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# Le microbiote buccodentaire humain : actualisation des méthodologies et des techniques de culture

Saber Khelaifia

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**THESE**  
**POUR OBTENIR LE DIPLOME D'ETAT**  
**DE DOCTEUR EN CHIRURGIE DENTAIRE**

Présentée et publiquement soutenue devant

Aix-Marseille Université  
(Président : Monsieur le Professeur Éric BERTON)

Faculté des Sciences Médicales et Paramédicales  
(Doyen : Monsieur le Professeur Georges LEONETTI)

Ecole de Médecine Dentaire  
(Directeur : Monsieur le Professeur Bruno FOTI)

***Le microbiote buccodentaire humain : actualisation des  
méthodologies et des techniques de culture***

Présentée par

**KHELAIFIA Saber**

Né(e) le 1 Septembre 1979

Thèse soutenue le **2 Février 2023**

Devant le jury composé de

**Président : Professeur TASSERY Hervé**

**Assesseurs : Professeur TERRER Elodie**

**Docteur ABOUDHARAM Gérard**

**Docteur BAUDINET Thomas**

**Invité : Docteur PILLIOL Virginie**



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Docteur en Chirurgie Dentaire  
Docteur de l'Université Aix-Marseille  
Professeur des Universités  
Praticien hospitalier des CSERD  
Sous-section Odontologie Conservatrice, Endodontie.
  
- **À Madame le Professeur Terrer Elodie**  
Docteur en Chirurgie Dentaire  
Docteur de l'Université Aix-Marseille  
Professeur des Universités  
Praticien hospitalier des CSERD  
Sous-section Odontologie Conservatrice, Endodontie.
  
- **À Monsieur le Docteur ABOUDHARAM Gérard**  
Docteur en Chirurgie Dentaire  
Docteur de l'Université Aix-Marseille  
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Praticien hospitalier des CSERD  
Sous-section Odontologie Conservatrice, Endodontie.
  
- **À Monsieur le Docteur Baudinet Thomas**  
Docteur en Chirurgie Dentaire  
Chef de clinique des universités-assistant des hôpitaux  
Sous-section Odontologie Conservatrice, Endodontie.
  
- **À Madame le Docteur Pilliol Virginie**  
Docteur en Chirurgie Dentaire  
Chef de clinique des universités-assistant des hôpitaux  
Sous-section Odontologie Conservatrice, Endodontie.



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## 1. Introduction

La cavité buccale humaine est un écosystème complexe favorable au développement et à l'installation de nombreuses espèces bactériennes, archaea, virales, champignons et eucaryotes unicellulaires [1]. En effet, ces différents règnes du vivant, cohabitent et évoluent en équilibre composant ainsi ce que l'on appelle communément " le microbiote " [2]. Il est représenté par une communauté complexe qui persiste et se développe sur les toutes les surfaces de la cavité buccale généralement sous forme d'un biofilm multi-espèces ou dans la salive [3]. Ce biofilm lorsqu'il se développe sur les dents est désigné par le terme générique "plaque dentaire" [3]. Les propriétés uniques de la cavité buccale et les organes qui la composent, rendent la composition du microbiote oral caractéristique du site qu'il colonise et distinctes de celui des écosystèmes voisins, tel que la peau, les voix respiratoires et digestives [4]. Ces propriétés jouent un rôle majeur dans la détermination du futur microbiote et de sa composition [4]. En effet, plusieurs paramètres rentrent en jeu pour déterminer quelles espèces peuvent coloniser, se développer et s'installer pour devenir une composante majeure ou mineure du microbiote, y compris la qualité et quantité de la salive, le biotype et phénotype du parodonte, la profondeur des poches parodontales autour des dents et la santé du tissu dentaire [5].

Le microbiote buccodentaire entretient une relation symbiotique avec son hôte [6]. Les microorganismes résidents de la cavité buccale participent à équilibrer la flore pathogène, régulent les réponses indésirables et potentiellement pro-inflammatoires et favorisent la santé buccodentaire par la voie salivaire des nitrates, nitrites et de l'oxyde nitrique [7]. En effet, selon Rogers *et al.*, 1990, le métabolisme de l'azote joue un rôle protecteur vis-à-vis du développement du processus carieux [8,9]. Le microbiote avec la capacité de métaboliser les composés azotés, assure une bonne protection contre la carie car les produits finaux peuvent

augmenter le pH de la plaque et ainsi exercer un pouvoir tampon sur l'environnement et par conséquent procure une protection contre les attaques acides [9].

En revanche, cette relation symbiotique demeure fragile et susceptible de changer en fonction de nombreux facteurs comme le tabagisme, et la consommation d'alcool ainsi que des facteurs sociaux, économiques, alimentaires, sédentaires, autonomie et éducation [5,8]. En effet, ces facteurs influencent directement non seulement la composition du microbiote, mais aussi son comportement et son évolution d'un microbiote commensal vers la pathogénicité [10].

Depuis l'apparition de la notion de maladie infectieuse, l'Homme a toujours tenté de classer les microorganismes en tant que pathogènes ou commensaux faisant partie intégrantes de la flore locale et résidante, évoluant en parfaite symbiose avec son Hôte [11]. On sait maintenant que certaines espèces ou même communautés bactériennes peuvent avoir des propriétés qui échappent à nos systèmes de classification [12]. La distinction entre bactéries pathogènes et commensales devient de plus en plus une tâche complexe et ambiguë, notamment lorsque les microorganismes rencontrés habituellement dans un état de bonne santé se retrouvent impliqués dans des interactions complexes entre l'hôte et le microbe conduisant à un état dit de maladie ou infection [12]. L'un des meilleurs exemples de ce paradoxe est celui des maladies infectieuses buccodentaires incluant la maladie carieuse et les maladies parodontales [12].

## **2. Background**

### **2.1. La carie dentaire, une maladie infectieuse non-transmissible et microbiote-dépendante**

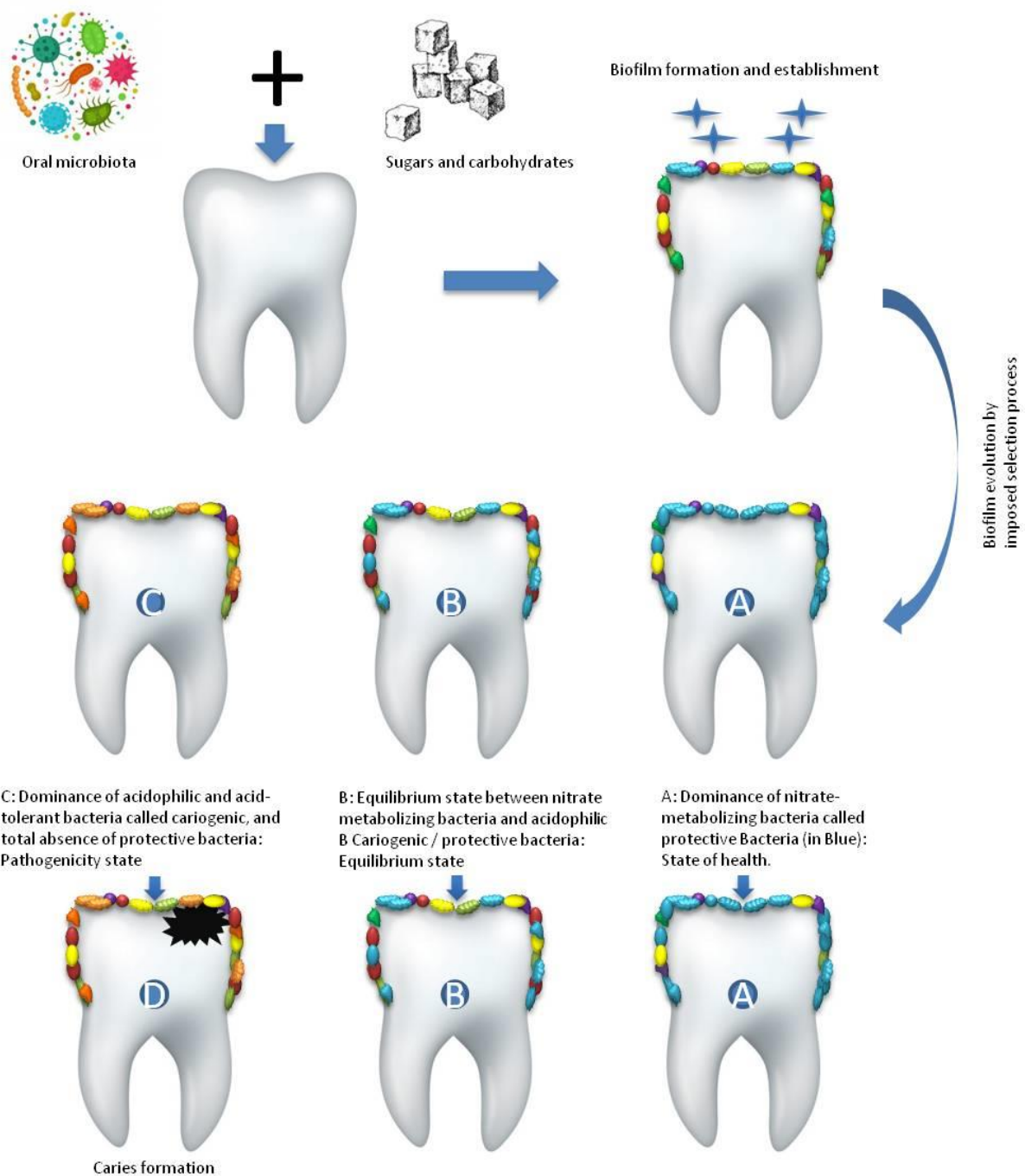
Pendant de nombreuses années, la carie dentaire était considérée comme une maladie infectieuse transmissible dont *Streptococcus mutans* est le principal agent pathogène causal [13]. La transmission de ces agents pathogènes était directement attribuée à la maman pendant la période d'allaitement [14,15]. En effet, ces bactéries étaient des agents infectieux que les nourrissons acquéraient de leur mère après l'éruption des dents temporaires [16]. Par conséquent, les stratégies cliniques visant à prévenir la transmission de ces organismes, consistaient en l'utilisation d'agents antibactériens pour éliminer ou prévenir la contamination [17]. Cependant, le paradigme selon lequel " un agent pathogène provoque une maladie " y compris pour la maladie carieuse a été abandonné et un nouveau concept holistique qui met en évidence l'implication d'une communauté microbienne comme entité de pathogénicité a vu le jour [18].

