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Etudes microbiologiques des parodontopathies

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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. Par ailleurs, 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

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Résumé

Les maladies parodontales, sont mondialement répandues touchant toutes les tranches d'âge. Ce sont des maladies à l'étiologie plurifactorielle et certains facteurs de risque comme le tabagisme sont très bien identifiés. Sur le plan microbiologique, elles se caractérisent par une dysbiose favorisant l'émergence de pathogènes, détruisant le tissu parodontal appelé parodonte par des processus inflammatoires chroniques. Depuis plusieurs décennies, il existe un panel de publications dans la littérature étudiant la composition de la flore orale chez des patients atteints de maladies parodontales. Cela a été possible grâce à la comparaison avec le microbiote sain notamment depuis l'avènement de l'amélioration des techniques de séquençage nouvelle génération (NGS). L'objectif de cette thèse est donc d'élaborer de nouvelles stratégies et d'utiliser de nouvelles techniques d'études pour étudier les potentiels acteurs dans l'apparition des maladies parodontales.

Dans un premier temps, une bibliographie exhaustive sur les maladies parodontales a permis la publication d'une revue avec pour objectif de mettre en évidence les dernières connaissances sur l'association entre la parodontite et la composition du microbiome, ainsi que les stratégies de diagnostic et les techniques de traitement récents. Cette revue a permis d'orienter l'étude de l'étiologie microbiologique, les pathogènes impliqués et les profils de microbiote associés aux maladies parodontales.

Dans une seconde étude, un protocole de culture, un nouvel outil d'identification par la réaction de polymérisation en chaine (q-PCR) et un système de typage *Multi-Locus Sequence Typing* (MLST) basé sur le génome ont été créés pour étudier l'implication du protiste *Trichomonas tenax* dans la survenue de la parodontite. En effet, il s'agit du seul protiste avec *Entamoeba gingivalis* à avoir été suspecté de participer au développement de la maladie. Les

résultats de biologie moléculaire démontrent un portage asymptomatique fréquent de *T. tenax* aussi bien chez les patients que chez les contrôles, un lien entre la présence du protiste et la sévérité de la maladie mais également l'existence de souches spécifiques associée à la gravité de la parodontite. Il important de noter l'originalité de ce travail de typage qui est basé d'abord sur la culture fastidieuse cet agent (et par la même la constitution de la plus grande collection du monde) puis du séquençage, certes grossier, qui a permis d'obtenir tout de même ce génome eucaryote de 46 millions de paires de bases en 4161 scaffolds.

Dans un autre travail, la combinaison de deux approches d'analyses, culturomique et métagénomique a permis de mettre en évidence un le lien éventuel entre la diversité du microbiote oral et les maladies parodontales. L'étude par culturomique reposant sur la multiplication des conditions de culture utilisée ainsi qu'une nouvelle approche d'analyse des résultats obtenus par pyroséquençage ont permis de mettre en évidence des espèces, des genres et des familles spécifiques chez les patients atteints de parodontites, de même que des bactéries dites « protectrices » présentent en majorité chez des contrôles ainsi que la détection d'une nouvelle espèce. Les bactéries retrouvées dans les formes sévères et avancées sont relativement comparables à ce qui a été déjà publié, même si certains agents considérés comme communs n'ont pas été retrouvés que ça soit en culture ou en métagénomique. En revanche, les formes modérées/débutantes permettent de détecter de nouveaux agents bactériens, notamment un groupe particulier de minimicrobes que nous suspectons d'être liés, voire responsables, avec l'apparition de la maladie.

Accessoirement, les nouvelles espèces bactériennes isolées par la technique de culturomique ont été étudiée par une approche taxonogénomique.

Mots-clés : parodontite, maladie parodontal, dysbiose, culturomique, métagénomique, *Trichomonas Tenax*, biologie moléculaire, microbiote oral, taxonogénomique.

Abstract

Periodontal diseases are worldwide prevalent in all age groups. These are diseases with a multifactorial etiology and some risk factors such as smoking are very well identified. Microbiologically, they are characterized by dysbiosis that promotes the emergence of a pathogen, destroying periodontal tissue called periodontal disease by chronic inflammatory processes. For several decades, there has been a panel of publications in the literature studying the composition of oral flora in patients with periodontal diseases. This was possible due to the comparison with healthy microbiota, since the inflow and advancement of next generation sequencing techniques (NGS). The goal of this thesis is to develop new strategies and use new advanced techniques to study the potential actors in the onset of periodontal diseases.

Initially, an exhaustive bibliography on periodontal diseases allowed the publication of a review, with the aim of highlighting the latest knowledge on the association between periodontitis and microbiota composition, as well as diagnostic techniques and recent therapeutic strategies. This review led to study the microbiological etiology, involved pathogens, and microbiota profiles associated with periodontal diseases.

In a second study, a culture protocol, a new chain polymerization reaction identification tool (q-PCR) and a genome-based Multi-Locus Sequence Typing (MLST) typing system were created to study the involvement of the protist *Trichomonas tenax* in the occurrence of periodontitis. Indeed, it is the only protist with *Entamoeba gingivalis* potentially associated with the development of the disease. Molecular biology results demonstrated frequent asymptomatic carriage mechanism of *T. tenax* and was identified in both patients and controls. Nevertheless, a link between the presence of the protist and disease's severity, also the existence of specific strains associated with the severity of periodontitis. It is important to note the originality of this

work, which is based first on the fastidious culture of this agent (and by the same constitution of the largest collection of the world) then sequencing, which allowed still obtain this eukaryotic genome of 46 million base pairs in 4161 scaffolds.

In another work, the combination of two approaches of analysis, culturomics and metagenomics, highlighted on a possible link between the diversity of the oral microbiota and periodontal diseases. Culturomics tool, based on exhaustive culture conditions, as well as results obtained by a new NGS data analysis strategy, allowed to identify specific bacterial species, genera and families in patients with periodontitis. In addition, this procedure identified bacteria called "protective" mostly detected in controls, as well as the isolation of a new bacterial species. Bacteria found in severe and advanced forms were relatively comparable to recent published data, even if some common microorganisms were not found using culture or metagenomics. On the other hand, the moderate / mild forms allowed the detection of new bacterial agents, especially “minimicrobes” a particular group of microorganisms suspected in relation with the occurrence of periodontal diseases.

Finally, the new bacterial species isolated by the technique of culturomics were studied by a taxonogenomic approach.

Keywords: periodontitis, periodontal disease, dysbiosis, culturomic, metagenomic, *Trichomonas Tenax*, molecular biology, oral microbiota, taxonogenomic.

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Introduction

Le microbiome humain est définit par un écosystème complexe constitués de micro-organismes vivants (bactéries, virus, archées, champignons et parasites). La présence des espèces bactériennes en relation symbiotique contribue à l'établissement d'un équilibre microbiologique et immunitaire bénéfiques. Cependant, des perturbations au sein des ces micro-environnements favorise la prolifération de pathogènes opportunistes entrainant des processus inflammatoires chroniques et excessifs détruisant progressivement le tissu. Ce déséquilibre révèle une cause majeure de diverses pathologies dont les maladies parodontales affectant la flore orale, sujet de cette thèse.

La parodontite est une maladie multifactorielle, affectant les adultes et dans certains cas les plus jeunes, entraînant la perte de dents avec complication systémique [1]. L'étiologie de la maladie se caractérise par un déséquilibre de l'écosystème sur le plan microbiologique qui s'accompagne de facteurs de risques génétiques ou environnementaux modifiables ou non [2].

L'avènement de nouvelles techniques NGS (Next Generation Sequencing) par séquençage à haut débit du gène de l'ARN ribosomal 16S a permis la description de divers microbiotes dans certaines pathologies par comparaison entre cohortes malade et contrôle dans le but d'identifier de potentiels acteurs impliqués dans les perturbations [3]. Néanmoins, la culture reste le moyen permettant l'isolement d'espèces à la fois vivantes et possiblement minoritaires du fait de la richesse bactérienne de certains prélèvements. La combinaison de ces deux techniques se révèlent donc nécessaire afin d'identifier l'étiologie associée aux pathologies et notamment aux maladies parodontales mais aussi d'étayer la description de la composition du microbiote. De nombreuses études métagénomiques ont été publiées dans la littérature [4–6] mais l'approche pluridisciplinaire de ce sujet reste encore inédite. Cette thèse

avait donc pour objectif l'usage de diverses approches et techniques pluridisciplinaires pour réaliser l'étude microbiologique des maladies parodontales.

Dans un premier travail, du fait de la quantité d'études publiées fréquemment sur les maladies parodontales, une mise à jour des dernières connaissances et découvertes sur le sujet était nécessaire. Une revue s'appuyant sur une bibliographie récente (avec des publications en majorité datant de moins de 10 ans) a été réalisé pour redéfinir les potentiels pathogènes impliqués dans la dysbiose auparavant centré sur le complexe rouge de Socransky (*Porphyromonas gingivalis, Treponema denticola* et *Tannerella forsythia*) [7]. Mais aussi les facteurs de risques précédemment identifiés, innés ou environnementaux tels que le tabagisme, le diabète, l'absence d'hygiène dentaire ou des facteurs génétiques et environnementaux [8–11]. Et enfin la description nouvelles stratégies de diagnostic par biomarqueurs [12] et techniques de traitements récents comme la vaccination [13]ou la thérapie par phage [14] et antioxydants[15]. Ce travail a été réalisé dans le but d'orienter ensuite l'étude de cette thèse.

Dans une autre étude, le protiste *Trichomonas tenax* proposé depuis les années 80 comme potentiel acteur dans le développement de la maladie a été étudié [16]. Plusieurs études ont été préalablement réalisées sur ce flagellé. La culture étant fastidieuse, la détection était réalisée par observation directe par microscopie mais aussi en absence de cohorte contrôle pour différencier une prolifération anormale d'une colonisation naturelle [17]. Nous avons donc au sein de notre laboratoire développé un nouveau protocole de culture de ce protiste ainsi que de nouveaux outils de biologie moléculaire de détection et d'identification par qPCR en temps réel mais aussi un système de typage pour mettre en évidence la présence éventuels de clones virulents. Une étude épidémiologique du portage de *T. tenax* dans deux cohortes respectivement malade et saine a été réalisé avec pour objectif de définir le rôle de *T. tenax* dans l'apparition ou la sévérité de la maladie.

Dans un troisième travail, nous avons utilisé le concept de « culturomique » mis en place au sein de notre laboratoire consistant à sélectionner les conditions optimales, associées aux techniques d'identification rapide (spectrométrie de masse), dans le but de décrire le plus fidèlement possible le profil bactérien de microbiote spécifique et d'identifier de nouvelles espèces [3,18]. Auquel nous avons associé des techniques complémentaires d'identification moléculaire et de génomique pour comparer la composition bactérienne de la plaque dentaire de patients atteints de parodontite avec celle de témoins sains (Publication 3). L'objectif étant d'identifier un agent pathogène ou un profil bactérien spécifique lié à l'apparition de la parodontite.

Dans la dernière partie de ce travail, nous avons utilisé l'approche taxono-génomique pour la description de nouvelles espèces bactériennes isolées par technique de culturomique. Cette approche proposée par Ramasamy et al. en 2014 se définit par une description du phénotype, une caractérisation génomique par similarité de séquences proches et d'une analyse du profil protéique par spectrométrie de masse MALDI-TOF (Matrix Assisted Laser Desorption/Ionization, Mass Spectrometry)[19]. La procédure est composée d'une succession d'analyse : suspicion d'une nouvelle espèce par un score MALDI-TOF MS < 2 et une similarité de séquence de l'ARNr 16S avec l'espèce apparentée la plus proche <98,65%. Mais aussi, la comparaison de génome avec les espèces les plus proches en termes de taille, de teneur en G+C, de pourcentage de séquences codantes, la description des caractéristiques et phénotypiques et enfin la résistance aux antibiotiques.

Le travail de ce manuscrit s'articule donc en 4 grands axes avec tout d'abord la mise à jour des dernières connaissances sur l'étiologie et les stratégies de diagnostic de traitements de la maladie, puis l'étude de la prévalence du protiste *Trichomonas tenax* dans la parodontite, également l'approche pluridisciplinaire pour décrire le profil microbien dans la maladie

parodontale et enfin la taxono-génomique bactérienne comme stratégie de description des nouvelles espèces.

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Partie 1

Définition et mise à jour des acteurs impliqués dans la parodontite

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Publication 1: Oral microbiota: update on the mystery of periodontitis

Sarah Benabdelkader, Michel Hosny, Gerard Aboudharam and Bernard La Scola

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Avant-propos

La parodontite est une maladie multifactorielle mondialement répandue affectant une grande partie de la population. L'apparition de microorganismes pathogènes suractivent le système immunitaire de l'hôte conduisant à une inflammation excessive du tissu parodontal. La dysbiose du microbiote de la plaque semble donc être l'étape initiale du développement de la parodontite. Certains les facteurs de risques génétiques ou environnementaux ainsi que le système immunitaire de l'hôte sont associés également avec l'apparition et la sévérité de la parodontite. Au cours de l'établissement de la maladie parodontale, des échanges entre l'hôte et les agents pathogènes peuvent entraîner l'apparition de biomarqueurs spécifiques utiles dans le diagnostic clinique de la maladie.

Ce travail bibliographique, à partir de publications pluridisciplinaires récentes de la littérature, met à jour l'association entre le microbiote oral et la maladie parodontale, les pathogènes impliqués dans la dysbiose, ainsi que les outils de prévention et de traitements de la parodontite.

Oral microbiota: Update on the mystery of periodontitis

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Abstract

Periodontitis is a multifactorial disease leading to tooth loss with systemic complication. Dysbiosis of commensal oral microbiota is an initial step of periodontitis occurrence, which interferes with host immune system and pathogenic microorganisms, leading to an excessive inflammation of periodontal tissue. Host immunity, composition of oral microbiota, several modifiable and non-modifiable risk factors, altogether, are associated with periodontitis severity. The diagnosis of periodontal disease is based on clinical characteristics; therefore, host-associated and pathogen-related biomarkers may also be useful, due to the occurrence of molecular changes during disease establishment. The specific objective of this review is to highlight latest knowledge on the association between the oral microbiota and periodontal disease, as well as recent diagnostic and treatment strategies.

Keywords: Periodontal disease, risk factors, microbiota, specific periodontopathogens, biomarkers, treatment.

Introduction

Periodontal disease is defined as an inflammation affecting periodontium tissue, caused by microorganisms of the dental plaque [1]. This disease is very common, affecting 30 to 90% of the population depending on studies and case definitions. Adults are most often affected, but also children and adolescents in few rare cases [2,3]. Periodontal disease has been recently classified into 4 levels (I to IV), estimated on the description (localized or generalized), severity and complexity of management and into 3 stages (A to C), based on direct or indirect evidence of the rate of progression [4]. Diagnosis of periodontal disease is based on specific clinical aspects: gum restriction, alveolar bone resorption, loss of dental junctions, occurrence of periodontal pockets and dental calculus; a microbial biofilm located between gingiva and tooth.

