A-Microbiological Analysis: Methanogenic Archaea by qPCR

DNA Extraction and PCR Assays

For DNA extraction, 0.2 g of each stool sample were mixed in 1.5 mL tubes with 500 µL of G2 lysis buffer from an EZ1®DNA Tissue Kit (QIAGEN, Hilden, Germany). Then, 0.3 g of acid-washed beads ≤ 106 µm (Sigma-Aldrich, Saint Quentin Fallavier, France) were added in each tube and shaken in a FastPrep BIO 101 device (MP Biomedicals, Illkirch, France) for 45 s for mechanical lysis before 10 min incubation at 100 °C. A 180 µL volume of the mixture was then incubated with 20 µL of proteinase K (QIAGEN, Hilden, Germany) at 56 °C overnight before a second mechanical lysis was performed. Total DNA was finally extracted with an EZ1 Advanced XL extraction kit (QIAGEN) and 50 µL eluted volume. Sterile phosphate buffered saline (PBS) (Fisher Scientific, Illkirch, France) was used as a negative control in each DNA extraction run. Extracted DNA was incorporated into real-time PCR performed using Metha_16S_2_MBF: 5′-CGAACCGGATTAGATACCCG -3′ and Metha_16S_2_MBR: 5′-CCCGCCAATTCCTTTAAGTT-3′ primers and the FAM_Metha_16S_2_MBP 6FAM- CCTGGGAAGTACGGTCGCAAG probe targeting the 16S DNA gene of methanogens, designed in our laboratory (Eurogentec, Angers, France) as previously described [31]. PCR amplification was done in 20 µL volume including 15 µL of mix and 5 µL of extracted DNA. Five microliters of ultra-pure water (Fisher Scientific, Illkirch, France) were used instead of DNA in the negative controls. The amplification reaction was performed in a CFX96 thermocycler (BioRad, Marnes-la-Coquette, France) incorporating a protocol with a cycle of 50 °C for 2 min, followed by 39 cycles of 95 °C for 45 s, 95 °C for 5 s and finally 60 °C for 30 s. Samples with a CT < 40 were considered positive. Gene amplification and PCR sequencing were performed as previously described [25,26,32–34].

Statistical Analysis

The responses for each quantitative parameter were described using median and 25–75 percentile (interquartile range, IQR) unless otherwise stated. Analyses were performed using the Wilcoxon test when two groups were compared, and the Kruskal–Wallis test when more than two groups were compared. The association between the different biomarkers of interest were analyzed, at each sampling time, using Spearman's correlation coefficient. The association profiles between different biomarkers were also analyzed using a principal component analysis method. Statistical analyses were performed at the conventional two-tailed α level of 0.05, using R 2.13.2 statistical software (R Foundation for Statistical Computing, https://www.r-project.org (accessed on 18 March 2022), Vienna, Austria).

3. Results

3.1. Demographic and Clinical Characteristics of Preterm Infants

The 43 preterm neonates included in our study had at birth an average weight of 1160.41 g (range 440–1750 g), an average gestational age of 29 weeks (range 24–32 weeks) and an average height of 37.11 cm (range 32–47 cm). Thirty-five (81%) were born by cesarean section and 8 (19%) by vaginal delivery. Only five mothers (11.6%) had received antibiotic therapy during the peripartum period. As part of the cohort follow-up, clinical evaluation (AF) was conducted at 1 year and assessed the presence or absence of health conditions, including atopic diseases. When necessary, allergy diagnosis was carried out according to current recommendations [35,36]. A total of nine children developed an atopic condition during the first year, manifested as asthma or cow's milk allergy (CMA) in eight and AD in three, with two patients presenting an association of AD, asthma, and CMA (Table 1).

Table 1. Clinical data for preterm infants investigated for the presence of fecal biomarkers. VD: vaginal delivery; CMA, cow's milk allergy; CS: cesarean section.