Des études menées sur des personnes de différents âges et ayant des régimes alimentaires différents dans le monde entier ont démontré des différences substantielles dans la composition du microbiote dans les biofilms recouvrant les lésions carieuses, avec un enrichissement d'espèces présentant un phénotype de production d'acide et de tolérance à l'acide [19,20]. Ainsi, le développement d'une lésion carieuse est associé à une modification de l'équilibre du microbiote dentaire résident imposé par la modification d'un facteur environnemental, conduisant à la domination de composants microbiotiques normalement minoritaires [19]. La consommation excessive et fréquente de sucre est le principal moteur de ce changement dysbiotique à ce jour mis en cause [20]. En effet, les acides organiques résultants du métabolisme des sucres pourraient produire un microenvironnement à pH acide dans le biofilm, provoquant ainsi une déminéralisation de l'émail et imposer un stress au

microbiote, entraînant le transfert de la communauté microbienne en équilibre vers un état cariogène imposé, principalement composée de bactéries productrices d'acides et de bactéries acidophiles [8,21]. Par conséquent, le microenvironnement à pH plus faible causé par le métabolisme des glucides joue un rôle important dans l'apparition et le développement des caries [8].

Selon Pang *et al* 2021, le processus carieux commence par la création d'un microenvironnement à pH faible causé par l'accumulation des acides organiques sécrétés par la microflore constituant le biofilm [8]. Ce microenvironnement va provoquer une déminéralisation de l'émail et imposer un stress au biofilm entraînant son passage vers un état dit cariogène. A ce stade un processus de sélection est établi par des paramètres physicochimiques y compris le pH acide pour qu'il ne puisse persister que les microorganismes acidotolérants ou acidophyles qui vont jouer un rôle important voir principal dans la continuité du processus de formation de la carie [8,21].

La carie dentaire peut donc être définie comme une maladie infectieuse non-transmissible d'étiologie bactérienne résultant d'un processus de sélection imposé par un microbiote acidophile et/ ou acidotolérant de la plaque dentaire en l'absence de bactéries qui métabolisent le nitrate, conduisant à la déminéralisation de l'émail (**Figure 1**).



**Figure 1:** Processus de sélection imposé par le microbiote acidophile et/ou acidotolérant de la plaque dentaire conduisant à l'état de pathogénicité cariogène. Blue: bactérie métabolisant le nitrate ; Autres couleurs: bactérie acidophile / acidotolérantes. (A) Etat de santé dentaire. (B) Etat d'équilibre (qui correspond aussi à une bonne santé). (C) Etat de pathogénicité (cariogène).

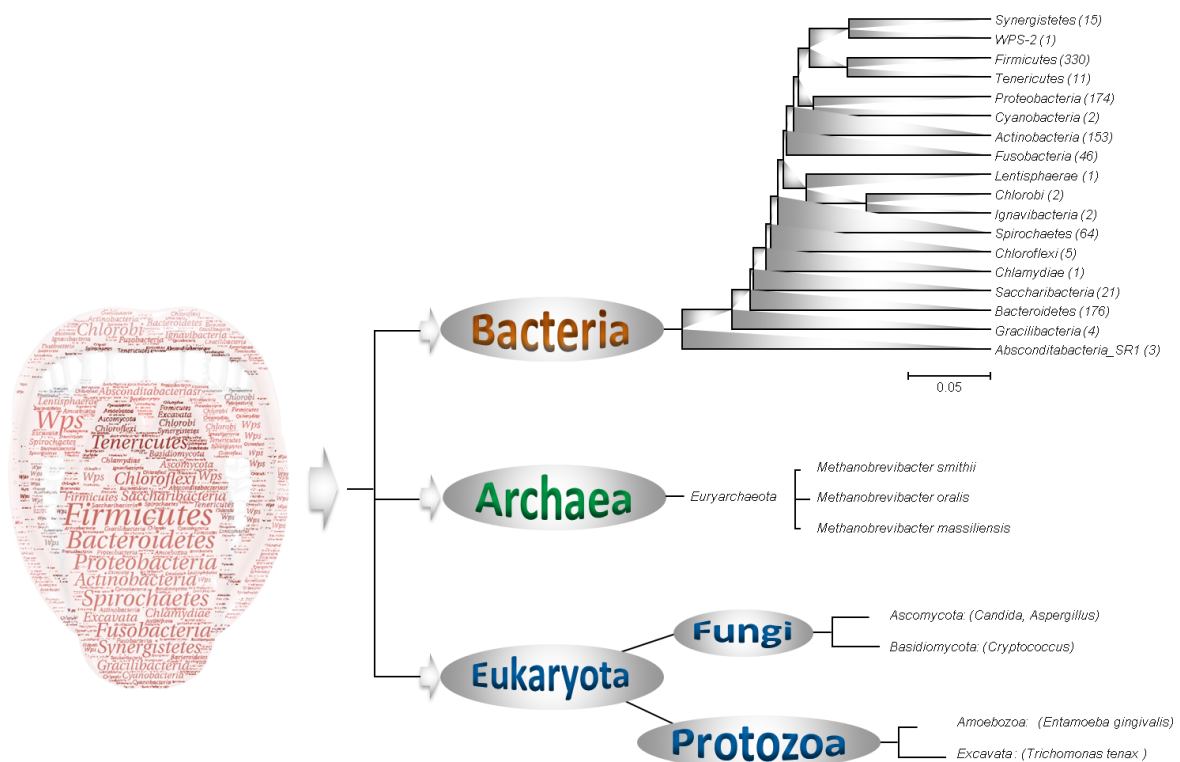
## 2.2. Les maladies parodontales, vers un processus polymicrobien

La parodontite a fait l'objet d'un historique de propositions d'étiologies microbiennes impliquant dans un premier temps une plaque dentaire non spécifique [8]. Ensuite et au fil des années, un nouveau concept impliquant des microorganismes spécifiques et variés a pu voir le jour [22,23]. La majeure partie des connaissances actuelles ainsi que notre appréciation de l'étiologie microbienne de la maladie parodontale découle d'études basées sur la culture microbienne et la caractérisation phénotypique conduites à la fin des années soixante-dix et au début des années quatre-vingt [24–26]. Ces études ont révélé des modifications significatives de la composition du microbiote entre l'état de maladie par rapport à l'état dit de santé.

Des travaux plus récents, ont permis de mettre en évidence une spécificité bactérienne dans l'étiologie de la maladie parodontale. Les auteurs ont constaté que le microbiote associé à la maladie contenait de nouvelles espèces pathogènes qui étaient soit absentes, soit à charge très faible dans un environnement sain [23,27]. En effet, Socransky *et al* 1998, ont caractérisé les communautés microbiennes parodontales sur la base d'un système de code couleur associant directement ces communautés et la gravité de la maladie [23]. Le complexe rouge était considéré comme le plus dangereux de ces groupes. Ce groupe est constitué de trois espèces comprenant *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia*, dont la détection était fortement associée les unes aux autres mais aussi aux sites malades [23].

Cependant, les approches moléculaires de détection des microbes se sont de plus en plus répandues et les études utilisant une méthodologie indépendante de la culture pour l'analyse du microbiote parodontal se sont multipliées [28]. Ainsi, de nouveaux concepts ont émergé, montrant que des organismes du complexe rouge tels que *P. gingivalis* peuvent être trouvés en l'absence de maladie, et avec des charges importantes. Cela constitue un argument contre le fait que ces microorganismes soient des pathogènes exogènes classiques et exclusifs [29,30].