Modifiable and non-modifiable risk factors have been increasingly associated with periodontitis, reinforcing the idea of a multifactorial disease. Thus, the combination of factors may affect the severity of illness. Recently, periodontal diseases have evolved towards association with the oral microbiota. Symbiosis of this complex ecosystem has been linked in establishing microbiological and immune equilibrium. Taxonomic, metagenomic and metabolic diversity depends on body sites and influences on human physiology. Dysbiosis of oral microbiota occurs after a microbial imbalance in biofilms. The damage of physical barrier of epithelial cells, leads to the incorporation of an extreme immune response, promoting the translocation of pathogenic and/or opportunistic microorganisms.

Periodontal disease is maintained by the microbial community inhabited within the dental plaque. Potential periodontopathogen and their virulence factors have been increasingly described; including Gram-negative bacteria, viruses, protists and many others. The ‘Red-complex’ which includes *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* was the most detected group during periodontitis. Socransky *et al.* identified this complex using DNA-DNA checkerboard hybridization technique [5]. In contrast, Gram-

389 positive species such as *Filifactor alocis* and *Peptostreptococcus stomati* and many others may
390 also occur [6] and ‘red complex’ has also been detected in healthy periodontium [7]. The loss
391 of alveolar bones and periodontal tissue restriction are irreversible clinical features described
392 in periodontitis. Healthy and pathogenic state of periodontal tissue are summarized in Figure 1.
393 The need for early diagnosis emerges, allowing rapid clinical intervention for precise treatment.
394 The evaluation of microbial community and associated virulence factors, thus molecular
395 biomarkers are the leading factors for the prognosis and diagnosis of periodontal illness [3,8,9].

396 This review provides an update on the definition of periodontitis, based on recent
397 multidisciplinary researches. We highlight the role of the oral microbiota and the presence of
398 specific microorganisms related to periodontal diseases. Finally, we focus on therapeutic
399 strategies to treat and prevent periodontitis.

1. Microbiota and periodontitis

The oral cavity represents one of the most remarkable body sites due to its microbial diversity. Although fungi, viruses, protozoa and archaea were found, bacteria were the most commonly detected microorganisms in the oral cavity. In detail, the oral cavity represents several niches, each of which shelters ecosystems very different from others such as, tongue, teeth, gums, jugal mucosa and inner surfaces of the lips. Saliva derived from the main salivary glands and ensures moistening of oral mucous membranes. Thus, teeth have a protective role but also constitute a support for microorganisms. One of the key elements of these ecosystems is aerobiosis or anaerobiosis: the presence of oxygen influences the composition of the oral microbiota. These ecosystems diverge according to age and pathologies. In addition to the anatomical complexity of the oral cavity, it is important to note that we are at the aerodigestive pharyngeal-laryngeal crossroads. The oral microbiome can therefore vary according to the pharyngeal-laryngeal or digestive flora [9].

1.1. Healthy microbiota

The oral microbiome represents a massive holobiont generating beneficial effects to the host including, resistance to potential pathogens, the immune response maturation, nutrients degradation from food and indirect role on adipocytosis and the synthesis of weight-regulating hormones [10]. As soon as life begins, the oral cavity is colonized. It is noted that the infant's microbiota undergoes a substantial reorganization, which mainly depends on the body site and not on the mode of delivery six weeks after delivery [11]. The oral cavity is colonized by a set of microorganisms with 700 predominant taxa identified within the human mouth and including 200 predominant species [12,13]. The microbiota is formed from birth through contact with the mother and nutritional strategy (abundance of *Streptococcus* et *Lactobacillus* spp. in breastmilk). Bacterial flora is constantly modified with age and influenced by environmental parameters [10,14] but is stable in adulthood [15]. Moreover, oral microbiota seems to be

affected with buccal hygiene. Toothpastes containing enzymes and proteins were associated with a better gingival health [16]. This type of toothpaste enhanced salivary immune system, promoting the establishment of beneficial bacterial community associated with healthy gum (*i.e. Neisseria* sp.) and decreased those linked to periodontal disease (*i.e. Treponema* sp.) [17]. Oral "core microbiome" was defined by Zaura *et al.*, predominantly composed by five genus: Firmicutes (*Streptococcus*, *Granulicatella* and family Veillonellaceae), Proteobacteria (*Neisseria* and *Haemophilus*) , Actinobacteria (*Corynebacterium*, *Rothia* and *Actinomyces*), Bacteroidetes (*Prevotella*, *Capnocytophaga* and *Porphyromonas*) and Fusobacteria (*Fusobacterium*) [18]. This finding was confirmed by several meta-transcriptomic and metagenomic studies presenting those same genus commonly found in healthy periodontium [19–22]. Specific species have also been associated with healthy periodontium such as *Streptococcus oralis, Streptococcus sanguinis, Streptococcus gordonii, Granulicatella elegans, Neisseria flavescens* and *Porphyromonas catoniae* [23].

1.2. Dysbiosis and periodontitis

Dysbiosis is the alteration of the microbiota that causes negative effects on metabolic activities, disrupting the balance between chronic inflammation and anti-inflammatory responses. Dysbiosis of microbial biofilm compromises the immune response resulting in the desorption of periodontium with the loss of dental junctions associated with the onset of periodontal pockets more or less significant, and in some forms of calcification [3]. Bacteria from subgingival biofilm could degrade glycoproteins, peptides and amino acids into fatty acids, ammonia and sulfur compounds inducing tissue inflammation and destruction leading to the periodontal disease [24]. The existence of specific interactions has been reported in periodontal biofilm: strains of high-virulence *P. gingivalis* W83 intensified by *P. intermedia*, induce effects on the symbiotic species by changing the metabolic pathways genes and quorum sensing. Also, *Fusobacterium nucleatum* and *Streptococcus mitis* had a strong correlation by

the co-adherence (a ‘bridge’ between early and later colonizers) and a biological interplay (metabolism of amino acid and nucleotide, membrane transport and genetic information processing) during the formation of dental plaque [25]. The biofilm generation can induce the establishment of resistance to different antimicrobial agents, (*i.e.* amoxycillin, doxycycline and metronidazole). The mechanism of resistance against antimicrobial can be explained by: low cell permeability, quorum sensing activity, efflux pumps, slow growth, stress response and the alteration of membrane composition [26]. Recent metagenomics studies demonstrated that the phyla Bacteroidetes, Candidatus Saccharibacteria, Firmicutes, Proteobacteria, Spirochaetes and Synergistetes are moderately associated with periodontitis and Actinobacteria associated with the healthy microbiome [27,28]. At the genus level, periodontopathogens like *Anaeroglobus*, *Bulleidia*, *Desulfobulbus*, *Filifactor*, *Mogibacterium*, *Phocaeicola*, *Schwartzia* or candidate phyla radiation TM7 were detected in relative abundance in patients with periodontitis [29]. At the species level, *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Fretibacterium fastidiosum* and *Filifactor alocis* have been recognized as pathogens strongly associated with periodontitis nowadays [23,28,30]. Additionally, pockets deepening was caused by several bacterial genera in chronic periodontitis (*i.e*. *Acholeplasma, Peptococcus* and *Mycoplasma*). Which is not the case in aggressive periodontitis, were *Corynebacterium* and *Klebsiella* were associated with pocket deepening [31]. Finally, a transcriptomic study of the oral microbiome identified an overexpression of putative virulence factors in periodontal samples for members of the "red complex" and candidate division TM7, strongly detected in the disease community [32].

2. Study of specific pathogenic microorganisms associated with periodontitis

While dysbiosis is increasingly being interpreted as the main microbial condition associated with periodontitis, various microorganisms have been detected during periodontal disease. We discuss here the specific infectious etiologies of periodontitis.

2.1. Bacteria

The human oral cavity contains an abundant and polymorphic flora of microorganisms. Bacterial diversity was higher in periodontal patients than in healthy subjects, which could be interpreted as the consequence of a nutritionally richer environment or a reduced immune competence. Several bacterial species were associated with periodontal disease: the original 'red complex', *Aggregatibacter actinomycetemcomitans, Prevotella intermedia* and TM7 [33].

a. *Porphyromonas gingivalis*:

P. gingivalis is a Gram-negative non-motile anaerobic bacterium and is one of the main etiological pathogens isolated in periodontal disease. This bacterial species was detected in 85.75% of subgingival plaque samples of patients with chronic periodontitis. In contrast *P. gingivalis* were previously identified in healthy controls. Inflammatory reaction and tissue damage caused by *P. gingivalis* are due to the presence of several virulence factors, especially lipopolysaccharides (LPS), capsules, fimbriae, teichoic acids, protease and many others [34]. The mechanism of host immune response modulation by *P. gingivalis* is summarized in Figure 2.

b. *Tannerella forsythia:*

Previously named *Bacteroides forsythus, Tannerella forsythia* is a Gram-negative anaerobic bacterium, second member of the 'red complex' isolated from periodontal pockets of patients with periodontitis. *T. forsythia* degrades collagen type I and III, which initiates the formation of the periodontal pocket, promoting the growth of anaerobic pathogens and aggravating the severity of periodontitis [35].

c. *Treponema denticola:*

T. denticola is a Gram-negative motile anaerobic bacterium, third member of the 'red complex' found in periodontal pockets. A recent review highlights the symbiotic relationship between *P. gingivalis* and *T. denticola*. This latter produces succinate for *P. gingivalis*, therefore *P. gingivalis* produces fatty acids necessary to *T. denticola* growth, inducing periodontal lesions [36].

d. *Aggregatibacter actinomycetemcomitans:*

A. actinomycetemcomitans is a Gram-negative non-motile facultative anaerobic bacterium. *A. actinomycetemcomitans* is known to be an etiological agent of aggressive periodontitis. This species activated the quorum sensing of *Streptococcus mutans*, a cariogenic acid-producing bacterium, inducing alveolar loss [37]. A recent study of biogeographic microbiome at the micron scale has shown a complex aggregation between these genera [37,38].

e. *Prevotella intermedia:*

Initially, *Bacteroides intermedius*, *P. intermedia* is a Gram-negative anaerobic pathogenic bacterium involved in periodontal diseases [36]. It has been reported that sever periodontitis is strongly associated with the combination of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* [39].

f. TM7

TM7, bacteria from the Saccharibacteria phylum and Candidate Phyla Radiation (CPR) predominantly prevalent in multiple sites within the human oral microbiome [40], were also associated with periodontal disease. Abundance increased by 1 to 21% in patients with various types of periodontitis [41]. Usually found by metagenomic tools, He et *al*. isolated the first cultivable TM7x on *Actinomyces odontolyticus* subsp. *actinosynbacter* strain XH001, this strain was a surface of interactions between both species [42]. CPR bacteria, particularly TM7,

modulate microbial ecology through parasitism and probably play a poorly understood role in the development and severity of periodontal disease [40].

2.2. Viruses

Viruses were also implicated in the occurrence of periodontal diseases, such as Herpes simplex viruses (HSV1), Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) [43,44]. Those periodontal viruses are identified in several syndromes associated with severe periodontitis such as Guillain-Baré syndrome (CMV and localized periodontitis) Kostmann syndrome (EBV and severe periodontal diseases) or Down syndrome (CMV, EBV, HSV and moderate to advanced periodontitis) [45]. It has been suggested that CMV and *P. gingivalis* have a synergistic effect on the appearance and development of periodontal diseases. In addition, Elamin *et al.* confirmed the coinfection of EBV or CMV with *A. actinomycetemcomitans*, leading to an increased risk of aggressive periodontitis[46].

2.3. Protists

The involvement of eukaryotes in the development of periodontal diseases has been suspected since the 1980s [47,48].

a. *Entamoeba gingivalis*:

The first amoeba described in human sites, *Entamoeba gingivalis*, was strongly detected in periodontal pockets compared to healthy sites in several studies with a heterogeneous prevalence of 6 to 69% [48–50]. In contrast, those articles only identified this species on healthy sites in periodontal patients as control cases. The real prevalence of this amoebae in healthy people remains unknown because of the absence of control cohorts. For this reason, the involvement of *E. gingivalis* in the disease and its mechanisms of action remain unclear.

b. Trichomonas tenax

Known as an harmless commensal [47] but identified recently as a parasite [51], the protist *T. tenax* has been detected in periodontal pockets, but a recent review of the literature has explained the heterogeneity prevalence (ranged from 0 to 94.1%) by different diagnostic methods and population diversity [52]. *T. tenax* induced an physiopathology effect against the human macrophage and deregulated the proinflammatory cytokines in periodontal disease [53] and an *in vitro* cytotoxic effect on mammalian cells [54]. A recent publication of our team demonstrated the existence of specific clones of *T. tenax* associated with periodontitis severity, using genomics-dependent and culture-based detection methods, and confirmed that this protist is part of the healthy periodontal microbiota [55]. The mechanism of host immune response modulation by *T. tenax* is summarized in Figure 2.

2.4. Other microbes

Bacteriophages are viruses whose bacteria are also common members of the oral microbiota and have been suggested to be associated with oral diseases. In subgingival biofilm, myoviruses are significantly more abundant in periodontal disease, suggesting that periodontitis favors lytic phage with an active role in modulating bacterial diversity [56]. A prophage induction in lysogenic of the periodopathogen *A. actinomycetemcomitans* in co-culture with fibroblast induced an important release of leukotoxin [57]. Also, a study reveals a subgingival colonization of some yeasts, especially *C. albicans*, associated with the severity of chronic periodontitis [58]. Finally, archaea were also linked to periodontal diseases. First, *Methanobrevibacter oralis* is correlated with periodontitis severity and can be used as a biomarker of periodontitis [59]. Also, a repertoire of archaea cultivated from severe periodontitis in our laboratory identified *Methanobrevibacter smithii* and a new species of *Methanobrevibacter* sp. strain N13 in a periodontitis patients [60].

3. Risk factors associated with periodontal disease

3.1. Diabetes mellitus

Diabetes mellitus is the first major risk factor confirmed for periodontitis. Type 2 diabetes, but also type 1 diabetes, increases the risk of developing periodontitis. The link between diabetes and peridontal disease is suspected to be associated with a specific inflammatory reaction. Patients present increased systemic levels of proinflammary mediators such as interleukine-6 (IL-6), C-reactive protein (CRP) and TNF-α enhancing insulin resistance [61]. A bidirectional relationship is definied between periodontitis and diabetes. Indeed, even if glycemic level in diabetes is a key to determin the risk for periodontitis, periodontitis has a negative impact on glycemic control management and the development of diabetic complications [62,63]. Futhermore, the oral microbiota composition differs between diabetic and non-diabetic periodontal patients. Recently, Miu *et al.* highlighted a less fequency of *Tannerella forsythia, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in peridontitis patients with type 2 diabetes mellitus compared to non-diabetic peridontitis patients, but the species were strongly associated with periodontitis [64]. Additionally, diabetes mellitus promotes the growth of some bacterial species and this factor must be included for specific treatment of diabetic patients with periodontitis.

3.2. Smoking

The smoking status is also a risk factor linked to periodontitis. Smoking habit increases the development, progression and the severity of the disease: the negative effects of smoking depends on frequency, dose and type of tobacco used on the tissue destruction and periodontal healing [65].