Code	Meconium (M)	Two-weeks (W2)	Four-Week (W4)	Six-weeks (W6)	Peripartum Maternal Antibiotic Therapy	Mode of Delivery	Gestational Age	Weight	Size	Asthma or CMA	Atopic Dermatitis
1	0	W2	W4	W6	No	VD	30	1275	37	Yes	Yes
2	M	W2	W4	W6	Yes	CS	27	925	34	Yes	No
3	M	W2	W4	W6	No	CS	26	565	31	Yes	No
4	0	0	W4	0	No	VD	25	820	34	Yes	No
5	M	W2	W4	0	No	CS	32	1260	39	Yes	No
6	M	W2	W4	W6	Yes	CS	27	680	31	Yes	No
7	M	0	W4	W6	No	CS	29	1565	42	No	No
8	M	W2	W4	W6	No	CS	28	890	33	No	No
9	0	0	0	W6	Yes	CS	30	1150	38	No	No
10	M	W2	0	0	No	CS	31	1570	43	No	No
11	M	W2	W4	0	No	CS	32	1575	44	No	No
12	M	W2	W4	0	No	CS	30	1360	39	No	Yes
13	M	W2	0	W6	No	CS	25	870	34	Yes	Yes
14	M	W2	W4	0	No	CS	32	1155	39	Yes	No
15	0	W2	W4	W6	No	CS	25	440	28	No	No
16	0	0	0	W6	No	VD	30	1590	41	No	No
17	M	W2	W4	W6	No	CS	24	530	31	No	No
18	M	W2	0	0	No	CS	26	925	35	No	No
19	M	W2	W4	0	No	VD	30	1480	41	No	No
20	M	W2	W4	0	No	VD	30	1460	38	No	No
21	M	W2	W4	W6	No	CS	29	880	35	No	No
22	0	W2	W4	W6	No	CS	28	840	35	No	No
23	M	W2	W4	0	Yes	VD	30	1670	43	No	No
24	M	W2	W4	W6	No	CS	31	1120	38	No	No
25	0	W2	0	0	No	CS	28	915	36	No	No
26	M	0	W4	W6	No	CS	26	925	35	No	No
27	M	W2	W4	W6	Yes	CS	30	1335	39	No	No
28	M	W2	W4	W6	Yes	CS	30	1355	47	No	No
29	M	W2	0	W6	No	CS	30	1480	39	No	No
30	0	W2	W4	W6	Yes	CS	28	1010	35	No	No
31	M	W2	W4	W6	No	CS	29	1050	39	No	No
32	M	W2	W4	0	No	CS	29	1190	38	No	No
33	0	W2	W4	W6	No	CS	30	1175	39	No	No
34	M	0	0	0	No	CS	32	1930	44	No	No
35	0	W2	W4	0	Yes	CS	29	1430	30	No	No
36	M	W2	W4	0	No	CS	30	1750	43	No	No
37	M	0	0	0	No	CS	27	600	29	No	No
38	M	W2	W4	W6	No	VD	25	750	32	No	No
39	M	W2	0	W6	No	CS	31	980	36	No	No
40	M	W2	0	W6	No	CS	31	1410	39	No	No
41	M	0	0	0	No	CS	30	770	33	No	No
42	M	0	0	0	Yes	VD	32	1568	41	No	No
43	M	0	0	0	No	CS	30	1680	39	No	No

3.2. Immune Profiling

3.2.1. Total Protein Determination

First, we measured the total protein content in all samples. The median concentration of fecal proteins was stable from birth to six weeks, ranging from 4.53 to 9.18 g/L ($p = 0.10$; Kruskal–Wallis) (Table 2).

Table 2. Determination of fecal immune biomarkers.

	Meconium		Two Weeks		Four Weeks		Six Weeks			
	n = 33		n = 33		n = 29		n = 24			
	n (%) Detectable	Median IQR	n (%) Detectable	Median IQR	n (%) Detectable	Median IQR	n (%) Detectable	Median IQR	*p*-Value (Frequency)	*p*-Value (Levels)
Total Proteins (g/L)	33 (100)	9.18 (4.51–13.54)	33 (10,055)	5. 4.23–6.05)	29 (100)	4.53 (3.00–5.52)	24 (100)	6.46 (5.39–7.76)	NS	0.10
Total IgE (µg/L)	30 (90.90)	7.3 (6.4–9.9)	32 (97)	8.47 (6.8–9.8)	27 (93.10)	9.74 (3.39–0.26)	24 (100)	115.08 (41.00–193.70)	0.41	<0.0001
Tryptase (µg/L)	3 (9.1)	<1	3 (9.1)	<1	4 (13.79)	<1	14 (58.33)	1.8 (0.0–3.4)	<0.0001	0.61
Calprotectin (µg/L)	33 (100)	310.4 (151.1–771.3)	33 (100)	291.23 (189.41–487.87)	29 (100)	402.44 (300.06–607.3)	24 (100)	422.37 (335.53–823.30)	NC	0.13
EDN (µg/L)	33 (100)	83.2 (19.3–165.0)	33 (100)	70.1 (17.8–152.5)	29 (100)	109.0 (44.2–200.0)	24 (100)	98.1 (57.5–200.0)	NC	0.21
TGF-β (pg/L)	24 (72.7)	121.3 (4.6–258.9)	30 (91)	267.43 (61.71–1000)	26 (89.65)	384.57 (129.60–936)	22 (91.66)	466 (104.36–1430.29)	0.09	0.014
IL-1β (pg/L)	13 (39.4)	0.12 (0.1–2.7)	28 (84.8)	1.53 (0.37–6.53)	25 (86.20)	3.27 (0.31–10.76)	22 (91.66)	6.23 (1.66–20.84)	<0.0001	0.001
IL-10 (pg/L)	4 (12.12)	3.9 (3.9–3.9)	6 (18.18)	3.9 (3.9–3.9)	5 (17.24)	3.9 (3.9–3.9)	5 (20.83)	3.9 (3.9–3.9)	0.85	0.53
IL-6 (pg/L)	25 (75.75)	11.6 (0.5–43.7)	7 (21.21)	0.2 (0.2–0.2)	20 (68.96)	0.2 (0.2–0.2)	20 (83.33)	3.77 (1.66–19.25)	<0.0001	<0.001

Concentrations are expressed as median and interquartile ranges (IQR). n (%): number of samples in which the biomarker was detected (relative frequency of detection). The median and IQR were calculated by restricting the results above the lower LOQ (limit of quantitation) for each analyte. Statistical test: chi-square (frequency), Kruskal–Wallis (concentration). NC, not calculable (calprotectin and EDN were detectable in all samples and at all sampling times).