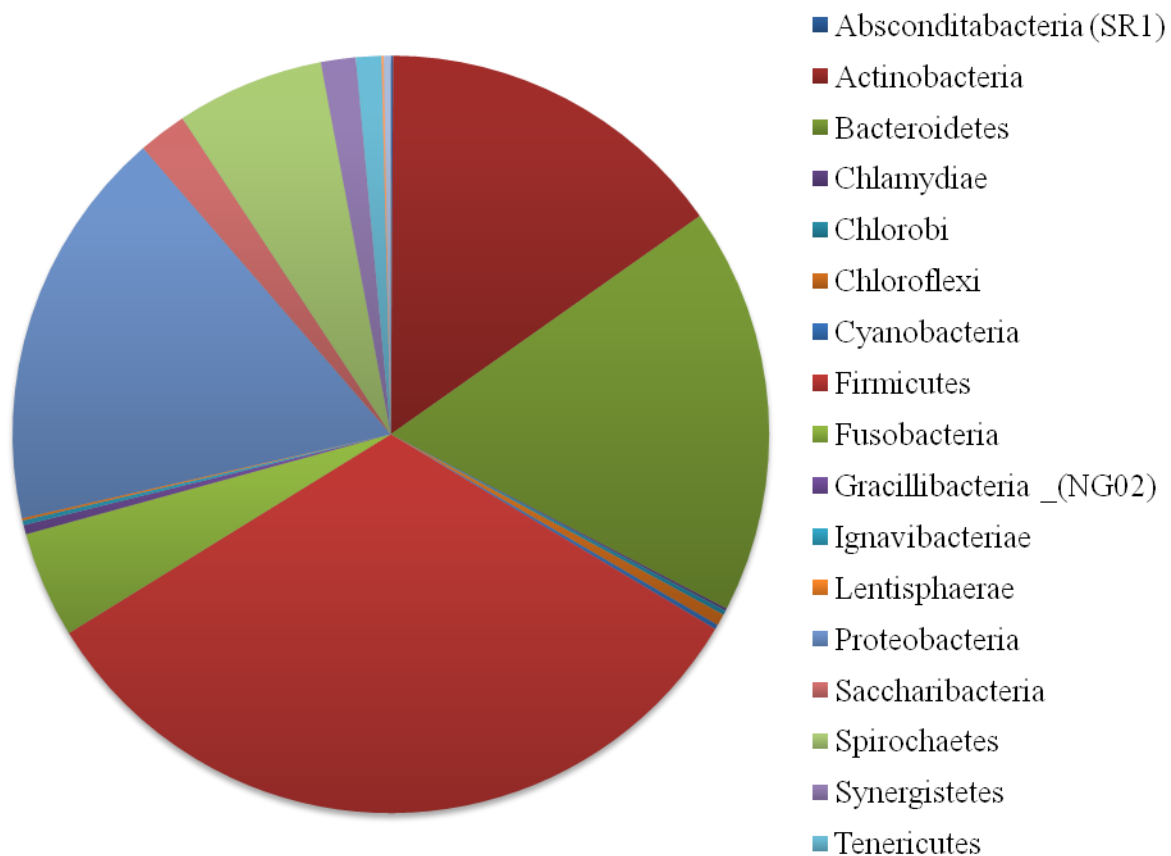
De plus, le microbiote parodontal est plus hétérogène et diversifié qu'on ne le pensait auparavant. La cavité orale humaine renferme le deuxième microbiote le plus important en terme de diversité après celui des intestins [31]. Sa structure ouverte crée un environnement particulier propice au développement d'une flore mixte, aérobie, microaérophile et anaérobie [31]. Le microbiote oral est composé de plus de 700 espèces différentes [32,33]. D'après les données de la Humen Oral Microbiome Database [https://www.homd.org/taxa/tax\\_hierarchy](https://www.homd.org/taxa/tax_hierarchy) sa composition s'élève à environ 774 espèces bactériennes et archaea (**Figure 2**).



**Figure 2:** Distribution phylogénétique des 19 phyla isolés ou détectés au moins une fois dans la cavité buccale humaine. Le nombre d'espèces par phylum est présenté entre parenthèses dans l'arbre phylogénétique. L'illustration à gauche de la figure a été réalisée à l'aide de l'outil en ligne wordle ([www.wordle.net](http://www.wordle.net)), la taille du nom de chaque phylum est proportionnelle au nombre d'espèces qu'il contient. L'arbre phylogénétique a été créé à l'aide du logiciel MEGA. La figure 2 a été construite d'après les données recueillies dans la base de données du microbiome oral humain ; <https://www.homd.org/>.



Actuellement, les estimations basées sur la métagénomique suggèrent un nombre globale tournant autour de 1200 espèces regroupées dans 19 phyla différents: Absconditabacteria (SR1), Actinobacteria, Bacteroidetes, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gracilibacteria \_(NG02), Ignavibacteriae, Lentisphaerae, Proteobacteria, Saccharibacteria, Spirochaetes, Synergistetes, Tenericutes WPS-2, Euryarchaeota, ([https://www.homd.org/taxa/tax\\_hierarchy](https://www.homd.org/taxa/tax_hierarchy)) [22]. La majorité des espèces cultivées sont essentiellement regroupées dans les phyla : Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes (**Figure 3**) [22].



**Figure 3:** Distribution quantitative de la diversité bactérienne cultivée et non cultivée composant le microbiote oral, représentée par le nombre d'espèces composant chaque phylum. Chaque phylum est représenté par une couleur différente comme indiqué à droite. (La figure 2 a été construite selon les données recueillies à partir la Human Oral Microbiome Database ; <https://www.homd.org/>).

Parmi les espèces qui composent cette grande diversité, environ 200 peuvent être rencontrées chez un même individu et 50 sont trouvées dans un même site [34]. Ces données vont à l'encontre de la notion des trois organismes qui composent le complexe rouge de Socransky défini en 1998 sur la base de techniques de culture basique et qui ne permettaient pas la culture de tous les microorganismes qui composaient cet écosystème [23,35]. En plus, la plupart des organismes nouvellement détectés par culture ou par biologie moléculaire, présente une haute valeur prédictive et une bonne corrélation avec la maladie parodontale, voire meilleure que celle du complexe du code couleur classique [22].

De plus, contrairement au " paradigme " selon lequel il existe une dominance des bactéries gram-négatives dans les sites atteints de parodontite, la détection des espèces anaérobies gram-positives est en augmentation significative dans ces mêmes sites avec des profondeurs de poches importantes par rapport aux sites sains, et avec des charges de détection plus importantes que celles des espèces gram-négatives dans certaines études [22,36].

Bien que des investigations supplémentaires soient nécessaires pour démontrer l'implication de ces microorganismes nouvellement détectés dans les maladies parodontales, il est actuellement évident que la notion de complexe rouge comprenant trois espèces comme représentant principal de l'étiologie de la maladie parodontale doit être actualisée [29]. En effet, de nouvelles techniques de détections basées sur le séquençage à haut débit de l'ADN d'une niche écologique complète (NGS) combinées à des techniques de détection basées sur la culture bactérienne comme le concept culturomics appliqué essentiellement au microbiote intestinal représentent une perspective d'avenir pour étudier le microbiote buccodentaire, afin d'élargir les connaissances concernant sa composition et étudier son rôle potentiel dans la santé et la maladie buccodentaire [8,35,37].



## **Culturing the Human oral microbiota, updating methodologies and cultivation techniques**

Saber Khelaifia<sup>1,2\*</sup>, Virginie Pilliol<sup>1,2</sup>, Souad Belkacemi<sup>1</sup>, Herve Tassery<sup>2</sup>, Elodie Terror<sup>1,2</sup>, Gerard Aboudharam<sup>1,2\*</sup>

<sup>1</sup>Aix-Marseille Univ, IRD, MEPHI, AP-HM, Institut Hospitalo-Universitaire Méditerranée-Infection, 19-21 Boulevard Jean Moulin, 13385, Marseille cedex 05, France

<sup>2</sup> Ecole de médecine dentaire, 27 Boulevard Jean Moulin, 13385, Marseille cedex 05, France

\*Corresponding author:

Saber KHELAIFIA : [khelaifia\\_saber@yahoo.fr](mailto:khelaifia_saber@yahoo.fr)

IHU Méditerranée Infection 19-21 boulevard Jean Moulin 13385 Marseille cedex 5 France

Tel: +33 (0)4 13 73 24 01 Fax: +33 (0)4 13 73 24 02

Gerard Aboudharam : [gerard.aboudharam@univ-amu.fr](mailto:gerard.aboudharam@univ-amu.fr)

IHU Méditerranée Infection 19-21 boulevard Jean Moulin 13385 Marseille cedex 5 France

Tel: +33 (0)4 13 73 24 01 Fax: +33 (0)4 13 73 24 02

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# Culturing the human oral microbiota, updating methodologies and cultivation techniques

Saber Khelaifia<sup>1,2\*</sup>, Virginie Pilliol<sup>1,2</sup>, Souad Belkacemi<sup>1</sup>, Herve Tassery<sup>2</sup>, Elodie Terror<sup>1,2</sup>, Gerard Aboudharam<sup>1,2\*</sup>

<sup>1</sup> Aix-Marseille Univ, IRD, MEPHI, AP-HM, Institut Hospitalo-Universitaire Méditerranée-Infection, 19-21 Boulevard Jean Moulin, 13385, Marseille cedex 05, France.

<sup>2</sup> Ecole de médecine dentaire, 27 Boulevard Jean Moulin, 13385, Marseille cedex 05, France.

\* Correspondence:

Saber Khelaifia : [khelaifia\\_saber@yahoo.fr](mailto:khelaifia_saber@yahoo.fr). IHU Méditerranée Infection 19-21 boulevard Jean Moulin 13385 Marseille cedex 5 France Tel: +33 (0)4 13 73 24 01 Fax: +33 (0)4 13 73 24 02.

Gerard Aboudharam : [gerard.aboudharam@univ-amu.fr](mailto:gerard.aboudharam@univ-amu.fr). IHU Méditerranée Infection 19-21 boulevard Jean Moulin 13385 Marseille cedex 5 France Tel: +33 (0)4 13 73 24 01 Fax: +33 (0)4 13 73 24 02.

## Abstract:

Recent years have been marked by a paradigm shift in the study of the human microbiota, with a re-emergence of culture-dependent approaches. Numerous studies have been devoted to the human microbiota, while studies on the oral microbiota still remain limited. Indeed, various techniques described in the literature may enable an exhaustive study of the microbial composition of a complex ecosystem.

In this article, we report different methodologies and culture media described in the literature that can be applied to study the oral microbiota by culture. We discuss the use of culture media to culture fastidious bacteria, methods which opened the door to the use of culture as a diagnostic tool in hospital laboratories. We also report on specific methodologies for targeted culture and specific culture techniques and selection methodologies for cultivating members of the three kingdoms of life commonly found in the human oral cavity, namely, eukaryota, bacteria and archaea.

This bibliographic review aims to bring together the various techniques described in the literature, enabling a comprehensive study of the oral microbiota in order to demonstrate its involvement in oral health and diseases.