The periodontal ecology is also modified by the habit of smoking, which leads to the alteration of the diversity of the oral microbiota incorporated in the biofilm associated with periodontitis [29]. Disease-associated genus such as *Parvimonas*, *Bacteroides* and *Treponema*

had higher prevalence in the microbial community of smokers with periodontitis compared to non-smokers [66]. The oral microbiota of smokers had increased microbial diversity, enriched with pathogenic species and fewer commensal bacteria similar to a disease-associated community, generating “at-risk-for-harm” microbiome promoting the susceptibility of the onset of periodontal disease [67]. The presence of methanogenic archaea, and in particular *Methanobrevibacter oralis,* has also been correlated with the severity of periodontal disease [59]. Statistical analyzes revealed a significant correlation between PCR detection of methanogenic archaea in oral fluid and smoking, especially *M. oralis* and *Methanobrevibacter smithii* that are transmitted by oral fluid in tobacco smokers [68].

3.3. Genetics and epigenetics

The persistence of periodontitis-associated pathogenic bacteria can be affected by genetic factors, increasing the risk of disease development and leading to early tooth loss [69]. A recent genome-wide association study (GWAS) of chronic periodontitis, exposed several potentiels loci associated with periodontal disease, including genes involved in the immune response and epithelial barriere function [70]. The genetic predisposition to the disease did not define a mononucleotide polymorphism but several genes potentially involved. In addition, epigenetics has regulated the levels of inflammatory markers during periodontitis through mechanisms such as histone acetylation and DNA methylation: virulence factors of pathogens in local biofilm can induce epigenetic changes in periodontal tissues [71].

3.4. Other factors

Other environmental factors, such as socio-economic status, may prevent patients from undergoing routine dental examination and maintenance [72]. Poor dental health or stress by immunosuppressive effect may be involved in the emergence of periodontal disease by decreasing adaptive and innate immune defence [3]. Obesity and overweight are correlated to

615 periodontitis : the chronic inflammation and oxidative stress leading to the insulin resistance,
616 increased glucose dyslipidemia and hepatic damage could impact the onset of the disease
617 [73,74]. Unhealthy diet with excessive carbohydrates poly-unsaturated fat, deficient protein and
618 vitamin C and B12 intake can affect gingival bleeding and destructive periodontal disease [75].
619 Indeed, patient with psychological disorders, such as stress, are more likely to develop the loss
620 of alveolar bones, by hyperproduction of IL-6 and decreased immunity against *P. gingivalis*. In
621 addition, specific systemic diseases were associated with periodontitis, especially neutropenia,
622 lazy leukocytes, Chediak-Higashi, Papillon Lefevre and Down syndromes[8].

623 ## 4. Molecular and cellular biology of periodontitis

624 ### 4.1. Microbial-associated genes profiling

625 Microbial communities generating biofilm induce the expression of several genes in
626 response to stress mechanism. In addition, recognized genes may have positive feedback and
627 affect the physiopathology mechanism of periodontal disease. Genes related to antibiotic
628 resistance have been reported in patients, including multidrug resistance efflux pumps, as well
629 as ABC transporters, conjugative transposons and beta-lactam degradation [22,23].
630 Additionally, genes associated with virulence have also been observed in relation to bacterial
631 motility (flagellar genes), transport and attachment (*i.e.* peptide and iron), as well as endotoxins
632 biosynthesis (lipid A component of Gram-negative bacteria). Conversely, potassium transport
633 and polysaccharide biosynthesis were down expressed during periodontitis [22].

634 ### 4.2. Periodontitis biomarkers

635 Early diagnosis is a major challenge in modern medicine. To this end, researches shift
636 towards the discovery of early disease biomarkers by evaluating the cellular biology at the onset
637 of the disease. Several biomarkers have been studied during the occurrence of periodontal

disease compared to healthy controls and other oral diseases. Signatures depend on the level of specific metabolic and immunological profiling.

Chen *et al.* identified high levels of noradrenaline, uridine, α-tocopherol, dehydroascorbic acid, xanthine, galactose, glucose-1-phosphate and ribulose-5-phosphate in gingival crevicular fluid of patients with generalized aggressive periodontitis. Contrariwise, decreased rates of thymidine, glutathione and ribose-5-phosphate was observed [76]. On the other hand, several salivary biomarkers were significantly associated with periodontal disease: Inflammatory signature caused by chronic bacterial infection and detected by an increase in fatty acids ω-6 and TNF-α [77,78]. Additionally, immunological systemic metabolic signatures were also identified during periodontal diseases as follow: immunoglobulins (variance in serum levels of *P. gingivalis* IgA and IgG subclasses) [79], pattern recognition receptors (Surfactant protein D; recognition of Gram-negative bacterial LPS) [80] and cytokines (MCP-1/CCL2; monocyte chemoattractant protein-1, MIP-1α/CCL3 and macrophage inflammatory protein-1 alpha) [81]. To support this idea, Maney *et al.* assessed polymorphism of Interleukins (IL) identified during aggressive periodontal diseases, where the IL-1 was the key cytokine implicated in the occurrence physiopathogenesis of periodontitis. However, the IL polymorphisms reported for aggressive periodontitis appear to be different from those associated with chronic periodontitis [82]. Urine biomarkers were also considered predictive factors in the Japanese society, where β_2-Microglobuline and Neutrophil gelatinase-associated lipocalin displayed correlation with clinical aspect of periodontitis [83]. Indeed, Suzuki *et al.* have suggested supposed molecular biomarkers for the diagnosis and prognosis of chronic periodontitis by *in silico* analysis of several transcriptomic datasets. Authors reported common pathological pathway including twelve genes: *CD53*, *CD19*, *CD79A*, *IL-8*, *IL-1β*, *IL-10Rα*, *C3* (*complement system*), *CXCL12* (*Chemokine C-X-C motif ligand 12*), *FCGR3B* (*Fc fragment of IgG, low affinity IIIb*), *FCGR2B* (*Fc fragment of IgG, low affinity IIb*), *SELL* (*Selectin L*) and *CSF3* (*Colony*

stimulating factor 3). In addition, *TNF* (*tumor necrosis factor*) and *FGF2* (*fibroblast growth factor 2*) were the common upstream regulators of the diagnosis biomarkers [84].

4.3. Genetics of periodontitis

Genetic research is the evaluation of genes to better understand the development of disease. It is based on the study of genetic variants within the human genome. Nevertheless, GWAS and independent GWAS have identified several genes potentially associated with aggressive periodontitis *i.e.*: *glycosyltransferase 6 domain containing 1*, *antimicrobial peptide genes defensin alpha 1*/*alpha 3*, *protein coding gene sialic acid binding Ig like lectin 5*, *platelet factor 4*, *pro-platelet basic protein*, *C-X-C motif chemokine ligand 5*, *UDP glucuronosyltransferase family 2 member A1 complex locus*, *plasminogen* and *antisense noncoding RNA in the INK4 locus*. In contrast, *neuropeptide Y* was noticed during severe chronic periodontitis [85]. Finally, the epigenetic study conducted on adult female twins revealed a significant link between the CpG site and gingival bleeding and specific DNA methylation changes associated with periodontal disease [86].

5. Therapeutic modalities and prevention of periodontitis

5.1. Vaccination

Immunisation remains a key therapeutic strategy to prevent infectious-associated diseases. As mentioned above, microbiological etiology was an exclusive cause of periodontitis. Several vaccination assays have been reported, especially for the major periodontopathogen *P. gingivalis*. The *in vitro* immunisation obtained on killed whole-cell *P. gingivalis* induced: the blockage of periodontitis and alveolar bones loss, followed by increased levels of IgA and IgG [87]. Thus, virulence factors of *P. gingivalis* antigens were a point of interest for the development of periodontal vaccines, including proteases, fimbriae, capsular polysaccharides (CPS), LPS and vesicles of external membranes. On the other hand, antigenic sequences of

687 phosphorylcholine, CPS and thermal shock protein were used as target antigens, possessing
688 genetic homology with that of other pathogenic proteins and would be adapted to polymicrobial
689 periodontitis[88]. Puth *et al.* described dysbiosis-linked periodontal mucosal vaccine. This
690 latter is a mix of fusion proteins from *Fusobacterium nucleatum* and *P. gingivalis*. Successful
691 assays were reported in both mucosal and systemic immune system [89].

692 **5.2. Antioxidants**

693 Decreased levels of micronutrients, such as vitamin C/E, carotenoids, coenzyme Q10,
694 polyphenols, flavonoids, melatonin and selenium, were associated in the occurrence of
695 periodontitis. Excessive immune response and the presence of reactive oxygen species (ROS)
696 are usually the keystone in tissue injury during periodontal illness. ROS are secreted on
697 osteoclasts surfaces, leading to the loss of alveolar bones. Talmaç *et al.* reviewed the impact of
698 concertation-linked antioxidants to prevent periodontitis through four mechanisms: direct effect
699 on free radical, decreasing ROS titration, primary radical neutralization and metal chelator
700 formation (albumin, ferritin etc.). Therefore, natural antioxidants as well as the administration
701 of nutrients containing vitamin C (grapefruit) showed a protective and beneficial effect on
702 decreasing sulcus bleeding score, thus reducing deed pockets. Furthermore, Coenzyme Q10
703 intensified gingival soft tissue by reducing inflammation reaction, the presence of
704 microorganisms, dental plaque, gingival index and deep pockets [90].

705 **5.3. Probiotic therapy**

706 During our decade, microbiota manipulation therapy expanded to treat dysbiosis-associated
707 diseases, particularly intestinal microbiota, despite the lack of researches regarding the
708 employment of probiotics in oral health. Several strains were used for this purpose, especially
709 from the genus *Lactobacillus*. As for dental diseases, the use of *L. rhamnosus* and *L. reuteri*
710 exhibited beneficial effect on *S. mutans* [91]. Recently, *Lactobacillus reuteri* shewing tablets

exhibited decreased in gingival inflammation and deep pocket during periodontitis in smoker population [92]. On the other hand, probiotics strains of Lactobacilli and Bifidobacteria modified adhesion and invasion processes of the periodontopathogen *P. gingivalis* [93].

5.4. Phage therapy

Various parameters disrupt antimicrobial therapy, particularly the biofilm matrix, preventing them from penetrating through the periodontal tissue and the presence of antibiotic resistance. However, the use of phage therapy seems to be an attractive selection and depends on the targeted periodontopathogen. Self-multiplication and low-cost production of phage are a keystone and play an important role during infection. To date, no interference with antibiotic resistance has been described. Phages were previously used to block the generation of dental carries, where phages associated with *Streptococcus sanguis* were employed in various ways (spray, food, dental products, pills etc.). Nevertheless, phage was also active on the periodontal pathogenic serotype b of *A. actinomycetemcomitans*. Finally, it is important to improve the safety of this strategy; the phage bacterial lysis mechanism produces endotoxins that induce an inflammatory response [94].

5.5. Symptomatic therapy

The use of medication allows either symptomatic treatment or causal agent eradication. The most common periodontitis antibiotics are Tetracycline, Doxycycline, Metronidazole, Amoxicillin, Amoxicillin-clavulanate, Ciprofloxacin and Macrolides [95]. In addition, Eugenol, a local antibacterial, anti-inflammatory, anaesthetic and analgesic component showed beneficial effect on periodontal tissue [96]. Periodontal surgery has been applied to treat periodontal tissue and alveolar bone loss. In parallel, platelet-rich plasma is an ongoing strategy employed to achieve tissue regeneration. The mechanism of this polypeptide growth factor is

734 to promote bone reconstruction, by amplifying the production of blood vessels and the process
735 of cell proliferation [97].

Future perspective

Oral bacteria are possible biomarkers for systemic diseases and oral microbial profiles could potentially be useful in assessing the risk of periodontal disease. Available data on oral microbiota reflect its protective or pathogenic feature and the complex structural organization that may emerge from its composition. It is essential to maintain normal microbiota inhabited in oral tissue to defend from opportunistic pathogens and polymicrobial dysbiosis. Prevention strategies, such as oral hygiene, lifestyle and patient education, are fundamental to maintaining a healthy oral cavity. Periodontitis remains a multifactorial disease encompassing certain specific microorganisms, genetic and environmental factors. There is therefore a real need to explore new periodontal pathogens and to understand the mechanism of pathophysiology. Future approach should include early diagnosis of periodontitis in high-risk subjects: a combination of biomarkers associated with the host stress response, finally, those related to oral dysbiosis and bacterial virulence factors.

Executive summary

Microbiota and periodontitis

- The oral microbiota symbiosis represents beneficial effects on host's immune system.
- Oral microbiota includes various microorganisms, generated since birth and varying according to several factors.
- Five genera are presented within oral microbiota ecosystem: Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria.
- Oral microbiota dysbiosis leads to metabolic and immune distortion.
- Biofilm of dental plaque is a niche of altered microorganisms causing periodontitis.

Study of specific pathogenic microorganism associated with periodontitis

- Specific microorganisms were associated with periodontitis: Bacteria, viruses, protists and many others.
- The presence of red complex was increasingly associated with the disease.
- *Porphyromonas gingivalis* was a major periodontopathogen.
- The identification of viruses was associated with some systemic diseases.

Risk factors associated with periodontal disease

- Diabetes mellitus and smoking habit seem to be major factors leading to periodontitis.
- Smoking promotes the establishment of dysbiosis and favours the overgrowth of methanogenic archaea associated with periodontitis.
- Genetic research exposed putative loci associated with periodontal disease.

Molecular and cellular biology of periodontitis

- Pathogenic virulence genes were detected in periodontal samples.
- Specific biomarkers from gingival crevicular fluid, saliva, urine and blood may lead researchers to diagnose periodontitis.
- Genetic and biomarkers were linked to immune response and inflammatory reaction.

Therapeutic modalities and prevention of periodontitis

- The usefulness of vaccination is suggested depending on the identified periodontopathogen.
- Recent research has focused on antioxidants to treat periodontitis, reducing the proliferation of reactive oxygen species.
- *Lactobacillus* species remains a major probiotic to treat gingival inflammation.
- Phages are new strategy to kill specific periodontopathogens, due to the absence of cross-resistance with antibiotic treatments.
- The platelet-rich plasma approach enabled the regeneration of alveolar bones.