3.2.2. Immune Cell Markers and Cytokines

Total IgE was detectable in over 90% of the samples at all ages, in increasing amounts between birth (meconium) and six weeks ($p < 0.0001$; Kruskal–Wallis).

Conversely, tryptase detection increased with sampling age, reaching 58% in samples at six weeks, up from less than 15% at earlier times ($p < 0.0001$; Chi-square). As most values were lower than the quantification limit, quantitative comparison was not significant ($p = 0.61$, Kruskal–Wallis) (Figure 2 and Table 2).

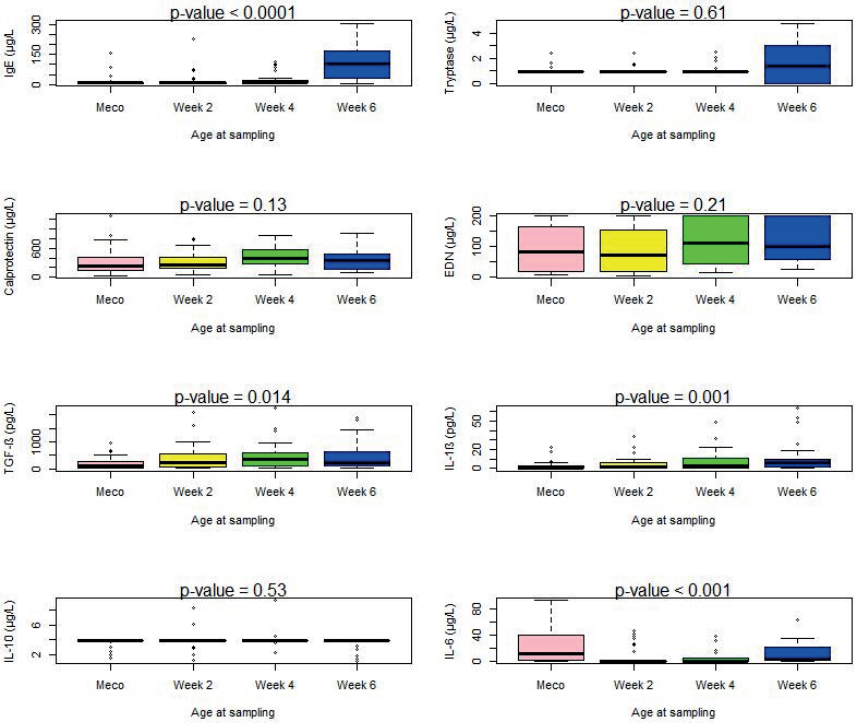


Figure 2. Statistical test: chi-square (frequency), Kruskal–Wallis (concentration). NC, not calculable (calprotectin and EDN were detectable in all samples and at all sampling times).

Calprotectin and EDN were detected in all samples at comparable levels irrespective of age ($p = 0.13$ and 0.21, Kruskal–Wallis) (Figure 2 and Table 2).

All cytokines except TNF-α were detectable in meconium and fecal samples. TGF-β and IL-6 were the most prevalent, detected in up to 90% of samples, while IL-10 was the less prevalent, found in 20% or less of the fecal samples. The frequency of detection of IL-6 and IL-1β increased with age ($p < 0.0001$; chi-square), although there was a sharp drop in IL-6 frequency of detection and measured levels between meconium (75%, median 11.6 pg/L) and samples at two weeks (21%, median 0.2 pg/L).

TGF-β and IL-1β median concentrations increased with age ($p = 0.014$ and 0.001, respectively; Kruskal–Wallis). The median level of IL-6 was the highest in meconium samples and increased again at six weeks ($p < 0.0001$; Kruskal–Wallis). IL-10 median concentrations did not vary with age (Figure 2 and Table 2).

Maternal antibiotic therapy and route of delivery did not significantly affect the meconium levels of cytokines, total IgE, tryptase, calprotectin, and EDN (Supplementary Table S1). However, analysis according to the development of atopic disease during the first year showed that meconium calprotectin levels were lower in neonates who subsequently developed asthma or CMA compared to those who did not ($p = 0.02$; Wilcoxon test) (Figure 2). Levels of other mediators and cytokines were not associated with the occurrence of an atopic disease (Table 3).

Table 3. Comparison of mediators and cytokines and the occurrence or absence of an atopic condition between years 0 and 1. Statistical test used: Wilcoxon test.