**Keywords:** culture, human oral microbiota, bacteria, archaea, fungi, culturomics.

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## 1. Introduction

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



biotype and phenotype of the periodontium, the depth of the periodontal pockets around the teeth and the quality of the dental tissue [5].

The oral microbiota maintains a symbiotic relationship with its host [6]. The resident microorganisms of the oral cavity help fight pathogens, down-regulate undesirable and potentially pro-inflammatory responses, and promote oral health [7,8]. However, this symbiotic relationship remains fragile and susceptible to change depending on social, economic, dietary and nutritional factors, as well as sedentary lifestyle, autonomy, hygiene compliance, smoking, and alcohol consumption [5,9]. These factors directly influence the composition of the microbiota and also its behaviour and its evolution from a commensal microbiota to pathogenicity [10]. Since the notion of infectious disease first appeared, humans have tried to classify microorganisms as pathogens or commensals as part of the local and resident flora, evolving in perfect symbiosis with their host [11]. It is now known that some bacterial species or even communities may have properties that escape our classification systems [12]. The distinction between pathogenic and commensal bacteria is becoming an increasingly complex and ambiguous task, especially when the microorganisms usually found in healthy states are involved in complex interactions with their host leading to a state of disease or infection [12]. Recent years have been marked by a paradigm shift in the study of the human microbiota, with a re-emergence of culture-dependent approaches [13–18]. Recent studies have been mainly devoted to cultivating the gut microbiota and studies on the oral microbiota remain very limited. The oral microbiome is highly diverse. Approximately 774 indigenous species are present in the oral cavity including 54% cultivated and named species, 14% cultivated but not yet named species, and 32% remaining uncultivated phylotypes [6] (the Human Oral Microbiome Database, <https://www.homd.org/>). Recently, molecular based studies have demonstrated that the oral microbiota is more diverse and complex and plays a vital role in the pathogenesis and development of many oral and systemic diseases [19,20].

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

## 2. Current strategies to study the human oral microbiota

### 2.1. Next-generation sequencing and metagenomics

Next-generation sequencing (NGS) is a new molecular approach applied to study the microbial diversity of diverse ecological niches [21,22]. NGS has enabled a more in-depth and non-specific exploration of the human microbiota by generating a huge amount of data leading to better understanding its composition, mainly through amplicon sequencing and whole-genome metagenomics [23,24]. NGS has enabled a rapid and comprehensive analysis of microbial diversity in various ecological niches, which has propelled it to the forefront to gradually replace tedious and time-consuming standard culture techniques [21,25].

However, the limitations of NGS were quickly identified. Indeed, the absence of a standardised protocol and the variability of the used DNA extraction techniques, as well as the variability of the primers used for amplification, create biases making the results dependent on the used technique [22]. In addition, the depth bias associated with all molecular methods, due to limited sensitivity, results in the neglect of minority species [26]. One of the major shortcomings of molecular techniques remains the lack of information related to the viability, adaptation, and availability of strains for further *in vivo* studies [15,18].

The limitations and lack of information generated by molecular techniques encourage us to return to the sources of the fundamental research in microbiology, namely microbial culture [15]. Indeed, microbial culture represents the basic component of microbiology, and has long been side-lined at the expense of molecular



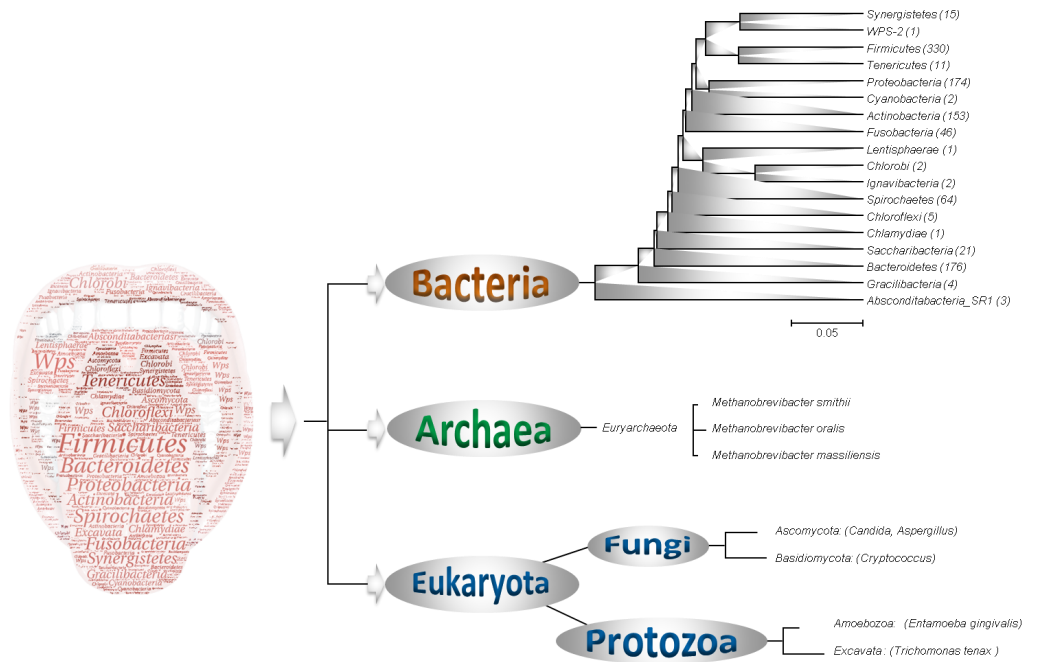
methods [15,21]. Much recent work underlines the need to combine molecular techniques such as NGS with recent and innovative approaches such as culturomics to overcome the lack of these techniques and to have a global and exhaustive view of the composition of the microbiota of a given ecological niche [18].

## 2.2. *Culturomics: a new concept applicable to oral microflora*

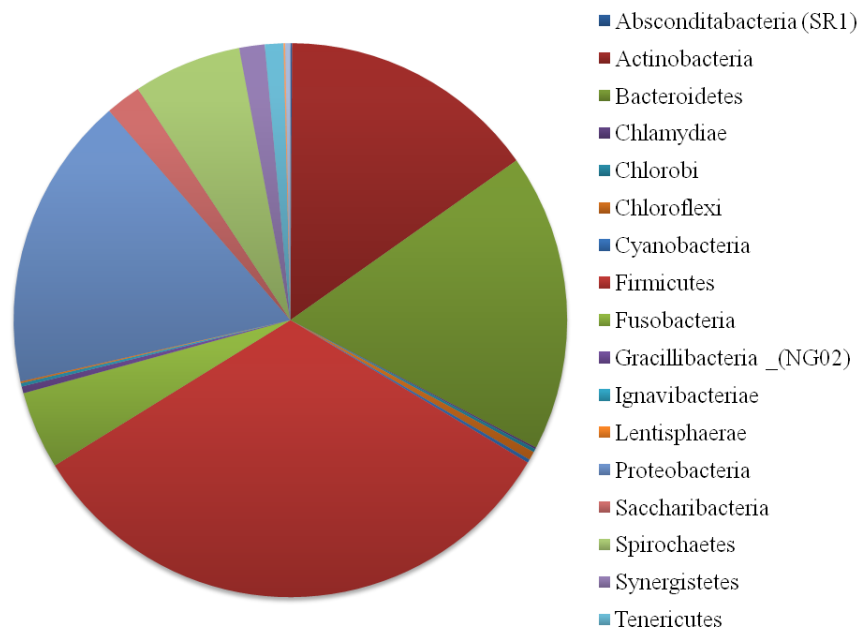
Culturomics is a high-throughput culture-based concept created in 2012 with the goal of isolating all species that make up the human digestive microbiota [27]. Since then, many studies have combined high-throughput culture and metagenomics with the goal of reducing the dark matter of the microbiota, consisting of unassigned and unmatched sequences, through the isolation and sequencing of previously uncultured species [17].

Culturomics has proven itself over the last ten years, and its application has been made possible by rapid colony identification using Matrix Assisted Laser Desorption Ionization/Time Of Flight Mass Spectrometry (MALDI-TOF MS) [27]. Culturomics has demonstrated its effectiveness and contribution to the exploration of the gut microbiota over the last decade by significantly expanding the field of knowledge regarding the repertoire of the human microbiota by isolating new or previously uncultured species [15–18,27]. Indeed, to date, more than 2,770 different species, including 800 new species, have been isolated by culturomics from human specimens from different sites including: human gut, skin, urinary tract, vaginal and respiratory microbiota [28].

Various studies throughout the world have shown the interest of diversifying culture media and conditions to broaden the scope of culture, in addition to the use of specific media and targeted approaches (**Table 1**). Indeed, the use of selective and non-selective media has allowed a continuous progression of knowledge concerning the diversity of the human microbiota [29]. The oral microbiota is undoubtedly very diverse, as shown by the Human Oral Microbiome Data Base (<https://www.homd.org/>), which currently contains more than 774 different species (<https://www.homd.org/>) (**Figure 1**) [30,31]. Currently, metagenomics-based estimates suggest a global number of around 1,200 species grouped into 19 different phyla, namely *Absconditabacteria* (SR1), *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Gracilibacteria* (NG02), *Ignavibacteriae*, *Lentisphaerae*, *Proteobacteria*, *Saccharibacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes* WPS-2, and *Euryarchaeota* ([https://www.homd.org/taxa/tax\\_hierarchy](https://www.homd.org/taxa/tax_hierarchy)) [19]. The majority of cultured species are grouped in only six phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, and *Spirochaetes* (**Figure 2**) [19].



**Figure 1:** Phylogenetic diversity of the 19 phyla isolated or detected at least once in the human oral cavity. The number of species per phylum is presented between parents in the phylogenetic tree. The illustration on the left of the figure was made using the online tool wordle ([www.wordle.net](http://www.wordle.net)); the size of the name of each phylum is proportional to the number of species it contains. The phylogenetic tree was created using MEGA software. Figure 2 was built according to data collected from the Human Oral Microbiome Database; <https://www.homd.org/>.