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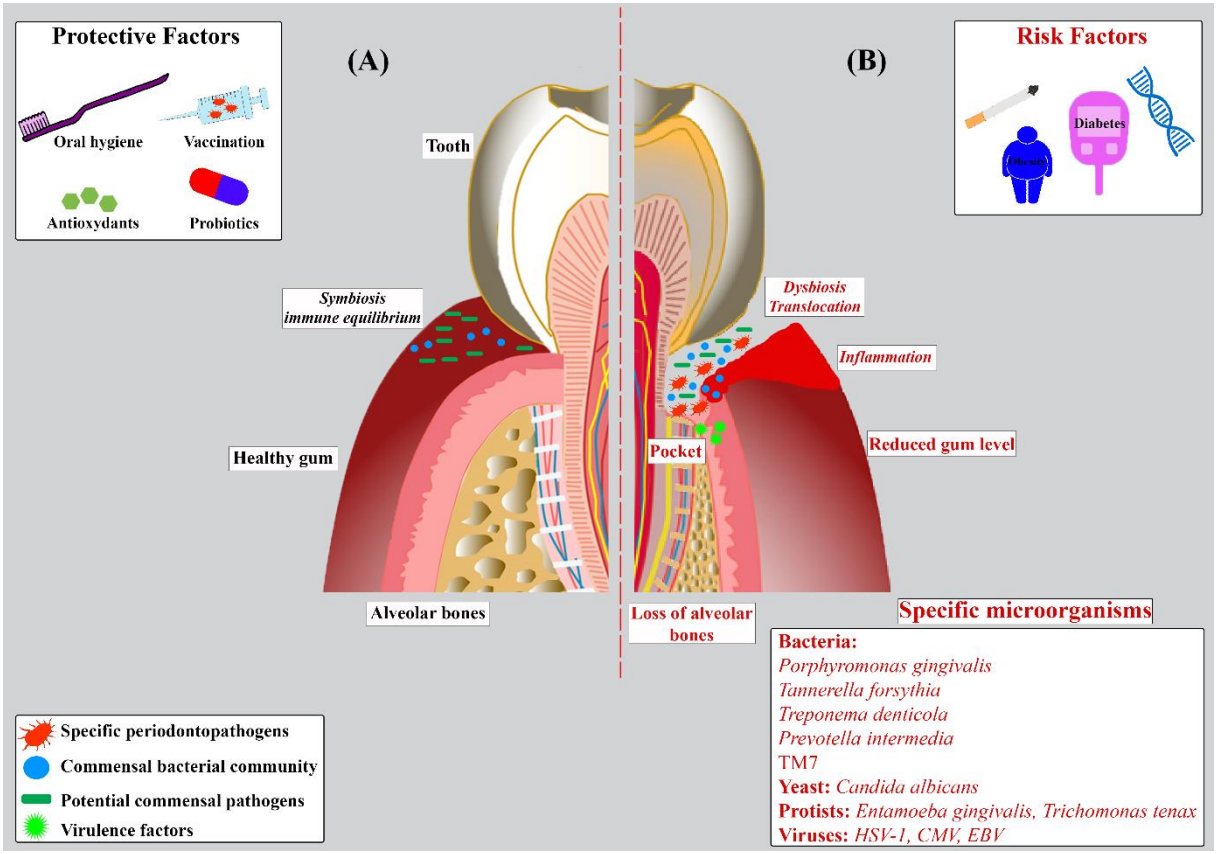

Figure 1: comparison between normal and pathological periodontal tissue.

(A) Healthy state of periodontal tissue includes healthy gum and the presence of alveolar bones. The establishment of this mechanism is due to the existence of symbiotic relation between commensal beneficial oral microbiota and oral cavity. (B) Dysbiosis, modifiable and non-modifiable risk factors are the keystone during the pathogenesis of periodontal diseases, allowing opportunistic bacteria overgrowth. Specific microorganisms were translocated by periodontal pockets, maintaining the occurrence of inflammation process.

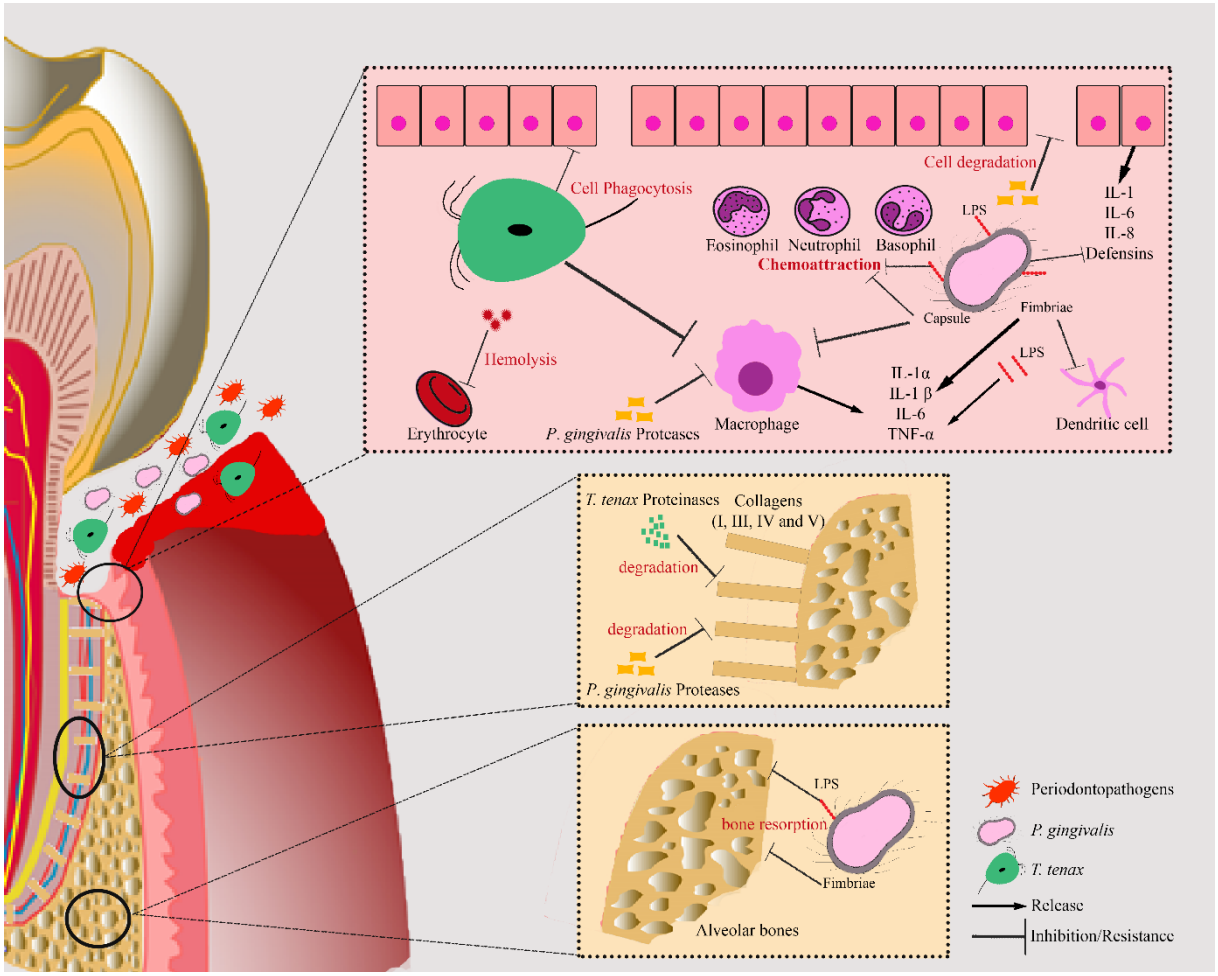

Figure 2: Periodontal innate immunity and virulence factors during the invasion of specific microorganisms.

Healthy periodontal tissue is inhabited by immune cells establishing immune tolerance to commensal microorganisms by secretion of anti-inflammatory components and the regulation of adaptative immunity. Cells invasion by periodontopathogens (*i.e. T. tenax and P. gingivalis*) is the initiative of extreme inflammatory response. The mechanism of physiopathology is associated with the presence of potential virulence factors, that modulate innate immunity.

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Partie 2

Etude de la relation entre *Trichomonas tenax* et la parodontite

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Publication 2: Specific clones of *Trichomonas tenax* are associated with periodontitis

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Avant-propos

Depuis des années, certains articles de la littérature suspectent une association entre la présence du protiste *Trichomonas tenax* dans la plaque dentaire et le développement de la parodontite. Cependant, la culture de ce protiste reste fastidieuse et les outils d'identification moléculaire sont peu fiables. Au cours de ce travail, nous avons créé un protocole de culture efficace, un nouvel outil d'identification par q-PCR en temps réel du pathogène et un système de typage MLST (Multi-Locus Sequence Typing) des souches obtenues par culture pour confirmer la pathogénicité de *T. tenax* dans la parodontite.

L'étude comporte deux cohortes respectivement, de 106 patients atteints de parodontite classés par stades de gravité et de 85 patients contrôles. Les résultats combinés des deux techniques démontrent que *T. tenax* est significativement détecté chez les patients atteints de parodontite par rapport à la cohorte saine. Également, le protiste est significativement plus fréquent chez les patients atteints de parodontite au stade sévère, ce qui établit un lien entre le portage de *T. Tenax* et la sévérité de la maladie. Le génotypage des 53 isolats de culture, indique la présence d'une diversité de souches de *T. tenax*. De plus, le typage MLST permet l'identification de certains clones du protiste associés à la parodontite. Ces données suggèrent donc un lien entre le portage de souches spécifiques et la sévérité de la parodontite. Plus fréquemment détecté dans les cas parodontaux, *T. tenax* est probablement lié à l'apparition et / ou à l'évolution des maladies parodontales.

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RESEARCH ARTICLE

Specific clones of *Trichomonas tenax* are associated with periodontitis

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Abstract

Trichomonas tenax, an anaerobic protist difficult to cultivate with an unreliable molecular identification, has been suspected of involvement in periodontitis, a multifactorial inflammatory dental disease affecting the soft tissue and bone of periodontium. A cohort of 106 periodontitis patients classified by stages of severity and 85 healthy adult control patients was constituted. An efficient culture protocol, a new identification tool by real-time qPCR of *T. tenax* and a Multi-Locus Sequence Typing system (MLST) based on *T. tenax* NIH4 reference strain were created. Fifty-three strains of *Trichomonas* sp. were obtained from periodontal samples. 37/106 (34.90%) *T. tenax* from patients with periodontitis and 16/85 (18.80%˚) *T. tenax* from control patients were detected by culture ($p = 0.018$). Sixty of the 191 samples were tested positive for *T. tenax* by qPCR, 24/85 (28%) controls and 36/106 (34%) periodontitis patients ($p = 0.089$). By combining both results, 45/106 (42.5%) patients were positive by culture and/or PCR, as compared to 24/85 (28.2%) controls ($p = 0.042$). A link was established between the carriage in patients of *Trichomonas tenax* and the severity of the disease. Genotyping demonstrates the presence of strain diversity with three major different clusters and a relation between disease strains and the periodontitis severity ($p<0.05$). More frequently detected in periodontal cases, *T. tenax* is likely to be related to the onset or/and evolution of periodontal diseases.

Introduction

Periodontal disease is a widespread oral disease affecting adults and younger people, characterized by an inflammatory reaction that affects periodontium tissue [1]. A new classification published in 2018 based on description (localized or generalized), severity and complexity of management divides periodontitis into 4 stages, including initial periodontitis (I), moderate periodontitis (II), severe periodontitis with potential for additional tooth loss (III) and advanced periodontitis with extensive tooth loss and potential for loss of dentition (IV) [2]. According to this classification, periodontitis is also graded in 3 levels estimated with direct or

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indirect evidence of progression rate: slow (A), moderate (B) and rapid (C). Periodontal disease is characterized by receding gums, alveolar bone destruction, loss of dental junctions associated with the apparition of periodontal pockets, and in some forms, dental calculus deposits. This promotes the establishment of an anaerobic microenvironment that allows the growth of anaerobic microorganisms [3]. The immunological process initiates the migration of microorganisms into tissues and disrupts the immune response, causing the periodontium to resorb [4,5]. Some host risk factors have now been clearly identified, including smoking [6] and diabetes mellitus [7], but other genetic factors require further study [8].

The human oral cavity contains an abundant and polymorphic microbiota. A set of bacterial complexes living in subgingival plaque has been identified by Socransky et al; with the initial establishment of Streptococcus spp. and their disappearance, multiple complexes defined by green purple or yellow colors have been described but only the orange and red complexes have been suspected of being associated with the development of periodontitis [3]. Metagenomic studies confirm the association of certain bacterial species found in the orange complex, such as *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum* [9,10]. But also the strong association of the disease with the three bacteria described in the red complex: *Pophyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* are also found in the periodontal pockets by metagenomic recent studies[11,12] and new species involved have recently been identified [13]. Nevertheless, the "red complex" theory remains debatable due to its isolation in healthy controls (30%, 3/10) [14]. An increase in lytic phages in pathological situations also disturbs the periodontal-associated bacteria present [15]. Similarly, meta-transcriptomics analysis showed that the transcription of bacterial virulence factors increased in patients with periodontitis compared to healthy individuals [16]. Viral etiology has also been suggested as being involved in the development of periodontitis, and different viruses have been involved including Herpesviruses (HSV-1), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) [17].

Since the 1980s, the implication of eukaryotes in periodontal disease has also been proposed [18], such as the protists *Trichomonas tenax* and *Entamoeba gingivalis* [19–21] and yeasts, such as *Candida* sp. [22]. However, until now, studies focusing on the association between protists and periodontal disease did not use groups of healthy controls to differentiate between abnormal proliferation and natural colonization [20]. Like its neighboring species, *Trichomonas vaginalis*, the vaginitis-inducing pathogen, *T. tenax* belongs to the Parabasalia phylum and to the *Trichomonadidae* family [23]. *Trichomonas tenax* can ingest bacteria and various particles by phagocytosis necessary for their development[24]. Initially identified as an harmless commensal [18], then known as a zoonotic parasite [25], this microorganism was detected in the periodontal pockets using mostly optical microscopy [26,27], with an occurrence in patients ranging from 0 to 94.1% depending on the country and the detection procedure [21]. *T. tenax* has occasionally been isolated in cases of the salivary glands, lymph nodes or respiratory tract infections [28][29][30]. Recently, *T. tenax* was found to be significantly more prevalent in patients with Down syndrome combined with periodontal lesions (14/52), using 18S rRNA gene PCR in comparison to control patients (5/52), with a non-significant difference in plaque indexes between the two groups [31].

In this cohort study, we sought to estimate the prevalence of *T. tenax* and establish a potential link with the periodontitis severity. We investigated the presence of *T. tenax* in periodontitis as compared to the healthy controls using culture and quantitative molecular detection systems. We also used a genome-based system of strain typing to investigate the possibility that a clone or a group of clones of *T. tenax* with particular pathogenicity are involved in periodontitis.

Materials and methods

Clinical sample collection and treatment

This study was carried out in accordance with the recommendations and approved by the clinical research ethics committee, IFR 48, Aix-Marseille University (protocol N° 2016–011). All subjects gave a written informed consent in accordance with the Declaration of Helsinki. One hundred and ninety-one adults were prospectively enrolled in this cohort, 106 adult patients with periodontitis and 85 healthy adult control patients, (**S1 Table**). The study took place in the Odontology Department of the Hospital La Timone, Marseille, France, between January 2015 and June 2016. The 106 patients with periodontitis could be separated in three classes of severity: 19 patients with mild periodontitis (M), 27 with moderate periodontitis (Mo) and 60 with severe periodontitis (S) according to a previously reported scale using various criteria evaluation: size of the probing depth and the attachment loss for each patient [32]. Smoking status was also collected (**S1 Table**). Subgingival dental plaque samples were collected from multiple periodontal pocket and combined into a single tube per patient for the group test and the same method was used for healthy gingival sites in the control group. The sample was collected using a sterile Gracey curette (HuFriedy, Rotterdam, Netherlands) and transported into 1 mL of transport medium (C-top Ae-Ana, Eurobio, France). The samples were analyzed according to the detailed protocol in **Fig 1**.