Variables	Allergic Condition (APLV and Asthma)	*p*-Value			
		Meconium	2 Weeks	4 Weeks	6 Weeks
IgE	Yes	0.27	0.06	0.12	**0.03**
	No				
Calprotectin	Yes	0.27	0.18	0.91	0.61
	No				
EDN	Yes	0.59	0.19	0.41	0.87
	No				
TGF-β	Yes	0.09	0.18	0.76	0.76
	No				
IL-1β	Yes	0.37	1.00	0.28	0.91
	No				
IL-10	Yes	0.62	1.00	0.89	0.13
	No				
IL-6	Yes	0.61	0.28	0.37	0.75

Comparing the different biomarkers according to the presence or absence of an atopic condition, we observed a significant difference at week six for IgE between the presence and absence of cow's milk allergy and asthma. No significant difference was observed for AD (Supplementary Table S2)

3.2.3. Correlation between Biomarkers

As an expected control, significant correlations were found between weight and height (R = 0.90; $p < 0.0001$), between gestational age and height (R = 0.75; $p < 0.0001$), and between gestational age and weight (R = 0.75; $p < 0.0001$).

Total IgE and tryptase levels were strongly correlated in samples taken at any age. IL-10 and IL-6 were correlated at all ages except at two weeks (Table 4).

Table 4. Result of the detection of methanogenic Archaea.

	Meconium (n = 33)		Two Weeks (n = 33)		Four Weeks (n = 29)		Six Weeks (n = 26)		*p*-Value (Frequency)	*p*-Value (CT)
	n (%)	Median IQR	n (%)	Median IQR	n (%)	Median IQR	n (%)	Median IQR		
CT qPCR	30 (90.9)	36.74 (33.85–38.24)	27 (81.81)	37.20 (36.07–38.33)	23 (79.31)	37.75 (36.13–38.50)	19 (73.03)	38.28 (37.27–39.96)	0.34	0.12

CT methanogenic Archaea are expressed as median and interquartile ranges (IQR). n (%): number of samples in which methanogenic Archaea were detected (relative frequency of detection). The

stool concentration factor and median and RDI were not included in our calculations, and the median and RDI were calculated by restricting the results above the lower LOQ (limit of quantitation) for each analyte. Statistical test: Kruskal–Wallis.

3.2.4. Meconium Samples

Tryptase levels were correlated to levels of IL-10 (R = 0.48, $p = 0.001$) and IL-6 (R = 0.46; $p = 0.001$). Tryptase levels were correlated to levels of IgE (R = 0.91, $p = 0.0001$), and strong correlations were observed between levels of calprotectin and IL-1β (R = 0.90; $p < 0.0001$). A negative correlation between total protein concentration and TGF-β (R = −0.36; $p = 0.01$) was observed (Table 3).

3.2.5. Samples at Two Weeks

Strong correlations were observed between levels of calprotectin and IL-1β (R = 0.90; $p < 0.0001$), tryptase and IL-10 (R = 0.88; $p < 0.0001$), and total IgE and IL-10 (R = 0.85; $p < 0.0001$), while total protein concentration and IL-6 were negatively correlated (R = −0.61; $p = 0.0002$) (Table 3).

3.2.6. Samples at Four Weeks

Again, calprotectin and IL-1β were strongly correlated (R = 0.74; $p < 0.0001$). IgE and total protein were also correlated (R = 0.38; $p < 0.04$) (Figure 3).

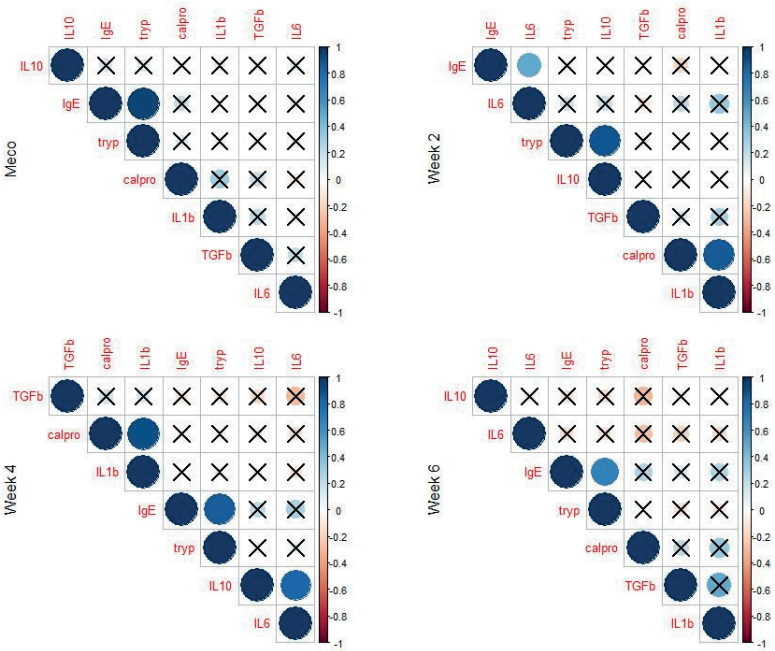


Figure 3. Correlation figure of fecal immune biomarkers.

3.2.7. Samples at Six Weeks

TGF-β was correlated with IL-1β (R = 0.49; $p < 0.01$) and with total proteins (R = 0.43; $p < 0.03$). Total proteins were correlated with IL-1β (R = 0.47; $p < 0.02$) and negatively with IL-6 (R = -0.68; $p = 0.0003$) (Figure 3).