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





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

### 2.3. Rapid culture of anaerobic bacteria using YCFA medium

YCFA (Yeast extract-Casein hydrolysate Fatty Acids) culture medium is a rich medium widely used in the literature to culture strict anaerobes [32,33]. It is composed of growth factors, antioxidants, volatile fatty acids and vitamins essential for their growth ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium1611.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1611.pdf)).

One very interesting approach to cultivate anaerobic bacteria in record time was proposed by Naud *et al.*, in 2020, using YCFA medium as well as the medium of blood culture bottles (BioMérieux, Marcy l'Etoile, France) supplemented with rumen [33]. The culture investigations, which were performed on only two stool samples, resulted in the isolation of 121 different bacterial species in only three weeks, of which 104 species were cultured within 24 hours of incubation (Table 1). This is an unprecedented technical achievement making it possible to use bacterial culture as a rapid and accurate diagnostic tool [33].

According to another study conducted by Browne *et al.*, the YCFA culture medium allowed the cultivation of 137 species, 68 of which were completely new species, and 63 of which belonged to the phylum *Firmicutes*, four to *Bacteroidetes* and one to *Actinobacteria* [32]. This medium also gave exceptional results during a study by Forster *et al.*, in 2019 on the diversity of the human digestive macrobiota by succeeding in cultivating 273 different bacterial species, of which 105 were new bacterial species distributed over three phyla; 91 species belonged to the phylum *Firmicutes*, 13 to the phylum *Bacteroidetes*, and one to the phylum *Proteobacteria* [34].

The oral microbiota consists of a large majority of anaerobic microorganisms [35,36]. YCFA medium is suitable for the culture of these oxygen-intolerant microorganisms and the studies described above demonstrate the high yield of culture using this medium to cultivate anaerobes [32,33]. Moreover, this medium is able to cultivate the most represented phyla in the human oral cavity, namely *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* [32,33]. The YCFA medium may constitute a good culture base for future investigations to explore the diversity of the oral microbiota. So far, and according to the sources we have consulted, no study exploiting the potential of the YCFA medium to study the diversity of the oral microbiota has been conducted.

### 2.4. Specific conditions and media for a targeted culture

#### 2.4.1. Spore-forming bacteria in the oral cavity

Recent studies have described the isolation and characterisation of spore-forming bacteria from the oral cavity in the context of pathogenicity [37] or as an integral part of the oral microbiota [38]. Despite these limited studies, the number of spore-forming bacteria colonising the oral cavity is limited to a few cultivated species, mainly from the genera *Bacillus* or *Peenibacillus* [38]. Numerous techniques have been optimised for the specific culture of spore-forming bacteria in various domains, including the intestinal microbiota (Table 1).

#### 2.4.2. Alcohol decontamination

In 2016, Browne *et al.*, described the use of improved pre-culture conditions that allowed for the targeted isolation of spore-forming bacteria [32]. This technique consisted of pre-incubating the sample in an equal volume of 70% alcohol for four



hours at room temperature under aerobic conditions (**Table 1**). A wash was then performed prior to plating on a rich culture medium under anaerobic conditions [32].

The alcohol selection or disinfection technique was then adapted and modified by the culturomics team in 2020 to specifically isolate spore-forming bacteria that could be used in bacteriotherapy trials for the treatment of *Clostridium difficile* infections from human stool samples [39]. A total of 254 bacterial species were identified, of which nine represented new species. 242 species isolated in this study have never been included in clinical trials and represent potentially interesting new candidates for bacterial therapy trials [39].

#### 2.4.3. Thermal shock

Thermal shock is a selective technique based on the use of high temperature for the isolation of spore-forming bacteria. One of the culture protocols validated by the culturomics approach is to expose the sample to a constant temperature of 80 °C in a dry bath for 20 minutes to select spore-forming or spore-producing bacteria. The exposed sample is then subcultured on Columbia agar plates in anaerobic conditions [15].

#### 2.4.4. The use of antioxidants to improve anaerobic conditions

Antioxidants have long been used to establish an anaerobic atmosphere or to contribute to its improvement [40,41]. Indeed, antioxidants such as sodium sulphide (Na<sub>2</sub>S) or amino acids such as cysteine are found in old culture media optimised for the culture of anaerobic bacteria but also for the culture of methanogenic archaea that sensitivity to oxygen is widely demonstrated [14,40,41]. Since then, many studies have focused on the beneficial effect of antioxidants for the culture of fastidious anaerobes. A more recent study conducted by La Scola *et al.*, in 2014, succeeded in cultivating six strictly anaerobic bacteria and seven aerobic bacteria aerobically on Schaedler agar culture medium supplemented with 0.1g glutathione and 0.5g ascorbic acid. Among the species cultivated during this study, the authors mention the *Fusobacterium necrophorum* and *Ruminococcus gravus* species, known for their extreme sensitivity to oxygen [42]. Their results suggest that using this unique medium it would be possible to cultivate anaerobic and aerobic bacteria aerobically in a standard incubator, enabling the renewal of the anaerobic culture [42].

In 2015, Dione *et al.*, were able to expand the spectrum of bacterial species grown with antioxidants by culturing aerobic, microaerophilic, and anaerobic bacteria on a single medium by a simple addition of antioxidant consisting of 0.1g glutathione + 1g uric acid + 1g ascorbic acid to Shedler agar medium [43]. This technical feat enabled the culture of 251 different bacterial species and showed that it was possible to perform antibiograms of anaerobic bacteria in aerobic conditions without affecting the sensitivity of the results [43]. This technique was then applied to methanogenic archaea culture to successfully isolate 13 strains of *Methanobrevibacter smithii* and nine strains of *Methanobrevibacter oralis* from 100 stools and 45 oral samples [13].

The work of Million *et al.*, in 2020 confirmed the beneficial effect of antioxidants on the culture of anaerobes [44]. In this case, antioxidants were able to decrease the oxidative stress experienced by anaerobic bacteria of the genus *Clostridium* and maintain an increased production of volatile fatty acids such as butyrate, isobutyrate and isovalerate when grown in the presence of three antioxidant molecules: glutathione, ascorbic acid and uric acid. Volatile fatty acids are known to contribute to the maintenance and active resilience of host-bacterial mutualism against mucosal oxygen and uncontrolled oxidative stress *in vivo* [44].

Recently, new molecules with antioxidant activities have been described in the literature, such as phenolics, flavonoids and carotenoids [45]. These molecules with antioxidant potential should be tested for their antioxidant effect in order to improve or even establish the anaerobic atmosphere necessary for the culture of anaerobic bacteria in the oral cavity.

#### 2.4.5. Bacterial co-culture



Co-culture has been widely used in recent years to make possible the culture of certain microorganisms remained uncultured so far. Co-culture is based on the capacity of some bacteria to produce or secrete active substances, cofactors or growth factors of interest to other species lacking them [13]. Numerous approaches have been used either to accelerate the bacterial growth, by the addition of missing unknown nutrients in the culture medium, or known nutrients not commercially available [13,46–48]. To illustrate the contribution of co-culture to the field of microbial culture, and in particular oral cavity microbes, we took the example of methanogenic archaea.

The culture of methanogenic archaea is fastidious and requires an external supply of H<sub>2</sub> and CO<sub>2</sub> required for their growth [14]. In 2016, Khelaifia *et al.*, succeeded in substituting the gaseous supply of H<sub>2</sub> and CO<sub>2</sub> by a co-culture with *Bacteroides thetaiotaomicron* used as the sole source of hydrogen and CO<sub>2</sub> in a dual chamber culture device. In this device, *B. thetaiotaomicron* were grown in the bottom chamber in Schaedler liquid medium supplemented by antioxidants producing enough hydrogen and CO<sub>2</sub> to allow growth of the methanogenic archaea *Mathanobrevibacter smithii* on solid medium and isolate viable colonies [13]. Previous studies have used cultured species to boost the growth of other fastidious bacteria using diffusion chambers or a double layer of agar separated by porous membranes [46,47]. Co-culture is therefore becoming a good alternative for laboratories lacking the technical means to culture fastidious bacteria and methanogenic archaea from all sources, whether biological or environmental [13].

#### 2.4.6. Selective media based on antimicrobial agents, antibiotics and phages

The ability of some microorganisms, including bacteria and archaea, to resist antimicrobial agents is one of the most important public health problems of recent years, but also poses an interesting possibility for microbiologists to decontaminate samples for the purpose of the specific isolation of these resistant microorganisms [49,50]. Methanogenic archaea are known to be resistant to the majority of antibiotics used in antimicrobial therapeutics and the treatment of infectious diseases except for metronidazole derivatives, chloramphenicol and fusidic acid [51,52]. Many studies have focused on this ability found in some microorganisms to facilitate their isolation from a complex environment. The culture medium optimised by Khelaifia *et al.*, in 2013, is now one of the most widely used media for the selective culture of methanogenic archaea [13] (**Table 1**). One of the peculiarities of this medium in addition to its complex nutrient composition (thus considered a rich medium), is the addition of a cocktail of antibiotics aimed to eliminating Gram-positive and Gram-negative bacteria, fungi and yeasts [13,51].

The disadvantage of the use of antibiotics is the obligatory selection of resistant strains [49,50]. Bacterial growth can also be inhibited by the use of phages, which reduces the potential development of resistance associated with the use of antibiotics [53]. Indeed, bacteriophages are viruses that specifically infect certain bacteria. In the case of lytic phages, host bacteria are destroyed by their phage through a process of cell lysis [15]. This specific lysis capacity can therefore be used to specifically eliminate majority bacteria in a polymicrobial culture of any origin, including the human oral cavity, in order to cultivate minority and slow growing bacteria.