Isolation of *Trichomonas tenax*

Twelve-and-a-half cm^2 vented flasks (Corning, NY, USA) containing 10 mL of liquid ATCC: 1171 TYGM-9 medium without rice starch were used to inoculate 250 μL of the clinical samples. The medium was supplemented with 100 Units/mL of Penicillin-Streptomycin Gibco (ThermoFisher, MA, USA) and 20 μg/mL Voriconazole (Sigma-Aldrich, United-States) to retard the bacterial and fungi growth that may interfere with the *T. tenax* development. Flasks were then incubated under anaerobic conditions using Anaerogen generators (ThermoFisher, MA, USA) at 35˚C. Growth was observed by optical microscopy examination. For the strains cryopreservation, cultures were centrifuged at 720 x g for 10 minutes. The final pellet was suspended in 1 mL of TYGM9 medium containing 1% of di-methyl-sulfoxide (DMSO) and placed in a Nunc cryotube, maintained at -80˚C for between 15 and 20 hours before being placed inside a -150˚C freezer.

Molecular identification and typing of isolates

Primer design. Based on the analysis of Malik *et al.*[33], we designed specific primers in the 3,048 bp sequence of the RNA polymerase II *rpb1* gene available on the NCBI website (accession number: HM016234.1 for *T. tenax* strain NIH4). Primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with standard parameters (**Fig 1F**).

Genome sequencing of *Trichomonas tenax*. Briefly, 15 vented Corning 75cm^2 (NY, USA) flasks containing LYI medium of *T. tenax* NIH4 reference strain (ATCC number 30207) were pelleted at 2000 g for 15 minutes, rinsed twice with the same centrifugation parameters in Page's amoeba Saline before final re-suspension in 1 mL of phosphate buffered saline. Concentrated cells were placed at -80˚C before DNA extraction and sequencing. Genomic DNA was sequenced using the Illumina MiSeq (Illumina, Inc, San Diego CA 92121, USA).

The gDNA was quantified by a Qubit assay (Life technologies, Carlsbad, CA, USA) to 6.3 ng/μL and dilution was performed requiring 1 ng of DNA as input. The genomic DNA was fragmented and tagged. Limited cycles of PCR amplification completed the tag adapters and introduce dual-index barcodes. After purification on AMPure beads (Life Technologies,

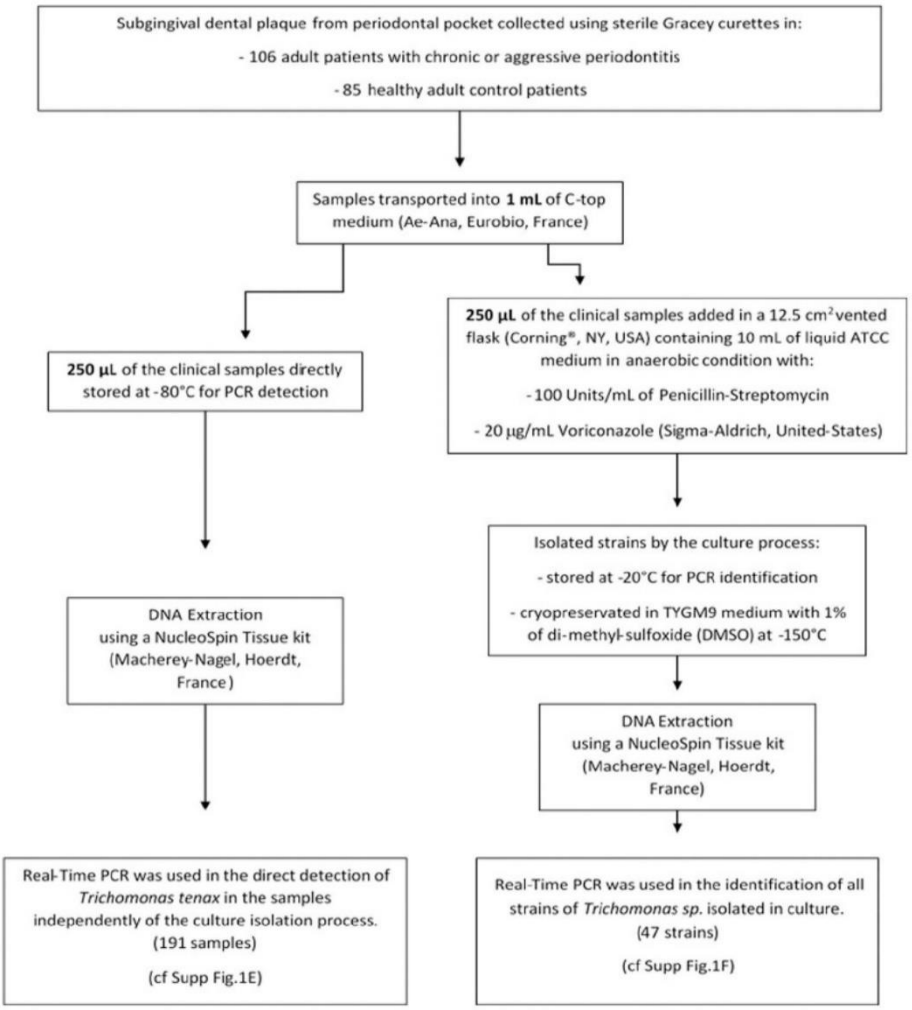

Fig 1. Schematic protocol for *Trichomonas tenax* studies.

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Carlsbad, CA, USA), libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina). Normalized libraries were pooled into a single sequencing library, the MiSeq. The pooled single strand library was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and paired-end sequencing with dual index reads was performed in a single 39-hour, 2x251- base pair run (bp). Total information of 10.5 Gb was obtained from a cluster density of 1,288,000 per mm^2 with 86.3% (20,305,000 clusters) of the clusters passing quality control filters. Within this pooled run, the index representation of *T. tenax* was determined to 39.43%. The 8,005,980 paired end reads were filtered according to the read qualities.

Multilocus sequence typing. The reads obtained were assembled using the CLC Genomics Workbench. Protein sequences were predicted using the Prodigal platform [34] and analysis. A local Blastp on the predicted proteins was performed on NCBI Blast (Basic Local Alignment Search Tool) against non-redundant protein sequences (nr) database using the standard parameters. The *T. tenax* NIH4 genome sequence was deposited on the EMBL-EBI website (Bioproject: PRJEB22701 and whole contigs under accession numbers OCTD01000001-OCTD01004161). Seven single-family household genes were selected ranging in length from 450 to 500 bp (**Table 1**) based on MLST designed for *Trichomonas vaginalis* [35].

Table 1. Primers used for multilocus sequence typing genes of *Trichomonas tenax*.

Genes	Forward	Reverse	Sequence length (bp)	No of alleles
Alanyl tRNA synthetase (ALTS)	5′-CCGTCCAGGATGGTGTCTTC-3′	5′-GTAACATCGAATGGCTGGCAC-3′	514	5
DNA mismatch repair protein (DMRP)	5′-ATTGGACAATGGAACCAGTCA-3′	5′-TGACCATATTTCGCACCACG-3′	516	5
Serine hydromethyltransferase (SHMT)	5′-GAGCGATGGAGGACATTTGAC-3′	5′-TTTGGTGAAGATGAGGACCACC-3′	452	4
Mannose-6-phosphate isomerase (M6PI)	5′-AGGTGTTGCAGAGGAGTTGG-3′	5′-TGCTATTTCGTTTGCAGGAACA-3′	421	7
Glutamine amidotransferase class-I (GAT1)	5′-TCTGTTGCACAAGGTCTCAA-3′	5′-TTGTGTAGCCGCCGTATTTG-3′	424	3
Histidyl tRNA synthetase (HIST)	5′-CGTCTCCATCGACACACCAG-3′	5′-TCCATCTCGTCGAGGACCTT-3′	658	8
Cysteinyl tRNA synthetase (CYST)	5′-GCCCGACTGTTTACTCGACA-3′	5′-CGAAGATCGATACCACCGCA-3′	657	0

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Single standard PCRs were performed to allow DNA amplification of each selected gene. Primer hybridization was conducted at 59˚C and amplified products were sequenced as described previously [36]. Sequences were corrected and assembled using the ChromasPro software version 1.71 (Technelysium, Australia). Obtained sequences were aligned using MUSCLE tool in the Molecular Evolutionary Genetics Analysis software (MEGA) version 7.0.18 (Pennsylvania State University, United-States) and, finally, phylogenetic trees were generated using the maximum-likelihood (ML) method within FastTree version 2.0 [37]. MLST nucleotide sequences data are available in the EMBL-EBI database under accession numbers LT934459 to LT934497.

Direct molecular detection from clinical samples

qPCR on the *rpb1* gene was used to confirm the presence of *T. tenax* directly from clinical samples using specific primers and probe (**Fig 1E**). Cross amplifications were prevented *in silico* and primers were tested on the DNA of *Trichomonas vaginalis* strain G3 (ATCCPRA-98). DNA extraction from our isolates and specimens were performed using the NucleoSpin Tissue kit (Macherey-Nagel, Hoerdt, France). The optimized 20 μL Quantitative Real-Time Polymerase Chain Reaction (qPCR) mix contained: master mix (10 μL), primers (0.5 μL, 20 nM), probe (0.5 μL, 5 nM), water (3.5 μL) and 5 μL of DNA. qPCR temperature cycle was: DNA activation at 50˚C for two minutes, denaturation at 95˚C for five minutes, followed by 40 cycles of 95˚C for one second and 60˚C for 30 seconds for the plate read. qPCR tubes were deposited in a CFX96 Touch thermal cycler (Bio-rad, France). The results were normalized by testing the gene encoding albumin in parallel. The primers used were: Forward: 5′- GCT GTCATCTCTTGTGGGCTG T-3′, Reverse: 5′- AAACTCATGGGAGCTGCTGGTTC-3′ and FAM probe: 6FAM- 5′ CCTGTCATGCCCACACAAATCTCTCC-3′ [38]. A ratio of the cycle threshold (Ct) obtained in q-PCR for the *rpb1* gene and the albumin gene was calculated. (**S3 Table**).

Statistical analysis

The statistical analysis was performed using "R" software (Version 3.5.1) using a ordinal logistic regression with a four-modalities qualitative variable to investigate the correlation between health status and detection methods (culture or PCR) with the existence of *T. tenax*.

χ^2 tests for sex and smoking status descriptive statistics and Fisher test for the phylogenetic tree analysis using *Statistical Package for the Social Sciences* (SPSS Inc, IBM Company).

Table 2. Frequency of *Trichomonas tenax* by qPCR and culture.

	Culture		qPCR		Culture or qPCR	
	Controls	Patients	Controls	Patients	Controls	Patients
Negative	69 (81,2%)	69 (65,1%)	61 (71,8%)	70 (66%)	61 (71.8%)	61(57.5%)
Positive	16 (18,2%)	37 (34,9%)	24 (28,2%)	36 (34%)	24 (28.2%)	45 (42.5%)
Total	85	106	85	106	85	106

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Results

Isolation of *Trichomonas* sp.

Fifty-three strains of *Trichomonas* sp. were obtained from periodontal samples, 37/106 from patients with periodontitis and 16/85 from control patients (p = 0.018) (Table 2, Table 3). Six isolates were lost before conservation. Protists were likely to be *T. tenax* based on their morphology as observed by microscopy. For a definitive identification, we first performed alignment of available *rpb1* gene using online Clustal Omega [39] with standard parameters for 11 sequences of *Trichomonas* spp. and we visualized it using MView online software (http://www.ebi.ac.uk/Tools/msa/mview/). Following the 100% consensus sequence available (S1 Scheme), we designed degenerated primers on conserved regions between the 11 different *Trichomonadidae* strains. In order to evaluate the intra-species diversity of the *rpb1* gene, we amplified and sequenced the *rpb1* gene from 15 randomly chosen strains isolated from our control and diseased patients. Of the 3,000 base pairs obtained, all sequences were 100% identical to the reference strain *T. tenax* NIH4, except one strain (number 13) which possesses two synonym single nucleotide polymorphisms (SNP). All remaining isolates were identified as *T. tenax* using our specific primers.

Detection of *T. tenax* using quantitative real time PCR

Sixty of the 191 samples were positive for *T. tenax*, 24/85 controls and 36/106 periodontitis patients (p = 0.089) (Table 2; Table 3). The standardized results using the average ratio of the *rpb1* gene on the albumin gene demonstrate the absence of a link between the amount of *Trichomonas* DNA and the pathological status (p = 0.087, χ^2) (S3 Table).

Combined results

A good correlation was observed between qPCR and culture, as 83% of positive cultures were also positive for qPCR and 88% of negative cultures were also negative for qPCR. By combining both results, 45/106 patients were positive by culture and/or PCR, compared to 24/85 of controls (p = 0.042, χ^2). No association could be found with the patient's gender and health status (p = 0.710, $\chi 2$) or with the detection of *T. tenax* (p = 0.485, χ^2). The carriage of *T. tenax* and smoking status are correlated regardless of health status (p = 0.001, χ^2). Regarding the periodontitis classification of each patient, *T. tenax* is significantly more detected in severe periodontitis than in mild or moderate periodontitis ($p<0.05$).

Table 3. Comparison between frequency of qPCR and culture.

		Culture		
		Negative	Positive	Total
Real-Time PCR	Negative	122 (88,4%)	9 (17%)	131
	Positive	16 (11,6%)	44 (83%)	60
	Total	138	53	191

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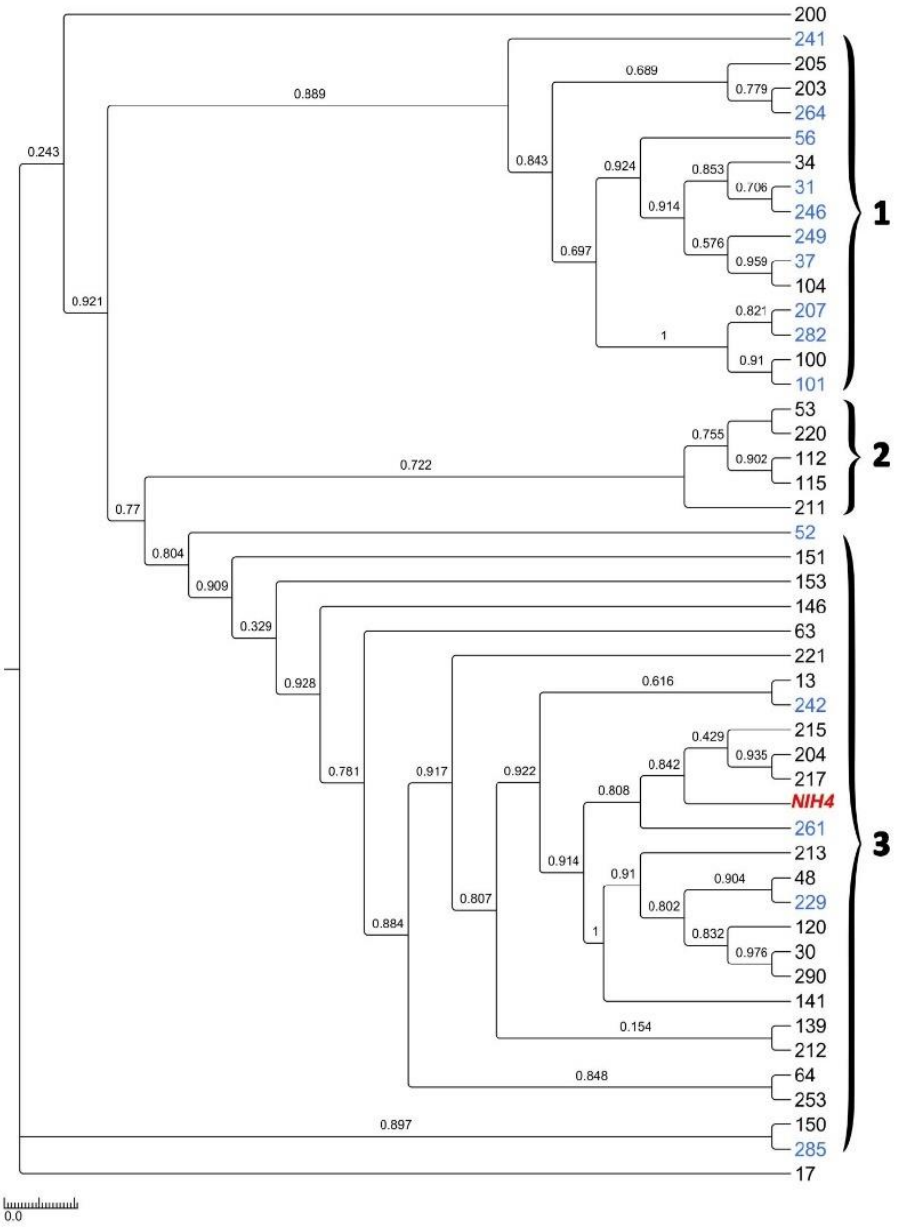

Fig 2. *Trichomonas tenax* relationship based on multilocus sequence typing phylogenetic analysis. Maximum likelihood phylogenetic tree of *T. tenax* strains. Colors legend: black for patients, blue for control and red for reference strain.