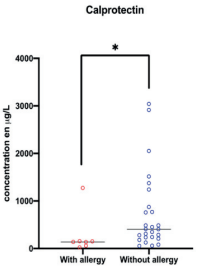
3.3. Frequency of Detection of Methanogenic Archaea and Relationship with the Subsequent Development of Atopic Diseases

Using real-time PCR with 16S rRNA archaeal gene PCR primers, we detected methanogenic Archaea DNA in 30/33 (90%) meconium samples, 27/33 (81%) two-week-old samples, 23/29 (79%) four-week-old samples, and 19/24 (73%) six-week-old samples, respectively. We found no significant difference in the frequency of detection nor in CTs according to age at sampling (Table 4).

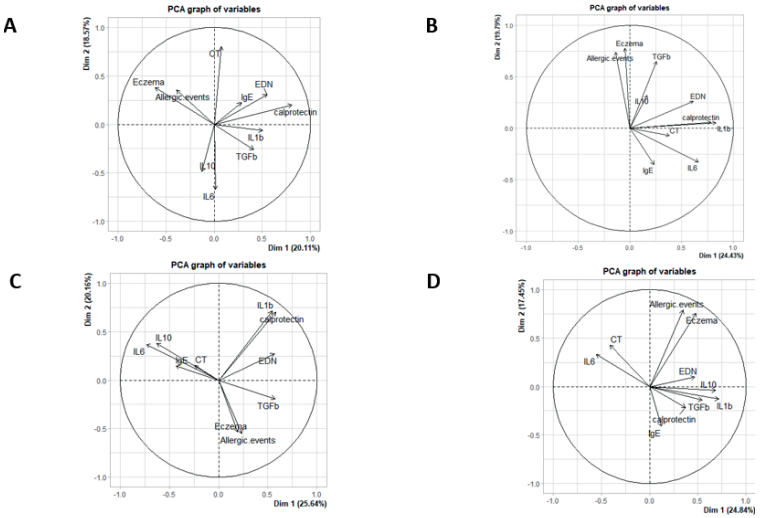
3.4. Unsupervised Analysis of Immunological Markers and Methanogenic Archaea Atthe Neonatal Period, and the Subsequent Occurrence of AD, Asthma and CMA during the First Year

We performed unsupervised analysis of the immunological data, CT of Archaea, and the clinical information of the occurrence of allergic events during the first year of life. Data were analyzed for each of the four sampling times.

For meconium, calprotectin, EDN, and IL-1β levels were negatively and significantly ($p < 0.001$) correlated (r = −0.64) with subsequent development of AD. Calprotectin, EDN and IL-1β had the largest and most significantly ($p < 0.01$) correlated positive correlation coefficients, which were 0.79, 0.53, and 0.51, respectively. No correlation was observed for the Archaea CT with the other parameters (Figure 4b(A)).



(a)



(**b**)

Figure 4. (**a**). Comparison of meconial calprotectin concentration according to the later occurrence of asthma or cow's milk allergy. (**b**). Principal component analysis of neonatal immune and archaeal biomarkers as a function of later occurrence of atopic conditions. (**A**) Negative and significant ($p < 0.001$) correlation (r = −0.64) between the occurrence of atopic dermatitis with calprotectin, EDN, and IL-1β. Strong positive and significant correlation ($p < 0.01$) between calprotectin (r = 0.79), EDN (0.53), and IL-1b (0.51). No correlation between Archaea TCs and other parameters. (**B**) Correlation between allergic events (asthma or cow's milk allergy) and atopic dermatitis. Strong positive and significant ($p < 0.001$) correlation between IL-1b (r = 0.88) and calprotectin (r = 0.82), positive correlation between IL-6 (r = 0.62) and EDN (r = 0.59), significant ($p < 0.001$). Low positive (r < 0.5) and significant correlation between Archaea TCs and biomarkers IL-1b, calprotectin, IL-6, and EDN. (**C**) Positive and significant correlation ($p < 0.001$) between atopic dermatitis, allergic events with the markers calprotectin (r = 0.61), IL-1b (r = 0.58), EDN (r = 0.57), and TGF-β (r = 0.57). Negative correlation between IL-6 (r = −0.71) and IL-10 (r = −0.61) with atopic dermatitis and allergic events. Positive and significant correlation between Archaea TCs with calprotectin, IL-1b, IL-6, and EDN. Negative correlation between Archaea Ct and allergic events. (**D**) Negative correlation between IL-6 (r = −0.51) with other biomarkers and allergic events. Strong positive and significant correlation ($p < 0.001$) between IL-1b (r = 0.68), IL-10 (r = 0.67), and atopic dermatitis (r = 0.58). Low correlation (r < 0.5) between Archaea TCs with IL-1β, calprotectin, IL-6, and EDN. Negative correlation between allergic events and TC of Archaea.