#### 2.4.7. The genomic-reverse culture technique.

Reverse genomics is one of the most promising new perspectives in the cultivation of uncultured microorganisms in all areas of life [54]. This technique exploits the ability of antibodies to bind specifically to a given organism, to isolate it by culture, and then sequence its genome from a simple culture or complex ecosystem [54]. Using this method, Cross *et al.*, successfully isolated and cultured three species of *Saccharibacteria*, including the TM7 strain known to be an integral part of the human oral microbiota [54]. They also isolated and cultured species from the human oral SR1 bacteria group, which are members of a previously uncultured bacterial lineage [54].



A more recent study has also demonstrated the potential of reverse genomics by identifying a common system capable of culturing all members of the *Saccharibacteria* group from any human sample [55]. This method is also applicable to any other sample or specimen of diverse origin [55]. In this study, the authors describe their success in obtaining *in-silico* specific epitopes for *Saccharibacteria* spp., distributed in four different transmembrane proteins. Subsequently, they suggest the use of all these epitopes to synthesise antibodies targeting the *Saccharibacteria* species in order to cultivate them. According to the authors, this strategy can also be applied to archaea, or to other phyla/taxa, such as the *Parcubacteria* phylum and the DPANN group (*Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota*, and *Nanohaloarchaeota*) [55]. Thus, the use of this methodology can help cultivate new microbes and enrich our knowledge of the composition of the human microbiota in general and oral microbiota in particular. Most microorganisms in the human oral cavity from all taxonomic levels remain uncultured to date [56]. Reverse genomics can be a significant asset for the cultivation of uncultured microorganisms and can be applied to all species from all known ecosystems [54]. These species, once isolated and cultured, can be characterised and studied to better understand the role they play in the oral microbiota but also their actual involvement in oral disease and health [57].

### 2.5. *Treponemes* of the oral cavity

*Treponemes* are spiral-shaped motile prokaryotes belonging to the genus *Treponema* and the order *Spirochaetales*, in the family *Spirochaetaceae* [58,59]. These microorganisms colonise the human intestinal [60], oral [61] and genital [62] microbiota, as well as those of animals. Most of these microorganisms are part of the commensal flora, but some species are opportunistic pathogens, such as *Treponema pallidum*, the causative agent of syphilis in humans [63]. Other species have been directly associated with a high prevalence in patients with periodontitis and gingivitis, such as *Treponema denticola*. Indeed, numerous studies have revealed the direct involvement of certain *Treponema* species in the severity and extent of periodontal lesions [64,65]. *T. denticola* is the most widely studied of these, and its virulence factors have been identified. This bacterium is a member of the Sokransky red complex [66] and has the ability to bind to fibroblasts and epithelial cells via dentilisin protease and thus becomes cytotoxic and causes cell death [67].

*Treponemes* are a typical example of fastidious microorganisms with complex nutritional requirements. They cannot grow on minimal media, and their culture requires special and complex nutrients. Most *Treponema* species grow at temperatures ranging from 37 °C to 40 °C, anaerobically and/or micro-aerobically [68]. Several culture media have been reported in the literature, specifically designed to support the growth of these bacteria, and several modifications have been made to one or more of these media, including the addition of vitamins, carbohydrates, volatile fatty acids and serum of animal or human origin (**Table 1**) [68].

The genus *Treponema* is the predominant genus of *Spirochaetaceae* colonising the oral cavity. Although these organisms are part of the normal flora, they have, to date, been neglected by microbiologists and the number of cultured species is so far limited to only ten species [68]. However, studies based on molecular methods by direct amplification of 16S DNA from oral cavity samples have revealed the existence of more than 80 different phylotypes belonging to the genus *Treponema* that remain uncultured so far [69]. Hence, there is a pressing need to develop new identification and culture methods specifically targeting *Treponema* from clinical samples, to allow the extension of the *Treponema* repertoire associated with human microbiota and, more particularly, the oral microbiota. This can be achieved by isolating new species to phenotypically characterise them and sequence their genomes in order to study their roles in human oral health and pathology to determine their role within this complex microbiota.



### 2.6. Archaea of the oral cavity

Archaea are unicellular prokaryotes forming a separate domain of life from bacteria, eukaryotes and giant viruses [70]. They were first isolated from extreme environments, such as hot springs [71] and salty lakes, but it is now known that they are ubiquitous and can be found in all known ecosystems, including living organisms [72,73]. In humans, methanogenic and some halophilic archaea species are principally found [74,75]. Archaea have colonised all human mucosa, particularly intestinal [76] and oral mucosa [77], and have been associated with dysbiosis [78] as well as abscessed pathologies [79].

Culturing methanogenic archaea is a long and fastidious process, due to their oxygen intolerance and specific nutritional needs [14]. Most methanogenic archaea require an external source of hydrogen and carbon dioxide for their growth [14]. In 1969, Hungate pioneered methanogenic archaea culture. He first did so in a strict anaerobic liquid medium, and then in a solid medium in Roll tubes, which allowed a control of the culture atmosphere in general as well as the addition of hydrogen and carbon dioxide at various concentrations in particular [41]. Since then, different media appropriate to the requirements of each known species have been optimised (**Table 1**) [14]. Recently, the SAB<sup>®</sup> medium has been developed and optimised in order to be versatile and enable the culture and isolation of most methanogenic archaea species [14]. The addition of antioxidants to agar SAB<sup>®</sup> medium also enabled the aerobic culture of methanogenic archaea, by placing the agar plates in the upper part of a double chamber, with a lower part containing liquid culture medium inoculated with *B. thetaiotaomicron* as the sole continuous source of hydrogen and carbon dioxide [13]. Subsequently, Guindo *et al.*, improved the double chamber culture technique by proposing a new concept, based on the production of hydrogen through a chemical reaction between acetic acid and iron filings [80].

In the oral cavity, methanogenic archaea are part of the commensal microbiota, especially in individuals who smoke [81]. They are also associated with periodontal pathologies and especially with periodontitis [82] and peri-implantitis [83]. However, they have also been detected in inflammatory and infected dental pulp [84]. *M. oralis*, *M. smithii* and *Methanobrevibacter massiliense* are the sole methanogenic archaea cultured from oral cavity specimens, mainly saliva and dental plaque (**Figure 1**) [82]. Furthermore, the discovery of *Nanopusillus massiliensis* in dental plaque samples and its co-culture with *M. oralis* revealed the *Nanoarchaeota* phylum in the human oral cavity [85]. Finally, other archaea species have only been detected by molecular tools, including *Methanobacterium congolense*, *Methanocellus bourgensis*, *Candidatus Nitrosphaera evergladensis*, *Methanosarcinia mazei* [77,86] as well as non-methanogenic species belonging to the phylum Thermoplasmata [87,88].

Despite recent investigations regarding the culture of archaea, as well as technological advances and new culture methods, there remains a significant discord between the diversity of archaea in the gut microbiota and that of the oral cavity. To date, only three archaea species have been isolated from the oral cavity compared to seven from the digestive tract. This difference may be explained by the interest accorded to the gut microbiota recent years, particularly in the context of the Human Microbiome Project, which has focused on characterisation of the human gut microbiota [89]. In contrast, the oral microbiota remains neglected by microbiologists, and a lack of investigation and research work is notable, especially in the field of the culture of methanogenic archaea [72,77]. Additional efforts are needed to extend the repertoire of these microorganisms by first using molecular detection and diagnostic tools and then culture-based methods available in the literature to isolate them in order to better understand their metabolism and their involvement in human health and pathologies.



### 2.7. Fungi and yeasts of the oral cavity through the example of *Candida*

Fungi are a minor component of the oral microbiota. The diversity of this component is mainly represented by three genera, namely *Candida*, *Aspergillus*, and *Cryptococcus* (Figure 1) [90]. These commensal microorganisms are regularly present in low concentrations without causing infection, but they can become opportunistic pathogens when their host is immunodeficient [91]. The majority of oral fungal infections are forms of candidiasis [92]. These infections may be superficial and affect the mucosa, or they may penetrate the epithelium and be disseminated through the haematogenous pathway with serious consequences. Mucosal infections are seen in neonates and the elderly, two groups with suboptimal immune function [90]. They also affect people with suppressed immune systems [92].

Since the discovery of the first microbes [93], many techniques have been developed to study and cultivate fungi and yeasts from the oral cavity. Indeed, multiple references are proposed by manufacturers to enable an easy and selective culture of these microorganisms, including Sabouraud dextrose agar, malt agar, potato dextrose agar, CZAPEK, Colombia agar, Dixon agar, modified Leeming Notman agar, YPD medium and glycine-vancomycin-polymyxin B agar [29].

Recently, a new culture medium called FastFung® was developed by the Culturomics, Fungi and Yeasts Team at the IHU Méditerranée Infection, Marseille, with the objective of cultivating fastidious fungi [94]. The FastFung® medium was compared to the standard Sabouraud agar medium for the culture of 98 fungal and 20 bacterial strains. The positive culture rate of fungal strains was 100% compared to 95% for Sabouraud, and the inhibition of bacterial strains was 100% compared to 20% for Sabouraud medium [94].

The FastFung® culture medium was subsequently tested for the specific culture of *Malassezia* yeasts [95]. The aim of this study was to compare the performance of three skin sampling methods and two culture media for the detection of *Malassezia* yeasts by culture from human skin samples. The FastFung® medium showed promising results for the specific culture of *Malassezia* yeast and was proposed for direct application in routine laboratory clinical diagnosis [95].