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Genome sequencing

The 46,742,176 base pair (bp) draft genome obtained contains 4,161 scaffolds with a N50 of 13,554 bp with a minimum of 4,002 bp to a maximum of 92,188 bp with a G+C content estimated of about 34.6% similar to *T. vaginalis* G3 (35.5%). The gene prediction determined 34,291 Open Reading Frame (ORF) includes 21,854 proteins that could be annotated and 12,437 ORFans. Of these, 21,193 proteins are in common with *T. vaginalis*, and only five with *Trichomonas gallinae*, four with *T. tenax* (sequences of the same strain already available on the nr database) and one with *Tritrichomonas foetus*. This high number of best hits shared with *T.*

vaginalis G3 strain is logical, as this species in the only species of *Trichomonas* spp. for which a draft genome is available. In the *T. vaginalis* G3 genome, about 250 genes were annotated as ribosomal proteins [40]. In *T. tenax*, we obtained in the draft 52 ORFs annotated as the 40S ribosome and 68 annotated as the 60S ribosome. We isolated the complete sequence of the *rpb1* gene measuring 4,962 nucleotides and, congruently, the alignment with the same strain in the database showed 61% of coverage with 100% identity. The alignment starts at position 229 and finishes at position 3,276 of the complete sequence and makes the 3'end of this gene available.

MLST typing

Four housekeeping genes were extracted based on the *T. vaginalis* typing system: Alanyl tRNA synthetase (ALTS), DNA mismatch repair protein (DMRP), Serine hydromethyltransferase (SHMT), and Mannose-6-phosphate isomerase (M6PI). For genes for which no homologs of *Trichomonas vaginalis* could be detected in our *T.tenax* draft genome, we chose three genes because of their functional similarity to complete the system: Glutamine amidotransferase class-I (GAT1), Histidyl tRNA synthetase (HIST), Cysteinyl tRNA synthetase (CYST) (Table 1). The 47 strains and the reference NIH4 strain are clustered into three major groups: strains obtained in the control group are significantly predominant in group 1 and strains isolated in periodontitis patients are significantly predominant in group 3 ($p < 0.05$, Fisher). Only periodontal patient strains constitute the cluster 2. (Fig 2). Phylogenetic tree analysis, based on each housekeeping gene, showed similar topologies. Indeed, six of seven genes also present three major clusters, namely the DMRP, SHMT, ALTS, SHMT, M6PI and GAT1 gene (**S1–S7 Figs**). Concerning the tree based on the CYST gene, all sequences are identical between controls and patients, highlighting the conservation and lack of variability in this portion of the gene. All strain sequences obtained for each gene portion were compared with the sequences of the reference strain *T. tenax* NIH4 to investigate the presence of a single nucleotide polymorphism (SNP) (S2 Table). Four isolates had the same genotype, while the others 43 isolates each had a specific genotype.

Discussion

In this study, by combining a polyphasic approach that associates culture and qPCR, we found a correlation between periodontitis and the presence of *T. tenax* ($p < 0.05$). Although *T. tenax* is more frequently detected by qPCR in patients than in controls, the difference is not significant ($p = 0.435$). A significant difference was observed using culture only ($p = 0.015$). By combining the culture and PCR results to neutralize the effect of false negative of each technique and evaluate the real prevalence of *T. tenax*, the difference is significant ($p = 0.042$). The probability of false positive/negative frequency is reduced due to the good correlation observed between both techniques: 83% of positive cultures also positive for qPCR and 88% of negative cultures also negative for q-PCR techniques. We believe that this good correlation indicates that the culture and handling protocols, including the transport medium specifically developed for anaerobic microorganisms, were highly efficient. However, as commonly observed in clinical microbiology, the higher sensitivity of the PCR suggests that some *T. tenax* did not grew in culture. The reasons are unknown but usually because microorganisms are dead at time of inoculation due to delayed inoculation between sampling and culture, quality of the operator or quality of the batch of transport or culture media. The 11.6% of the positive samples in culture not identified by real-time PCR shows that false positive occur also with molecular amplification, usually as a consequence of inhibitors.

Furthermore, positive culture in controls underlines the difficulty of having a true negative control group. We first suspected that a higher rate of positive culture in periodontitis patients could have been the result of a higher concentration of protist in these samples. But the absence of such differences observed using quantitative PCR disproves this hypothesizes. Indeed, no difference in the amount of *T. tenax* DNA in controls and patients is demonstrated after normalization of the results by the albumin gene. A recent review reports higher prevalence of *T. tenax* occurrence in gum diseased (gingivitis and periodontitis) in most studies present in the literature and explains that the heterogeneity of the prevalence observed may be due to the different methods used to detect the protist (majority of microscopic observation, insufficient use of molecular biology) and the studied population diversity [21]. The same limits may be pointed out in another study reporting the absence of trichomonas in healthy sites in periodontitis patients [27]. Theses discrepancies, as compared to our study, could be related with false positive of our PCR procedure. However, the high level of positive culture herein is not in agreement with this hypothesis and the detection of trichomonads from healthy sites in controls with no periodontal disease indicated that *T. tenax* carriage remains common in the oral cavity.

A correlation could also be established between the severity of periodontitis and the presence of protists ($p<0.05$). *T. tenax* is found in severe periodontitis differing from other periodontitis, by the depth of the pocket as well as the loss of attachment. The environment of severe periodontitis would therefore be more favorable to the development of the protist and other bacteria species leading to serious lesions and inflammatory responses.

We now suspect that the specific periodontal microbiota associated with periodontitis could promote the growth of *T. tenax*. Indeed, when inoculated in the culture medium, the sample also contains bacterial microbiota of dental pockets. Indeed, periodontitis is suspected to be due to an inflammatory response to microorganisms [1,8]. Finally, no single microorganism is implicated but rather a combination of microorganisms act synergistically [3,17,20]. Investigating the difference in microbiota between healthy individuals and patients with mild and moderate periodontitis compared to severe periodontitis would highlight the combined role of protist and oral microbiome.

Several studies have demonstrated the efficacy of PCR in detecting *T. tenax* by employing rRNA sequencing [41]. Likewise, oral cavity metagenomics studies have identified significant *T. tenax* rRNA intergenic spacers [42]. Nonetheless, these systems come up against identification limits. Designing primers based on the *rpb1* gene according to Malik *et al.* [33], allowed us to create a highly specific and sensitive system: this gene is both discriminatory between different species and genotypes and is highly conserved within the same species [43,44]. All isolated strains of *Trichomonas sp.* were identified as *T. tenax*, once again demonstrating the effectiveness of this primer system and the extreme conservation of *T. tenax* based on the *rpb1* gene.

Finally, the MLST system made it possible to investigate the clonal relationship between the protist and periodontitis based on the system created for the closely studied neighboring species, *T. vaginalis* [35]. The data obtained reveal the existence of three clusters grouping *T. tenax*, suggesting genetically diverse strains affecting the periodontium tissue. A significant association could be observed between clustering and the occurrence of periodontitis. The possibility of finding new virulence factors in common between strains in future work could confirm the virulence of diseased strains related to the severity of the periodontal disease. Recently, a physiopathology mechanism of *T. tenax* has been described in the periodontitis disease: *T. tenax* induced an effect against the human macrophage and deregulates the proinflammatory cytokines [45]. Furthermore, *in vitro* studies showed that *T. tenax* had cytotoxic effects on mammalian cells [46]. An animal model reproducing periodontitis suggested in

these recent studies could be used to explore the potential pathophysiological role of *T. tenax* [21,47].

Conclusion

A high prevalence of *T. tenax* in both controls and patients is detected using genomic-dependent and culture-based methods of detection. *T. tenax* was more frequently associated with severe periodontitis. Three clusters of strains were highlighted by the MLST genotyping system, two were significantly associated with periodontitis. *T. tenax* appears to be associated with the onset or/and evolution of periodontal diseases. However, although these differences are statistically significant, it is impossible to determine whether they are a cause or a consequence of the disease.

Supporting information

S1 Scheme. Quantitative Real-Time PCR (q-RT PCR) specific for *Trichomonas tenax*.
(DOCX)

S1 Table. The table below presents the classification of each patient. The numbers correspond to a patient and preserve anonymity. The discontinuity of the numbering is linked to the change of operator in the laboratory. In black: patients with periodontitis.—In bold: the patient controls.
(DOCX)

S2 Table. Sequence types of *Trichomonas tenax* based on single-nucleotide polymorphism against the *Trichomonas tenax* reference strain. In bold: the number of sequences types.
(DOCX)

S3 Table. The table below presents the cycle threshold obtained in q-PCR for the *RPB1*gene and the albumin gene for the standardization. In black: periodontitis patients. In bold: controls.
(DOCX)

S1 Fig. *Trichomonas tenax* phylogenetic analysis based on Alanyl tRNA synthetase (ALTS) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S2 Fig. *Trichomonas tenax* phylogenetic analysis based on DNA mismatch repair protein (DMRP) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S3 Fig. *Trichomonas tenax* phylogenetic analysis based on Serine hydromethyltransferase (SHMT) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S4 Fig. *Trichomonas tenax* phylogenetic analysis based on Mannose -6-phosphate isomerase (M6PI) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S5 Fig. *Trichomonas tenax* phylogenetic analysis based on Glutamine amidotransferase class-I (GAT1) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S6 Fig. *Trichomonas tenax* phylogenetic analysis based on Histidyl tRNA synthetase (HIST) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

S7 Fig. *Trichomonas tenax* phylogenetic analysis based on Cysteinyl tRNA synthetase (CYST) gene. In black: patients with periodontitis. In blue: the patient controls.
(TIFF)

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Partie 3

Etude du rôle du microbiote oral dans la survenue de la parodontite

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Publication 3 : Repertoire of the periodontitis ecosystem using a multidisciplinary approach

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Avant-propos

L'association entre la présence d'espèces bactériennes spécifique dans l'écosystème de la plaque dentaire et l'apparition de maladies parodontales a été progressivement rapportée. L'objectif de ce travail est de s'appuyer sur une combinaison de diverses techniques complémentaires pour compléter la description de l'écosystème de la plaque dentaire déjà étudiée dans la littérature. L'utilisation de la méthode culturomique associée à la métagénomique de l'ARNr 16S, ainsi que la réaction en chaîne de la polymérase spécifique ont permis de comparer la composition du microbiote de la plaque entre deux cohortes de patients atteints de parodontite et de témoins en bonne santé. Cette étude par combinaison des deux techniques a détecté 308 espèces bactériennes, dont 15,8% exclusivement chez les patients et 22,7% chez les témoins. Des espèces bactériennes telles que *Prevotella phoceensis*, *Eubacterium infirmum* et *Slackia exigua* (culturomique), *Eggerthia catenaformis* et *Leptotrichia shahii* (métagénomique) ont été associées de manière significative à la cohorte atteinte de parodontite. Ce travail a également montré une absence de significativité pour des parodontopathogènes classiques tels que *Porphyromonas gingivalis*. Enfin, l'approche différente des résultats de métagénomique ont permis d'identifier de nouveaux groupes de bactéries d'intérêt, appartenant à des espèces non encore décrites ou cultivées de genres connus ou même inconnuschez les patients (Bacterial Group Of Interest in Patients, BGOIP) et chez les contrôles (Bacterial Group Of Interest in Controls, BGOIC).

Repertoire of the periodontitis ecosystem using a multidisciplinary approach

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Running title : Periodontitis and multidisciplinary approach

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Abstract

The association between the presence of various bacterial species in oral samples and the occurrence of periodontal diseases has been progressively reported. Multidisciplinary approaches were employed to study this relationship. The use of culturomics method allowed to compare oral microbiota composition between two cohorts of periodontitis patients and healthy controls. 16S rRNA metagenomic, as well as specific polymerase chain reaction were incrusted to confirm and enrich the microbiological results. By culture-based method 165 bacterial species were identified and 223 bacterial species using Illumina Miseq technology. The combination of both techniques revealed the detection of 303 bacterial species, including 15.2% exclusively in patients and 21.1% in controls. Bacterial species such as *Prevotella phoceensis*, *Eubacterium infirmum* and *Slackia exigua* (culturomics), *Eggerthia catenaformis* and *Leptotrichia shahii* (metagenomics) were significantly associated to periodontitis cohort. In contrast, no significant relationship was noticed in the case of standard periodontopathogens like *Porphyromonas gingivalis*. Overall, the identification of non-standard bacteria called BGOIP (bacterial groups of interest in patients) and BGOIC (bacterial groups of interest in controls) by a new approach of 16S rRNA metagenomic analyse occurred in periodontitis, opens new opportunities in terms to predefine periodontitis microbiota.

1. Introduction

Periodontitis is a chronic multifactorial disease characterized by microbially-associated host mediated inflammation that results in progressive destruction of the supporting structures of the teeth (Papapanou et al., 2018). Several risk factors, like smoking status or diabetes, have been associated with more disease progression or less responsiveness to bacterial reduction therapies and are integrate into grading of periodontitis (Tonetti et al., 2018). The NAHNES 2009-2012 report estimated that 44,7% of adults aged 30 years and older in the United States had periodontitis with 8,9% having severe periodontitis (Eke et al., 2015). Its high and constantly increasing prevalence, combined with its impact on quality of life, oral and general health, makes periodontitis a major public health problem.