At two weeks, IL-1β (r= 0.88) and calprotectin (r = 0.82) had a strong positive correlation with each other, followed by IL-6 (r = 0.62) and EDN (r = 0.59). These biomarkers were significantly ($p < 0.001$) associated. Archaea CTs had a weak (r < 0.5) but positive and significant ($p < 0.01$) association with the biomarkers IL-1β, calprotectin, IL-6, and EDN (Figure 4b(B)).

At four weeks, later occurrence of AD, CMA, and asthma was positively correlated with calprotectin (r = 0.61), IL-1β (r = 0.58), EDN (r = 0.57), and TGF-β (r = 0.57), and negatively correlated with IL-6 (r = −0.71) and IL-10 (r = −0.61). Calprotectin, IL-1β, and EDN were significantly associated with each other ($p < 0.001$). Archaea CTs were positively associated with calprotectin, IL-1β, IL-6, and EDN with a significant correlation ($p < 0.001$),

however, they were inversely correlated with allergic events, although the correlation coefficient was low (Figure 4b(C)).

At six weeks, only IL-6 correlated negatively (r = −0.51) with the other biomarkers and allergic events. IL-1β (r = 0.68), IL-10 (r = 0.67), and AD (r = 0.58) showed the strongest positive correlations, with AD significantly ($p < 0.001$) associated with IL-1β and IL-10. Archaea CTs were weakly correlated (r < 0.5) with IL-1β, calprotectin, IL-6, and EDN. However, allergic events were negatively associated with CT, and AD had almost no correlation (Figure 4b(D)).

4. Discussion

In this study, we describe a non-invasive screening method for profiling neonatal immunity and its validation in a preterm neonate cohort as a predictive tool for subsequent development of atopic diseases. The method was also applied to meconium samples, which reflect intrauterine processes and contain almost 1000 identified proteins with important functions [37]. The clinical endpoints of this study were evaluated at the age of one year, while the total duration of cohort follow-up will be three years.

Total IgE was detected in over 90% of the samples, at increasing concentrations with age. Transplacental delivery of allergens and preterm sensitization have long been recognized, possibly inducing sensitization and detectable meconial IgE [38,39]. Transplacental transport of maternal IgE able to sensitize fetal mast cells has been recently demonstrated [40], but its role in neonatal immune defenses or subsequent immune disorders is only speculative.

Addressing mast cell tryptase in meconium and later samples, we found that it was detectable only in a minority of meconium samples and during the first month of life, however, it became a common finding at the end of the neonatal period, represented by samples collected at six weeks. Fecal tryptase and IgE levels were strongly associated at each of the studied time points. These results suggest that gastrointestinal mast cells, as opposed to skin mast cells [40], are mostly recruited postnatally, and mature after birth with IgE levels exerting a positive effect. Conversely, maturity of gastrointestinal mast cell populations might be attained during late pregnancy. Tryptase is a serine protease able of autocrine activation of mast cells and induction of proinflammatory effects such as proteolytic cleavage and activation of PAR2 receptors and inactivation of VIP (Vasoactive Intestinal Peptide), associated with smooth muscle relaxation [41]. Through PAR-2 activation, luminal tryptase can contribute to the dysfunction of the gut epithelial barrier [42]. The presence of tryptase in stool samples has been associated with food allergic diseases, dietary exposure and/or mast cell stimulation or increased intestinal mast cell count [43,44]. In addition, fecal tryptase has also been shown to be associated with inflammatory bowel disease and irritable bowel syndrome [45,46].

Focusing on two secreted biomarkers of innate immune cells, neutrophil-derived calprotectin, and eosinophil-derived EDN, we found that fecal samples at all studied ages contained detectable and stable levels of both biomarkers. The levels measured in our preterm cohort were much lower than the reference values [47], but similar to those reported during the first postnatal month in another preterm cohort [48]. These results suggest that the preterm gut contains small numbers of granulocytes, or that such granulocytes are not activated. Indeed, lower neutrophils were reported in preterm infant cord blood [49]. An association between low levels of fecal calprotectin and adverse health conditions, including obesity and sepsis, by age two has been suggested [48].

Proinflammatory and anti-inflammatory cytokines were detected in the meconium and later samples of preterm infants, with the notable exception of TNF-α which was not demonstrated in any sample. Different temporal patterns were demonstrated: IL-6 levels were higher in the meconium than in later samples, while IL-10 was seldom detected and TGF-β and Il-1β displayed a progressive increase between birth and six weeks. Mostly undetectable IL-10 levels were also reported in a pilot study of fecal biomarkers in preterm infants [8]. Although we did not determine the cellular source of fecal cytokines, a shift in

immune cells lining the intestine has been demonstrated for macrophages, with resident fetal macrophages being replaced after birth by bone-marrow-derived macrophages [50]. Macrophage cytokine production, most notably of proinflammatory IL-6 and IL-1β, can be persistently altered by metabolic conditions [51]. The increase in TGF-β levels from birth to six weeks might provide a counter-acting mechanism in a proinflammatory environment. Allergic events (asthma or cow's milk allergy) and atopic dermatitis were also positively correlated with EDN, IL-1β, IL-10, and IL-6 at four and six weeks. The high production of fecal EDN, IL-1β, and IL-10 during the first weeks of life may therefore be an indicator for later risk of allergic diseases.