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

### 2.8. Protozoa of the oral cavity: through amoebae and trichomonas

Protozoa are microscopic, unicellular eukaryotes. Each protozoan typically exists as an independent cell, living freely as a phagotrophic microorganism [96]. In some species, the cells join together to form colonies. Many protozoa are pathogenic and can cause diseases such as malaria, while others are commensals in the digestive tract of ruminants and wood-eating insects [96].

Protozoa are also part of the oral microbiota, of which *Entamoeba gingivalis* is the most frequent representative, followed by *Trichomonas tenax* (Figure 1) [97]. Oral protozoan infections have been shown to be mainly associated with periodontal disease [98]. Indeed, their prevalence is much higher in patients with periodontal disease than in those with healthy periodontium [97]. Currently, the detection of oral cavity protozoa is mainly performed by direct microscopy or by PCR targeting the 18S gene [98,99]. Culture-based approaches applied to these organisms are very rare in the recent literature. The few studies which have been published use a minimal culture medium with an agar base comprised between 1.5% and 2% [100]. The ATCC 1171 TYGM-9 culture medium (<https://www.atcc.org/products/30956>) adopted from the work of Clark *et al.* in 1992 and 1997, remains the reference medium for the culture of protozoa from the human oral cavity [101–103]. New investigations are, therefore, necessary to improve the culture of these fastidious organisms and new



culture media must be optimised to broaden the research spectrum and isolate new species that have remained uncultured.

**Table1:** Update and description of culture techniques and their research perspectives.

Techniques	Usual denomination	Applications and perspectives	References
<b>Culturomics</b>	Culturomics	Culture based study of the microbial diversity of the human microbiota	[15-17,27]
<b>Rapid culture of anaerobic bacteria using YCFA medium</b>	Rapid culture	Culture based study of stool samples	[51]
<b>Blood culture bottles supplemented with rumen</b>	Rapid culture	Culture based study of stool samples	[51]
<b>Alcohol decontamination</b>	NO	Spore-forming bacteria	[49,55]
<b>Thermal shock</b>	NO	Spore-forming bacteria	[37]
<b>Antioxidants</b>	NO	Improvement of anaerobic conditions for aero-intolerant bacteria culture	[59-62]
<b>Co-culture</b>	NO	Improvement of fastidious bacteria culture	[61,64-66]
<b>Media supplemented with antibiotics and/or phages</b>	Antibiotics and phages	Selective culture of antibiotics and phase resistant organisms	[47,69,71]
<b>The genomic-reverse culture technique</b>	Genomic-reverse	<i>Saccharibacteria</i> TM7 group	[72,73]
<b>Oral <i>Treponema</i> enrichment</b>	T-Raoult medium	<i>Treponema</i> genus	[85]
<b>Hungate roll tubes</b>	Roll tubes	Methanogenic archaea culture and strictly anaerobic bacteria	[57]
<b>Double chamber technique</b>	SAB-medium	Methanogenic archaea culture	[61]
<b>Modified double chamber technique</b>		Methanogenic archaea culture	[97]
<b>FastFung®</b>	FastFung®	Fungi and yeasts	[111]
<b>The ATCC 1171 TYGM-9 culture medium</b>	ATCC 1171	Protozoa of the oral cavity	[115, 117]

NO: unspecified

### 3. Discussion

According to Jakubovick *et al.*, in 2010, the colonisation of oral surfaces by microorganisms occurs in a sequence of steps that is characteristic and subject to possible external intervention [121]. The process begins with the adhesion of bacteria to host receptors on epithelial cells in the periodontium or in the salivary pellicle covering dental surfaces [121]. Intercellular binding interactions between bacteria facilitate the adhesion of different microorganisms to available surfaces and increase the diversity of the adherent microbial population [122]. Microbial growth in oral biofilms is influenced by the exchange of chemical signals (quorum sensing), metabolites and toxic products between neighbouring cells [123]. Bacterial cells present on the surface of teeth, commonly known as dental plaque, produce extracellular polymers such as complex carbohydrates and nucleic acids [124]. These large molecules form a protective matrix that contributes to the development of dental caries and, eventually, periodontitis [124].

Since the discovery of the first microbes by Anthonie van Leeuwenhoek in the 17th century (1683) [93], microbial culture was one of the first methods used to study the human microbiota [15]. Since then, microbial culture has greatly contributed to improving scientific and medical knowledge of the microbes studied, as well as the diseases they provoke [16]. Microbial culture has long been considered tedious, costly



and time consuming [17,18]. As a result, microbiologists have gradually abandoned it in favour of new molecular approaches such as PCR techniques and metagenomics [17,18]. Indeed, metagenomics was considered as one of the most promising techniques for studying microorganisms without culturing them. It was first applied in 2005 to study the human intestinal microbiota with the prospect of revealing all remaining uncultured microbes [15]. Subsequently, culture-based studies were gradually abandoned and metagenomics became the reference method used by microbiologists to study complex ecosystems [21]. However, this technique presented many biases related to the extraction methods and the type of primers used for the amplification of genomic DNA, which made the results which were obtained non reproducible [104]. In addition, a depth bias makes all species with a population below  $10^5$  or even  $10^6$  per gram of sample undetectable by this technique [16,21,104]. Nevertheless, metagenomics has contributed to the expansion of knowledge about the uncultured part of the human microbiota, which does not correspond to any known microbe, commonly referred to as “dark matter” [16,17]. It is a question of an uncountable number of sequences not assigned to any known microorganism, which metagenomics is unable to identify [16,17].

In recent years, microbial culture has experienced a re-emergence thanks to environmental microbiologists who have developed culture conditions close to the original environment of bacteria in order to facilitate their cultivation [15]. Numerous approaches have subsequently proven their value in microbial culture such as microbial culturomics, which has made considerable progress in the field of culture and especially in the repertoire of human microbiota, enabling the culture of more than 2,770 species of bacteria, archaea, fungi and yeasts, as well as unicellular eukaryotes [28]. In contrast, few studies have focused on the oral microbiota by culture and studies performed in this field remain limited. Indeed, the oral cavity hosts a complex microbiota composed of more than 1,200 different bacterial species colonising the surface of the teeth and the oral mucosa [19]. After the gut microbiota, it is the second microbiota in humans in terms of number and diversity [19].

The study of the human microbiota has become a major public health issue given its involvement in human health and disease, which goes beyond our understanding. Several studies in the literature have clearly demonstrated its involvement not only in diseases but also in human health. Its involvement in the regulation of the response to anti-cancer treatments has been demonstrated [105–107]; as well as the participation in the metabolic homeostasis of certain species like *Akkermansia muciniphila* which could have a protective effect against metabolic disorders [108]. Indeed, the involvement of *Faecalibacterium prausnitzii* and *A. muciniphila*, two of the most abundant bacteria in the human gastrointestinal tract, in the marked attenuation of colitis, weight loss, and decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines in patients with chronic inflammatory bowel disease has been demonstrated [109,110]. In the oral cavity, numerous studies have described a protective role attributed to the oral microbiota. According to Sekirov *et al.*, 2010, the microbiota may provide its host with a physical protective barrier against incoming pathogens by competitive exclusion [7]. Indeed, many actions of the microbiota can have a beneficial effect on its host, such as the occupation of attachment sites, the consumption of nutrient sources and the production of antimicrobial substances [7]. The microbiota also acts on the immune system of its host by stimulating the production of various antimicrobial compounds such as defensins, cathelicidins and C-type lectins, known to be produced generally in the digestive tract of mammals [7,111]. The oral microbiota has also been incriminated in certain infectious and tumoral pathologies of the oral cavity as well as in numerous systemic pathologies such as diabetes, cardiovascular diseases and certain distant tumour illnesses [20].

It now seems clear that the oral microbiota plays an essential role in the maintenance of oral health in particular, and human health in general. The alteration





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

#### 4. Patents

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

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#### Conflicts of Interest:

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



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### 3. Discussion

Depuis la découverte des premiers microbes par Anthonie van Leeuwenhoek au cours du 17<sup>ème</sup> siècle (1683) [38] la culture microbienne a été parmi les premiers outils utilisés pour étudier le microbiote humain [37]. Elle a grandement participé à l'amélioration des connaissances scientifiques et médicales concernant les microbes étudiés, ainsi que les maladies qu'ils provoquent [35]. La culture microbienne a longtemps été considérée comme fastidieuse, lente, chronophage et coûteuse [39,40]. C'est pour cela que les microbiologistes l'ont petit à petit abandonné suite à l'apparition des nouvelles approches basées sur la biologie moléculaire comme les techniques de PCR et la métagénomique [39,40]. En effet, la métagénomique était considérée comme une des techniques prometteuses pour étudier les microorganismes en se passant de leur culture. Elle a été appliquée pour la première fois en 2005 pour étudier le microbiote intestinal humain [37]. Elle avait pour perspective de découvrir tous les microbes restant encore non-cultivés [37]. Par la suite, les études basées sur la culture ont peu à peu été abandonnées pour laisser place à la métagénomique devenue la méthode de référence utilisée par les microbiologistes pour étudier un écosystème complexe [41]. Cependant, la métagénomique a montré de nombreux biais liés aux techniques d'extraction et aux choix des amorces utilisées pour l'amplification de l'ADN génomique, qui rendaient les résultats obtenus non reproductibles [42]. Ajouté à cela, un biais de profondeur qui fait que toutes les espèces présentant une population inférieure à  $10^5$  ou même  $10^6$  par gramme d'échantillon restent indétectables par cette technique [35,41,42]. Néanmoins, la métagénomique a contribué à l'élargissement des connaissances concernant la partie non cultivée du microbiote humain et surtout qui ne correspond à aucun microbe connu, communément nommée la « *dark matter* » [35,39]. Il s'agit de d'un nombre incalculable de séquences non-assignées à aucun microorganisme connu dont la métagénomique est dans l'incapacité de les identifier [35,39].