The oral cavity is normally colonized by a personalized set of microorganisms with 700 bacterial species, identified in the human mouth; more than 250 were isolated, cultivated and classified (Dewhirst et al., 2010). In recent years, a large amount of data about microbiota of periodontitis patients was accumulated by using metagenomic analysis with next-generation sequencing (Griffen et al., 2012; Liu et al., 2012; Tsai et al., 2016). Several microorganisms were frequently suspected as being associated to periodontitis (bacteria, protists, yeast, viruses, and archaea), like the red complex (*Pophyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*), *Prevotella* sp., *Trichomonas tenax*, *Entamoeba gingivalis*, *Candida albicans*, Herpes Simplex Virus-1, Epstein-Bar Virus and *Methanovibracter* sp.(Bisson et al., 2018; Bonner et al., 2014; Cappuyns et al., 2005; Lyons et al., 1983b; Wade, 2013). If metagenomic approach allowed the description of a vast panel of microbiota of periodontitis, a large part of microorganisms identified by molecular tools remained fastidious and uncultivable (Thompson et al., 2015). Culture-based technique named “culturomics” has transfigured the concept of fastidious microorganisms. This advanced tool boosts bacterial multiplication by using various culture conditions, where obtained colonies are rapidly identified by MALDI-

TOF and 16S rRNA (Lagier et al., 2016). The implication of this technique in clinical study allowed to study microbiota composition and to isolate new bacterial species (Lagier et al., 2016). The complementarity of culturomics and metagenomics have been already proved (Lagier et al., 2015). Currently, one of the major limitations of culturomics is its fastidiousness as in the time where several hundreds of samples can be analyzed by a researcher per year using metagenomics, only a few dozen will be using culturomics.

In this work, we conducted multidisciplinary evaluation of bacterial community on two cohorts of healthy and periodontitis subjects. Culturomics technique was used to isolate rare and non-detectable microorganisms by conventional methods. Molecular biology tools were employed to target previously described periodontopathogens, completing the procedure by metagenomics approach by identifying potential pathogens implicated in periodontitis.

2. Materials and Methods

2.1. Study population and clinical sample collection

The subject population was enrolled at the Odontolology Departement of La Timone Hospital, Marseille, France between November 2016 and April 2018. After a medical questionnaire and a periodontal examination, subjects were divided into two groups: the periodontitis group, in which the periodontitis has been classified on the basis of the severity and extent of clinical attachment loss (AL) and probing depth (PD) into mild, moderate and severe, and the healthy control group (Eke et al., 2012) (Table S1).

The mild periodontitis subjects were included when ≥2 interproximal sites with AL ≥3 mm, and ≥2 interproximal sites with PD ≥4 mm (not on same tooth) or one site with PD ≥5 mm. The moderate periodontitis subjects were included when ≥2 interproximal sites with AL ≥4 mm (not on same tooth), or ≥2 interproximal sites with PD ≥5 mm (not on same tooth). The severe periodontitis subjects were included when ≥2 interproximal sites with AL ≥6 mm (not on same tooth) and ≥1 interproximal site with PD ≥5 mm.

The healthy control subjects were included when no clinical attachment loss was detected and bleeding score on probing was < 10% with pocket depth < 3mm.

Non inclusion criteria for both groups were minors, orthodontic patients and pregnant or lactating women.

One tube per subject of subgingival dental plaque was collected by using a sterile Gracey curette (HuFriedy, Rotterdam, Netherlands): healthy gingival sulcus from healthy controls and most periodontal pockets from periodontitis subjects. Samples were transported into 5 mL of C-top medium (C-top, Ae-Ana, Eurobio, France) inside Hungate tubes containing microbeads allowing the dissolution of dental calculus. This work was approved by the clinical research ethics committee, IFR-48, Aix-Marseille University (protocol N° 2016-011). Written informed consent in accordance with the Declaration of Helsinki was obtained from all subjects.

2.2. Culturomics and identification

The fresh samples were first enriched in eight different conditions or directly seeded without enrichment. Anaerobic enrichment process was done on bottles of liquid anaerobic hemoculture; (1) 0.2 µm filtered fresh rumen, (2) 5% sheep blood, (3) rumen and 5% sheep blood, (4) 0.2 µm filtered saliva and (5) a last condition without enrichment treat. Aerobic enrichment was conducted on bottles of liquid aerobic hemoculture; (6) 0.2 µm filtered fresh rumen, (7) 5% sheep blood, (8) rumen and 5% sheep blood. Each enriched sample was serially diluted with 0.1 M PBS (Dulbecco's Phosphate-Buffered Saline, ThermoFisher Scientific, Paisley, USA) and inoculated on 5% Columbia sheep blood agar (bioMérieux, Marcy l'Etoile, France) in aerobic and anaerobic conditions. The culture and subculture media were incubated at 35°C, close to the average oral temperature (Sund-Levander et al., 2002). Bacterial growth was monitored on various days (1, 3, 7, 10, 15, 21 and 30). Isolated bacterial species were identified on MALDI-TOF/MS (Bruker Daltonics, Billerica, Mass., Germany), then the amplification and sequencing of 16S rRNA gene was used to confirm this identification, as

previously described (Lagier et al., 2015). A cut-off of 4 was used to select bacterial categories identified in high prevalence between controls and periodontitis subjects.

2.3. Direct molecular detection

Molecular analysis was performed directly on clinical samples. DNA was extracted using the EZ1 DNA Tissue Kit (Qiagen, Germany). Several of the most cited potential periodontopathogens were chosen from the literature and specifically detected: *Trichomonas tenax, Entamoeba gingivalis, Treponema* sp., *Porphyromonas gingivalis, Methanovibracter* sp. and TM7 bacteria. The qPCR was used for *T. tenax* detection as previously described (Benabdelkader et al., 2019). For the other pathogens, standard PCR was employed, and temperature cycle was respected for each couple of primers (Table S2) (Benabdelkader et al., 2019; Grine et al., 2018; Huo et al., 2017; Park et al., 2011; Sizova et al., 2015; Takenaka et al., 2018; Trim et al., 2011). PCR mix (45 µL) contained Roche mix (25 µL), primers forward/reverse (10 µM, 1.5 µL), water (17 µL) and DNA (5µL). PCR tubes were placed in a 2720 thermal cycler (Applied Biosystems®, United States). Amplified DNA was visualized on 2% agarose gel electrophoresis and purified using NucleoFast® plates (Macherey-Nagel, France). Sequencing process was performed using BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems®, USA) on 16 capillary sequencer 3130 XL (Applied Biosystems®, United-States). In brief, 2 µL of purified DNA were loaded out in a total of 10 µL mixture: bigdye (4 µL), primer forward or reverse (10 µM, 0.5 µL) and water (3.5 µL). Standard PCR were done using the following temperature cycle: one cycle of 96°C for 1 minute, 40 cycle (96°C for 10 seconds and 50°C for 5 seconds), followed by a final extension cycle at 60°C for 3 min. Sequences were corrected and assembled using ChromasPro software version 1.71 (Technelysium, Australia) and identified on "NCBI Blastn" (Basic Local Alignment Search Tool).

2.4. MiSeq 16S rRNA sequencing

The total DNA from each samples were extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Hoerdt, France) and eluted in 50 µL. Metagenomic DNA was amplified for the "V3-V4" of the 16S rRNA, pooled, barcoded, then sequenced on MiSeq technology using the paired-end strategy (Illumina, Inc, San Diego CA 92121, USA). In brief, 45 PCR cycles to amplify the "V3-V4" regions, using the Kapa HiFi Hotstart ReadyMix 2x (Kapa Biosystems Inc,Wilmington, MA U.S.A), and the surrounding conserved region V3_V4 primers with overhang adapters (FwOvAd_341F-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG ; RevOvAd_785R-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC After purification on AMPure beads (Beckman Coulter Inc, Fullerton,CA,USA), concentration was measured using High sensitivity Qubit technology (Beckman Coulter Inc, Fullerton, CA, USA) and diluted to 3.5 ng/µl. Dual-index barcodes were added to the amplicon. The global concentration was quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA). Before loading for sequencing on MiSeq (Illumina Inc, San Diego, CA, USA) the pool was diluted at 8pM. Automated cluster generation and paired-end sequencing with dual index reads was performed in a single 39-hours run in a 2x250bp. The paired reads were filtered according to the read qualities. The raw data were configured in Fastq files for R1 and R2 reads.

2.5.Metagenomic analysis

Metagenomics procedure is illustrated in the **Scheme 1.** Those sequences were submitted to European Bioinformatics Institute (EMBL-EBI, http://www.ebi.ac.uk/). The paired-end R1 and R2 sequences files were assembled with a quality score cut-off value of 33% and filtered by length using PEAR (included reads: 200 < reads < 1000 nucleotides) (Zhang et al., 2014). A quality filter with a cut-off value of 28% was applied on reads assembled by

GALAXY tool (Giardine et al., 2005). Chimeric sequences were discarded by UCHIME (Edgar et al., 2011). Reads were clustered in operational taxonomic unit (OTU) at 97% identity using UCLUST (Edgar, 2010). A local blastn was performed against 16S rRNA RefSeq Version 15.1 Human Oral Microbiome Database (HOMD, www.homd.org) using the standard parameters. Hits with < 40% of coverage and duplicates were also removed.

Beside detection of known species, to have an approach to detect non cultured bacteria / not yet described species associated with periodontitis and allowing the comparison of the OTU between periodontitis subjects and controls, we tried to develop an innovative algorithm for clustering. A selection by identity was applied on blast results: hits > 98.5% were classed in known species level. For OUT we used the following procedure: for hits <98.5% and > 95% sequences were classified in unknow species identified to genus level; for hits < 95%, sequences were classified in unknow species identified to family level. Hits of unknown species identified at the genus level was established for each individual and coded as *genus C* for controls (C1 to C10), *genus P* for patients (P1 to P10) followed by a number corresponding to a unique sequence or OTU. i.e. the first sequence of unknown species identified at the genus level for control 1 is named *genus C1.1* The same nomenclature was used for unknown species identified at the family level. Hits of unknown species identified at the genus level and hits of unknown species identified at the family level were then clustered in bacterial groups of interest using the ProteinOrtho software at default parameters in order to identify an association of the groups to the status of individuals (controls, perionditis subjects, as severity in periodontitis) (Lechner et al., 2011). Arbitrarily, the bacterial groups of interest in periodontitis subjects (BGOIP, those suspected of being causal) were defined as those found to be 4 more occurrences than controls. The bacterial groups of interest in controls (BGOIC, those suspected to be protective) are those found to be 6 more occurrences than patients. Those BGOIP are separated in 3 categories: bacterial species of interest in periodontitis (BSGOIP), bacterial genera group of interest in

periodontitis (BGGOIP) and bacterial family group of interest in periodontitis (BFGOIP). Similarly, BGOIC were separated in bacterial species of interest in controls (BSGOIC), bacterial genera group of interest in controls (BGGOIC) and bacterial family group of interest in controls (BFGOIC).

2.6.Statistical analysis

The comparisons were performed using the Chi^2 test and the Fisher exact test for categorical variables. A *p* value of < 0.05 was considered statistically significant. Statistical analyses were performed with the R statistical package (version 3.1.2, Vienna, Austria). The principal component analysis was performed with XLSTAT 2017 (Addinsoft, Paris, France).

3. Results

3.1. Clinical sample collection

In the present study, 12 females and 8 males were recruited: 4 females and 6 males with periodontitis, 8 females and 2 males in healthy controls. The average age of subjects with periodontitis was 56.2 years (41-64) and the average age of controls was 41.8 years (25-66). The cohort was classified into age categories: 4 subjects in the class [21-30] years, 1 subject in the class [31-40] years, 5 subjects in the class [41-50] years, 5 subjects in the class [51-60] years and 5 subjects in the class [+61] years. Subjects characteristics are listed in Table S1.

3.2. Culturomics analysis

A total of 23,616 colonies were spotted by culturomics approach: 165 bacterial species were identified (**S1 Figure**). Seventy-one species (43%) were shared by the two groups, 46 species (27.9%) were found only in periodontitis group and 48 species (29.1%) exclusively in control group. At the species level, *Bifidobacterium dentium* (4/10 periodontitis), *Eubacterium infirmum* (7/10 periodontitis, 1/10 controls), *Olsenella uli* (8/10 periodontitis, 4/10 controls), *Prevotella buccae* (8/10 periodontitis, 4/10 controls), *Prevotella phoceensis* (7/10 periodontitis, 2/10 controls), *Pyramidobacter piscolens* (4/10 periodontitis), *Slackia exigua* (7/10 periodontitis, 2/10 controls) and *Streptococcus anginosus* (9/10 periodontitis, 5/10 controls) are predominant in the periodontitis group and *Abiotrophia defectiva* (4/10 controls) in majority in the control group. At genus level, Bifidobacterium, Eggerthella, Eubacterium, Olsenella, Pyramidobacter and Slackia genera were predominantly presented in periodontal group unlike genera Actinomyces, Capnocytophaga and Lactobacillus higher in the control group. Moreover, a new bacterial species, *Corynebacterium dentalis* strain Marseille P4122 (16S rRNA gene accession number: LT897837) was isolated in the periodontitis group.

Difference lists in the microbiota composition between the two groups are grouped in **Table 1**. Analysis by genus level for each subject is described in **S2 Figure**. All strains have been

deposited in the bank collection of our laboratory: The Collection de Souches de l'Unité des Rickettsies (CSUR).

3.3. 16S rRNA metagenomics analysis

We consider hits with a percentage of identity greater than or equal to 98.5% of identity as species after the blastn results. Using the Miseq technology, 223 different species were identified. One hundred and sixty-nine (75.8%) were found in both groups, including 36 (16.1%) only in the control group and 18 (8.1%) exclusively in periodontitis group (**S3 Figure**). Species like *Lachnoanaerobaculum saburreum* (5/10 controls) and *Fusobacterium hwasookii* (5/10 controls) were found only in the control group. Species such as *Fusobacterium naviforme* (2/10 periodontitis, 7/10 controls), *Haemophilus parahaemolyticus* (1/10 periodontitis, 5/10 controls), *Lepotrichia hongkongensis* (3/10 periodontitis, 7/10 controls) *Prevotella loescheii* (2/10 periodontitis, 6/10 controls), *Prevotella nanceiensis* (2/10 periodontitis, 6/10 controls) and *Streptococcus gordonii* (1/10 periodontitis, 5/10 controls) were identified predominantly in control group. Species like *Eggerthia catenaformis* (6/10 periodontitis, 1/10 controls), *Lepotrichia shahii* (6/10 periodontitis, 1/10 controls), *Neisseria perflava* (5/10 periodontitis, 1/10 controls), *Neisseria subflava* (7/10 periodontitis, 3/10 controls), *Oribacterium assacharolyticum* (5/10 periodontitis, 1/10 controls), *Prevotella baroniae* (9/10 periodontitis, 5/10 controls), and *Prevotella dentalis* (9/10 periodontitis, 5/10 controls), were in majority identified in periodontitisgroup (**Table 1**). At genus level, metagenomic analyzes allowed us to detect 10 BGGOIP (7 exclusively present in periodontitis group) and 11 BGGOIC (4 exclusively present in control group). At family level, 10 BFGOIP (4 exclusively in periodontitis group) and 21 BFGOIC (11 exclusively in control group) were detected (**Table 1**).