The neonatal period is paramount for the establishment of the intestinal microbiota. Intrauterine life is associated with low levels of maternal microbial translocation [52]. However, we have recently demonstrated the presence of the viable methanogenic Archaea *Methanobrevibacter smithii* in the meconium, suggesting intrauterine colonization of the fetus by this microorganism [26]. Here, we provided evidence for postnatal persistence of methanogenic Archaea in fecal samples and suggest a possible role in the orientation of intestinal immunity, supported by the negative association between Archaea abundance (inversely proportional to CT values) and the concentrations of EDN, IL-1β, and IL-6. We also found that Archaea abundance at four and six weeks was positively associated with later occurrence of allergic events. Archaea have been shown to produce SCFA which induce regulatory T cell differentiation, downregulate proinflammatory cytokines, and may protect against the occurrence of atopic conditions [19,53–57] However, in a cohort study, the protective effect of methanogenic Archaea was restricted to the species *Methanobrevibacter stadtmanae* [58]. A decrease in the load of beneficial methanogenic Archaea during the first years of life could therefore favor the occurrence of allergic events during the first years of life.

A third line of contribution to protection or increased risk of developing atopic conditions is the genetic background. As an example, a del/del genotype (−2549 −2567 del18) of Vascular Endothelial Growth Factor (VEGF) has been associated with asthma occurrence and irreversible bronchoconstriction [59].

The strengths of our study are methodological and medical:

(1) miniaturization and standardization, using small quantities of stool (1 g) and small volumes of extraction buffer (2 mL). The dilution of the samples was corrected by the freeze-drying process, as the lyophilizates were contained in 1 mL of buffer.
(2) prevention, thanks to the use of protease inhibitors, of the risk of potential contamination of the handler.
(3) suitability for a microarray platform yielding patterns of immune responses rather than individual measurements.
(4) suitability for combined immune and microbiological assessment.
(5) proof of concept of the immune profiling of fecal mediators in meconium and neonatal samples as predictors of later development of atopic disorders.
(6) proof of concept for non-invasive investigation of the immune status of preterm neonates.

The main weakness of this study is the lack of microbiological data outside Archaea. Further studies are warranted for longitudinal immuno-microbiological profiling of meconium and neonatal samples, in preterm and at-term infants. Its validation as a non-invasive diagnostic method will be in line with the currently unmet needs in terms of non-invasive diagnosis of allergy.

5. Conclusions

This study allowed us to highlight the presence of mediators in the meconium and feces of preterm infants. We provide proof of concept of the feasibility and value of a standardized fecal mediator assay for non-invasive profiling of neonatal immunity. Such assays can be used for early characterization of the immune status of a newborn. Technical

optimization for a multiplex assay could facilitate the implementation of fecal immune profiling in clinical and research laboratories. We report evidence of a correlation between the meconial and neonatal load of methanogenic Archaea and selected fecal biomarkers, and later occurrence of atopic conditions in preterm children.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm11102749/s1, Table S1: Comparison of biomarkers and CT of Archaea according to the presence or absence of maternal antibiotic therapy and according to the mode of delivery, Table S2: Comparison of median CT of Archaea in preterm infants according to the presence or absence of allergic events during the first year of life, Table S3: Comparison of median CTs of Archaea in preterm infants according to the presence or absence of atopic dermatitis during the first year of life.

Author Contributions: Y.S. developed the stool preparation method, performed the immunology experiments, analyzed the data, and wrote the first draft of the manuscript. M.M. and S.M. performed the experiments, analyzed the data, and proofread the manuscript. Y.S., C.O.G., L.K. and G.G. performed the microbiology experiments and analyzed the data. T.M. analyzed the data. G.G. and T.M. proofread the manuscript. J.-L.M., T.A.T., P.C., A.F. and J.V. designed the study and supervised the experiments. T.A.T. and A.F. collected and analyzed the clinical data. A.F. included patients and performed clinical examination. T.A.T., P.C., A.F. and J.V. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from both parents at infants' birth.

Data Availability Statement: Not applicable.

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Conflicts of Interest: J.V. reports speaker and consultancy fees in the past five years from Astra Zeneca, Meda Pharma (Mylan), Novartis, Sanofi, Thermo Fisher Scientific, outside the submitted work. The other authors declare no competing interests in relation to this study.

Abbreviations

AD: atopic dermatitis; CMA, cow's milk allergy; CT, cycle threshold; IQR, interquartile range; SCFA, short chain fatty acid; EDN, eosinophil-derived neurotoxin

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Résumé

Les archaea méthanogènes (appelées méthanogènes dans ce document) sont les plus prévalentes parmi les archaea des microbiotes oral et digestif humains. Dans le microbiote digestif humain, les archaea méthanogènes ressortissent des genres *Methanobrevibacter* et *Methanomassiliicoccus*. Ces microorganismes consomment l'hydrogène issu des fermentations bactériennes anaérobies pour la production du méthane comme produit métabolique, d'où leur nom.