Depuis quelques années la culture microbienne a connu une réémergence grâce aux microbiologistes environnementaux qui ont développé des conditions de culture qui se rapprochent de l'environnement d'origine des bactéries afin de faciliter leur culture [37]. De nombreuses approches ont par la suite fait leurs preuves dans le domaine de la culture microbienne comme le culturomics qui a réalisé en une dizaine d'années une avancée considérable dans ce domaine et surtout au niveau du répertoire du microbiote humain en permettant la culture de plus de 2770 espèces de bactéries, archaea, champignons et levures ainsi que des eucaryotes unicellulaires [43]. En revanche, très peu d'études se sont intéressées au microbiote buccodentaire par culture et les travaux réalisés dans ce domaine restent limités. En effet, la cavité buccale héberge un microbiote complexe composé de plus de 700 espèces bactériennes différentes qui colonisent la surface des dents et les muqueuses buccales [22]. Il s'agit du deuxième microbiote chez l'Homme en terme de nombre et de diversité après le microbiote intestinal [22].

L'étude du microbiote humain est devenue un enjeu de santé publique majeure au vu de son implication dans la santé et les maladies humaines qui a dépassé notre entendement. Plusieurs travaux dans la littérature ont démontré clairement son implication dans les maladies mais aussi dans la santé humaine. Son implication dans la régulation de la réponse aux traitements anti-cancer a été démontrée [44–46] ; ainsi que la participation à l'homéostasie métabolique de certaines espèces comme *A. muciniphila* pourrait avoir un effet protecteur contre les troubles métaboliques [47]. En effet l'implication de *F. prausnitzii* and *A. muciniphila*, deux bactéries des plus abondantes dans le tube digestif humain, dans l'atténuation marquée de la colite, de la perte de poids, de la diminution des cytokines pro-inflammatoires et augmentation des cytokines anti-inflammatoires chez les patients atteints de maladies inflammatoires chroniques de l'intestin [48,49].

Dans la cavité buccale, de nombreux travaux décrivent un rôle protecteur attribué au microbiote oral. D'après Sekirov *et al.*, 2010, le microbiote peut fournir à son hôte une barrière protectrice physique contre les pathogènes entrants par exclusion compétitive [50]. En effet, de nombreuses actions du microbiote peuvent avoir un effet bénéfique pour son hôte comme l'occupation des sites d'attachement, la consommation de sources de nutriments et la production de substances antimicrobiennes [50]. Le microbiote agit aussi sur le système immunitaire de son hôte en stimulant la production de divers composés antimicrobiens tels que les défensines, les cathélicidines et les lectines de type C, connues pour être produites généralement dans le tube digestif des mammifères [50,51]. Le microbiote oral a aussi été incriminé dans certaines pathologies infectieuses et tumorales de la cavité orale mais aussi dans de nombreuses pathologies systémiques comme le diabète, les maladies cardiovasculaires ainsi que certaines atteintes tumorales à distance [52].

Il semble maintenant évident que le microbiote oral joue un rôle essentiel dans le maintien de la santé buccodentaire en particulier et humaine en général. L'altération de ce microbiote a souvent été associée aux pathologies bucco-dentaires et systémiques. Cependant, le rôle que joue le microbiote oral dans l'apparition et le développement de ces maladies reste encore non élucidé et des investigations supplémentaires sont donc nécessaires pour le caractériser et élucider son implication dans les pathologies humaines et orales. Les approches d'études basées sur la culture demeurent une bonne perspective pour étudier le microbiote afin d'obtenir des isolats cliniques pour étudier leur métabolisme et leur comportement vis-à-vis des agents antimicrobiens utilisés en pratique clinique, ainsi que pour séquencer leur génome pour mieux comprendre leur implication dans le processus des maladies infectieuses buccales.

#### **4. Conclusion**

Dans ce manuscrit, nous avons rapporté les différentes méthodologies et milieux de culture décrits dans la littérature potentiellement applicables à l'étude du microbiote oral. Nous avons rapporté l'utilisation de certains milieux de culture utilisés pour la culture des bactéries de culture difficiles comme le YCFA et les flacons d'hémocultures supplémentés par du rhumén et du sang de mouton, utilisés au cours d'une étude conduite par l'équipe culturomics à l'IHU de Marseille en combinaison avec une technique de culture dite rapide pour cultiver les bactéries fastidieuses en moins de 24h rendant ainsi l'utilisation de la culture comme un outil de diagnostic, possible dans les laboratoires de microbiologie hospitaliers. Nous avons aussi abordé des méthodologies spécifiques pour une culture ciblée ainsi que des techniques de culture spécifiques des trois règnes du vivant généralement rencontrés dans la cavité buccale humaine, Eucaryote, Bactérie, Archaea.

Cette revue bibliographique a pour but de présenter dans ce seul manuscrit les outils nécessaires et indispensables à l'étude du microbiote buccodentaire par approche de culture afin de démontrer son implication dans les maladies et la santé buccodentaire. En effet, la plus grande majorité des pathologies buccodentaires rencontrées en pratique clinique sont des pathologies infectieuses et inflammatoires d'étiologie microbienne. Par conséquent, connaître ces microbes semble être le moyen le plus efficace pour lutter contre les maladies infectieuses de la cavité buccale afin d'améliorer la prise en charge des patients.

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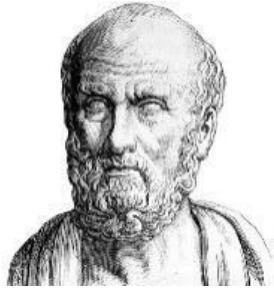
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## SERMENT MEDICAL

*En présence des Maîtres de cette Faculté, de mes chers condisciples, devant l'effigie d'HIPPOCRATE.*

*Je promets et je jure, d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la Médecine Dentaire.*

*Je donnerai mes soins à l'indigent et n'exigerai jamais un salaire au-dessus de mon travail, je ne participerai à aucun partage clandestin d'honoraires.*

*Je ne me laisserai pas influencer par la soif du gain ou la recherche de la gloire.*

*Admis dans l'intérieur des maisons, mes yeux ne verront pas ce qui s'y passe, ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs ni à favoriser le crime.*

*Je ne permettrai pas que des considérations de religion, de nation, de race, de parti ou de classe sociale viennent s'interposer entre mon devoir et mon patient.*

*Même sous la menace, je n'admettrai pas de faire usage de mes connaissances médicales contre les lois de l'humanité.*

*J'informerai mes patients des décisions envisagées, de leurs raisons et de leurs conséquences. Je ne tromperai jamais leur confiance et n'exploiterai pas le pouvoir hérité des connaissances pour forcer les consciences.*

*Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je n'entreprendrai rien qui dépasse mes compétences. Je les entretiendrai et les perfectionnerai pour assurer au mieux les services qui me seront demandés.*

*Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leur père.*

*Que les hommes m'accordent leur estime si je suis fidèle à mes promesses. Que je sois déshonoré et méprisé de mes confrères si j'y manque.*

**KHELAIPIA Saber** : Le microbiote buccodentaire humain, actualisation des méthodologies et des techniques de culture

Th. : Chir. dent. : Marseille : Aix –Marseille Université : 2023

Rubrique de classement : Odontologie Conservatrice

**Résumé :**

Ces dernières années ont été marquées par un changement de paradigme dans l'étude du microbiote humain, avec une réémergence des approches dépendantes de la culture. De nombreuses études ont été consacrées au microbiote intestinal humain alors que les études sur le microbiote oral restent encore limitées. En effet, différentes techniques décrites dans la littérature peuvent permettre une étude exhaustive de la composition microbienne d'un écosystème complexe.

Ici, nous avons rapporté différentes méthodologies et milieux de culture décrits dans la littérature qui peuvent être appliqués pour étudier le microbiote oral par culture. Nous avons discuté de l'utilisation de milieux de culture pour la culture de bactéries fastidieuses qui ont rendu possible l'utilisation de la culture comme outil de diagnostic dans les laboratoires hospitaliers. Nous avons également présenté des méthodologies spécifiques pour une culture ciblée ainsi que des techniques de culture spécifiques et des méthodologies de sélection pour cultiver les membres des trois règnes de la vie que l'on trouve couramment dans la cavité buccale humaine, à savoir les eucaryotes, les bactéries et les archaea.

Cette revue bibliographique vise à rassembler les différentes techniques décrites dans la littérature permettant une étude complète du microbiote oral afin de démontrer son implication dans la santé et les maladies buccales.

**Mots clés:** Culture, microbiote oral humain, bacteries, archaea, champignon, culturomics

**KHELAIPIA Saber** : The human oral microbiota, updating methodologies and culture techniques

Th. : Chir. dent. : Marseille : Aix –Marseille Université : 2023

Category : Conservative dentistry

**Abstract:**

Recent years have been marked by a paradigm shift in the study of the human microbiota, with a re-emergence of culture-dependent approaches. Numerous studies have been devoted to the human microbiota, while studies on the oral microbiota still remain limited. Indeed, various techniques described in the literature may enable an exhaustive study of the microbial composition of a complex ecosystem.

In this article, we report different methodologies and culture media described in the literature that can be applied to study the oral microbiota by culture. We discuss the use of culture media to culture fastidious bacteria, methods which opened the door to the use of culture as a diagnostic tool in hospital laboratories. We also report on specific methodologies for targeted culture and specific culture techniques and selection methodologies for cultivating members of the three kingdoms of life commonly found in the human oral cavity, namely, eukaryota, bacteria and archaea.

This bibliographic review aims to bring together the various techniques described in the literature, enabling a comprehensive study of the oral microbiota in order to demonstrate its involvement in oral health and diseases.

**Key words:** culture, human oral microbiota, bacteria, archaea, fungi, culturomics