Les archaea méthanogènes sont présentes dans le microbiote humain dès la naissance avec une diversification au fil des années. Cependant, le répertoire complet, les sources et les dynamiques des méthanogènes restent méconnus chez l'Homme. Dans le cadre de cette Thèse, nous nous sommes intéressés à l'étude des sources et de la dynamique d'acquisition des méthanogènes chez l'Homme. Dans une première partie, nous avons revu la littérature décrivant l'ensemble des rôles physiologiques des méthanogènes associés au microbiote humain. Notre revue a mis en évidence l'énorme déficit de connaissances et des informations partiellement contradictoires concernant les méthanogènes humains, ainsi que la nécessité d'étudier la dynamique des populations de méthanogènes et leur rôle exact au sein du microbiote humain. Dans la seconde partie de nos travaux de Thèse, nous avons optimisé et développé de nouveaux protocoles pour l'identification et la culture en routine des méthanogènes d'intérêt clinique dans le laboratoire de microbiologie clinique. Nos travaux expérimentaux ont eu pour résultat de réduire le temps d'isolement par culture et de sous-culture de *Methanobrevibacter smithii* et de *Methanobrevibacter oralis*, d'isoler par culture une nouvelle espèce de méthanogène que nous avons nommée *Methanomassiliia massiliensis* à partir du fluide buccal et enfin, d'identifier les méthanogènes d'intérêt clinique par spectrométrie de masse MALDI-TOF- MS. Concernant la dynamique des méthanogènes chez l'Homme, nous avons montré par une approche polyphasique incluant la PCR-séquençage et les microscopies, que la période intra-utérine constitue le premier moment d'acquisition de *Methanobrevibacter smithii* avec la détection sans précédent de ce méthanogène dans le méconium de nouveau-nés prématurés. Également, nous avons ouvert la possibilité de transmission zoonotique des méthanogènes en montrant que les contacts étroits avec des animaux domestiques ainsi que la consommation du lait et des produits laitiers de certains animaux sont associés à la diversification des méthanogènes chez l'Homme.

Les résultats de notre Thèse constituent des avancées majeures dans la compréhension des sources et de la dynamique d'acquisition des méthanogènes du microbiote humain et invitent à développer un milieu biphasique standard pour l'isolement et la culture rapide des méthanogènes directement à partir des échantillons cliniques, permettant de poursuivre des investigations pour isoler par culture les méthanogènes détectés dans le méconium et dans les produits laitiers.

Mots-clés : Archaea, méthanogènes, Sources des méthanogènes, dynamique des méthanogènes, *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanomassiliia massiliensis*, microbiote humain, liquide buccal, méconium, produits laitiers, animaux domestiques, *Tetragenococcus halophilus*.

Abstract

Methanogenic archaea (referred to as methanogens in this document) are the most prevalent archaea in human oral and digestive microbiota. In the human digestive microbiota, the methanogenic archaea belong to the genera *Methanobrevibacter* and *Methanomassiliicoccus*. These microorganisms consume hydrogen from anaerobic bacterial fermentations to produce methane as a metabolic product, hence their name.

Methanogenic archaea are present in the human microbiota from birth with diversification over the years. However, the complete repertoire, sources and dynamics of methanogens remain unknown in humans. In the framework of this thesis, we are interested in the study of the sources and the dynamics of methanogen acquisition in humans. In the first part, we reviewed the literature describing the physiological roles of methanogens associated with the human microbiota. Our review highlighted the huge knowledge gap and partially conflicting information regarding human methanogens, as well as the need to study the population dynamics of methanogens and their exact role within the human microbiota. In the second part of our thesis work, we optimized and developed new protocols for the routine identification and culture of methanogens of clinical interest in the clinical microbiology laboratory. Our experimental work resulted in reducing the time of isolation by culture and subculture of *Methanobrevibacter smithii* and *Methanobrevibacter oralis*, isolation by culture a new species of methanogen that we have named *Methanomassiliia massiliensis* from oral fluid and finally, to identify methanogens of clinical interest by MALDI-TOF-MS mass spectrometry. Concerning the dynamics of methanogens in humans, we have shown by a polyphasic approach including PCR-sequencing and microscopies, that the intrauterine period constitutes the first moment of acquisition of *Methanobrevibacter smithii* with the unprecedented detection of this methanogen in the meconium of preterm newborns. Also, we opened the possibility of zoonotic transmission of methanogens by showing that close contact with domestic animals as well as the consumption of milk and dairy products from certain animals are associated with the diversification of methanogens in humans.

The results of our thesis constitute major advances in the understanding of the sources and the dynamics of methanogen acquisition in the human microbiota and invite the development of a standard biphasic medium for the isolation and rapid culture of methanogens directly from clinical samples, allowing further investigations to isolate by culture the methanogens detected in meconium and in dairy products.

Keywords: Archaea, methanogens, methanogen sources, methanogen dynamics, *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanomassiliia massiliensis*, human microbiota, oral fluid, meconium, dairy products, domestic animals, *Tetragenococcus halophilus*.