3.4.Combined results (culturomics and metagenomics analyses)

Overall, 303 species were identified using culturomics and metagenomics techniques: 193 (63.7%) species were shared by the two groups: 64 (21.1%) were exclusively detected in control group, 46 (15.2%) only in periodontitis group. (**Figure S3**). The one hundred and ninety species shared by the both groups define a "core" common microbiome of subgingival plaque. Common species identified in equal proportions are listed in **Table 2**. Additionally, the two groups did not show any differences in the presence of specific periodontopathogens or potential periodontopahtogens such as *Porphyromonas gingivalis* (7/10 periodontitis, 8/10 controls), *Tannerella forsythia* (10/10 periodontitis, 9/10 controls), *Treponema denticola* (10/10 periodontitis, 8/10 controls) and *TM7* (10/10 periodontitis, 9/10 controls) ($P > 0.05$, Chi^2 test). The **Figure 1** illustrated combined results of bacterial species identified by culturomics and metagenomics.

3.5. Relative frequency distribution

By comparing the relative frequency, 14 bacteria identified in this study are significantly over-expressed in periodontitis subjects (**Figure 2A**). We find 3 species cultivated: *Prevotella phoceensis* ($p<0.001$), *Eubacterium infirmum* ($p<0.05$) and *Slackia exigua* ($p<0.05$) and 2 species identified by metagenomics: *Eggerthia catenaformis* ($p<0.05$) and *Leptotrichia shahii* ($p<0.05$). Also, 5 BGGOIP were found linked to periodontitis: Desulfovibrio sp. IHU Group 1 ($p<0.001$), Peptoniphilaceae gen. sp. IHU Group 2 ($p<0.001$), Fretibacterium sp. IHU Group 2 ($p<0.001$), Porphyromonas sp. IHU Group 1 ($p<0.05$) and Filifactor sp. IHU Group 2 ($p<0.05$). Finally, 4 BFGOIP were associated to periodontitis: Selenomodaceae gen. sp. IHU Group 1 ($p<0.0001$), Saccharibacteria gen. sp. IHU Group 1 ($p<0.001$), Synergistaceae gen. sp. IHU Group 1 ($p<0.05$) and Muribaculaceae gen. sp. IHU Group 1 ($p<0.05$). Furthermore, the BGGOIC Capnocytophaga sp. IHU Group 1 and the BFGOIC Neisseriaceae gen. sp. IHU Group 1, Fusobacteriaceae gen. sp. IHU Group 2, Flavobacteriaceae gen. sp. IHU Group 5 were

strongly associated to the healthy periodontium ($p<0.0001$). Twenty-nine other groups were also over-expressed in control groupand were listed in **Figure 2B.**

3.6.Principal component analysis

Principal component analysis (PCA) revealed a relationship between the periodontitis status and cultivated species *Slackia exigua, Streptococcus anginosus, Prevotella phoceensis* and: the *Prevotella dentalis* species identified species by NGS. Thus, correlation with the BGGOIC Porphyromonas sp. IHU Group 1, with the BFGOIC Leptotrichiaceae gen. sp. IHU Group 1, Saccharibacteria gen. sp. IHU Group 1 and Selenomonadaceae gen. sp. IHU Group 1 (**Figure 3**). The PCA performed between identified microorganisms and the disease's severity highlighted on specific bacteria associated with the periodontitis different stages. *Fretibacterium* sp. IHU Group 2, *Neisseria perflava*., Saccharibacteria gen. sp. IHU Group 1 and Veillonellaceae sp. IHU Group 1 were associated to the mild periodontitis. The cultivated species *Eubacterium infirmum, P. phoceensis, N. subflava* and Filifactor sp. IHU Group 2 and *Prevotella nanceiensis* from Illumina sequencing as linked with moderate periodontitis. Finally, several BGOIP and BGOIC are linked to the severe periodontitis: Leptotrichiaceae gen. sp. IHU Group 4 and Capnocytophaga sp. IHU Group 1 over-expressed in controls and Spirochaetacea gen. sp. IHU Group 1, Peptoniphilaceae gen sp. IHU Group 2, and Peptostreptococcaceae sp. IHU Group 1 and Selenomonas sp. IHU Group 1 over-expressed in periodontitis (**Figure 4**). The age class 41-50 and 51-60 were clustered with specific BGOIP such as Filifactor sp: IHU group 2, Fretibacterium sp. IHU Group 1, Spirochaetaceae gen. sp. IHU Group 1 and Peptoniphilaceae gen. sp. IHU Group 1, genera families from patient's cohort. The ages classes of 21-30 and 31-40 were associated with the healthy periodontal status and specific microorganisms in left side, **Figure 4**. No link between the sex of the patients and the healthy periodontal status was noticed.

3.7.Direct detection

To complete the microbial repertoire obtained in culture, specific bacterial species with a fastidious culture and protists possibly related to periodontitis were targeted by molecular assays (**Table 3**). No differences were detected between periodontitis subjects and controls in the carriage of those pathogens linked to periodontitis ($p > 0.05$, Chi^2 test).

4. Discussion

Our study was the first, to our knowledge, to describe the microbiome of healthy periodontium and periodontitis using a multidisciplinary approach of culturomics and metagenomics. We found that the composition of the subgingival microbiome differed according to the periodontal status and identified specific bacterial species, genera and families associated with healthy or periodontitis conditions. This study confirmed previous findings that some species are more commonly found in periodontitis, but the dual approach provided a very large-scale picture of the whole subgingival microbiome, thereby identifying new potential periodontopathogens species.

We found a total of 165 bacterial species, including 18.2% newly detected in oral microbiota, by culturomic approach and a total of 223 bacterial species using Illumina Miseq technology. Among them, 11 (*Actinomyces mediterranneense, Actinomyces timonensis, Bacillus sinesaloumensis, Brachymonas massiliensis, Collinsella massiliensis, Drancourtella massiliensis, Eggerthella timonensis, Jeddahella massiliensis, Marseillibacter massiliensis, Olsenella phoceensis, Olsenella timonensis*) were previously described as new bacterial species by our laboratory as part of the Culturomics Project (Lagier et al., 2015). A new bacterial species, *Corynebacterium dentalis* strain Marseille P4122 has also been isolated, but its implication in periodontal diseases needs to be established by epidemiologic and physiopathological investigations.

This study revealed a large range of bacterial species associated with periodontitis. Some of our results agree with previous studies that have explored the subgingival microbiome using High-Throughput Sequencing of the 16S rRNA Gene. At the genus level, we found that Filifactor, Porphyromonas and Peptonipholus were associated with periodontitis, that has been

previously described by Griffen et al. (2011). Several species and families from Firmicutes phylum like *Eubacterium infirmum, Eggerthia catenaformis,* Filifactor sp. group, Selenomonadaceae sp. group, or Synergistaceae sp. group, were found correlated to periodontitis in our study. This phylum was one of those found increased in case of periodontitis by Abuslem et al. (2013). It was also interesting to note that some of the species we identified predominantly in periodontitis like *Bifidobacterium dentium, Prevotella buccae* or *Streptococcus anginosus,* have been previously described as periodontitis-associated species in others studies which supports their link to the disease (Abusleme et al., 2013; Hong et al., 2015; Diaz et al., 2016).

Overall, the identification of non-standard bacteria called BGOIP (bacterial groups of interest in patients) and BGOIC (bacterial groups of interest in controls) by a new approach of 16S rRNA metagenomic analyse occurred in periodontitis, opens new opportunities in terms to predefine periodontitis microbiota. Previous study has estimated that more than half of the bacteria in the oral cavity are uncultivable (Paster et al., 2006). The metagenomics approach has allowed to identify some of them in this study like the BFGOIP Saccharibacteria gen. sp. (TM7) and the BGGOIP Fretibacterium sp. These uncultivable bacteria have been associated with gingivitis and chronic periodontitis (Brinig et al. 2003; Huang et al. 2016). In a recent study, Khemwong et al. (2019) used qPCR to show that levels of TM7 sp. HOT 356 and Fretibacterium sp. HOT 360 were higher in chronic periodontitis subjects than healthy controls although only the increased amount of Fretibacterium sp. HOT 360 was significantly correlated with percentage 4mm PD and bleeding on probing score. These clinical evidences have placed several TM7 taxa in the core microbiome associated with periodontitis (Abusleme et al., 2013). Similarly, several Fretibacterium taxa has been found as periodontitis-associated species (Kirst et al., 2015; Diaz et al. 2016). In our study, the 16S sequences of Saccharibacteria gen. sp. group

was identified exclusively in periodontitis samples and totally absent in controls, highlighting on a new potential link associated with the disease. Some authors suggested that they might have a role in the development of the microbiome community in health and disease by interaction with the core members of the microbiome, such as Actinomyces, and may be with specific periodontitis-associated species as well (Bor et al., 2019). To date, no causative relationship between these two uncultivated bacteria and periodontal diseases has been established and theirs characteristics and virulent factors are still unknown, which warrant further studies.

In this study, we also explored the existence of community types within periodontitis and found that the overexpressed bacteria differed according to periodontitis severity. In the mild stage, the identified bacteria might play a role in dysbiosis and periodontal tissue breakdown, leading to a completely different bacterial signature in severe stage. Among species associated with mild periodontitis, it was interesting to find Saccharibacteria gen. sp. group as some taxa of TM7 have been described as gingivitis associated species (Diaz et al., 2016). The chronological timeline has previously been explored by Hong et al. (2015) who proposed a model of temporal shift in the subgingival microbiome from health to periodontitis with gingivitis-associated and core species as mediators of transitions. They defined two periodontitis clusters: cluster A, enriched for species from the genera Campylobacter, Corynebacterium, Fusobacterium, Leptotrichia, Prevotella, Tannerella and TM7 species, and cluster B, associated with greater periodontitis severity and enriched with the red complex, *Filifactor alocis*, Treponema spp., Fretibacterium spp. and other species strongly associated with periodontitis. In our study, the chronological timeline leads to a decrease of bacterial diversity, highlighted by the combined techniques employed in this study.

Our study also focused on identifying species common to periodontal health and periodontitis conditions. These species constituted the "core species" of the subgingival microbiome. We identified 69 core species by culturomic method and 167 by methagenonic method. Some of them, like *Fusobacterium nucleatum, Campylobacter gracilis, Veillonella parvula, Pseudomonas pseudoalcaligenes* and *Prevottella nigrescens,* have already been identified in the core species of previous studies (Abusleme et al., 2013; Hong et al., 2015; Diaz et al., 2016). Their ability to thrive under both health and periodontitis nutritional and environmental conditions might be explained by a versatile metabolism and synergistic interactions with health and periodontitis associated species. Diaz et al. (2016) hypothesized that core species act as metabolic cornerstones for the whole microbial community and that they might play a role in the microbiome shifts from health to periodontitis. For example, *Fusobacterium nucleatum* has been described as the most abundant core species (Abusleme et al., 2013). This Gram-negative anaerobe seems to be capable to co-aggregate with a large range of oral species and to adapt to aerobic conditions by metabolizing oxygen via enzymatic activities such as that of NADH oxidase (Kolenbrander et al., 1989: Diaz et al., 2002).

The two groups presented no significant differences *in T. forsythia, P. gingivalis,* and *T. denticola* prevalence ($P > 0.05$, Chi2 test). These three members of the "red" complex have been widely investigated in numerous cross-sectional studies, with different age and ethnic groups, and have been closely associated to the presence of chronic periodontitis. For example, prevalence of *P. gingivalis* ranged from 29,6 to 97,5% in subgingival plaque of patients with chronic periodontitis (Hayashi et al., 2012; Moon et al., 2013; Puig-Silla et al., 2017). These bacteria were also found in subgingival plaque of periodontally healthy subjects although in a smaller percentage and seemed to increase the risk of an unfavourable evolution of the periodontal status (Puig-Silla et al., 2017). In 2013, Kato et al. found *P. gingivalis* in 40% of

the subgingival plaque samples from healthy Japanese adults. In 2017, Puig-Silla et al. used PCR to show that *P. gingivalis, T. forsythia and T. denticola* were present in 23.3%, 21,7% and 13,3% of healthy subjects respectively. These differences with our results might be explained by a smaller number of subjects in our study and should be explored in further studies that include a larger proportion of subjects.

Similarly, T. tenax was expressed in the same frequency between patients and healthy controls. Those microorganisms, evaluated by metagenomics and molecular microbiology. We previously inspected the relationship between *T. tenax* and periodontitis using culture-based and genomics techniques, where the mechanism of asymptomatic carriage was noticed by the identification of this protist in healthy controls. Furthermore, specific *T. tenax* clones were identified in patients and associated with periodontitis severity (Benabdelkader et al., 2019). A recent review highlights on the heterogeneity of populations and the low diversity of techniques used on *T. tenax* study (Marty et al., 2017).

The improvement of techniques used in the detection of bacterial species has opened a new way in the understanding and knowledge of the subgingival microbiome of periodontitis. However, caution should be exercised in interpreting the results of these studies, keeping in mind the “causal versus casual” concept. Indeed, finding a microorganism in higher levels in periodontitis than in health is not sufficient to determine whether it has initiated the pathological process or was favoured by the new nutritional and environmental conditions of the periodontal pockets. Recently, different theories have supported the issue of this concept by agreeing on the reciprocal interactions between the environment and the microbiome (Marsh, 2003; Hajishengallis et al., 2011; Hajishengallis and Lamont, 2012). One of these hypothesis has suggested that certain known periodontal pathogens, called “keystone pathogens”, have the capacity to modulate the host response in ways that impair immune surveillance, elevate the

virulence of the entire microbial community and thereby, mediate the shift from homeostatis to dysbiosis (Hajishengallis et al., 2011).

The culturomics technique is an efficient tool that enhance the oral microbiota repertoire. It was previously employed to characterize microbiota composition in several gastrointestinal disorders (i.e. obesity, colonic cancer, necrotizing enterocolitis, and Clostridium difficile infections), as well as skin diseases (i.e. psoriasis, atopic dermatitis and diabetic foot infections (Cassir et al., 2016; Gardiner et al., 2017; Hosny et al., 2017; Rosenthal et al., 2011). Culturomics and metagenomics are complementary approaches, by the important difference observed in the microbiota composition, when compared with results using each method. By combining both techniques, the identification of species missed by the other method was possible. Since, the putative periodontopathogen *P. phoceensis* was detected only by culturomics, while like *L. shahii* only detected by metagenomics. Culture-based method revealed the metagenomics bias, by identifying germs with "read-free" detection. This can be justified by the loss of genetic material during DNA extraction or sequencing procedure. Miseq sequencing technology depends on the quantity of initial DNA presented in the sample, for this reason, we chose to keep all good quality 16S rRNA reads. This protocol is enough for epidemiological study (presence/absence), instead of standard metagenomics studies that analyses in terms of reads number. An original microbiological vision was given by this analysis. Also, bacteria identified by genomic approach and owning a non-cultivable criterion, exhibited growth competition between species presented within gingival plaques of the periodontal pockets. New conditions would therefore be necessary to promote the growth of missed microorganisms and masked by predominant species whose growth is enriched by the selected growing conditions. The principal drawbacks of this study were the fastidious methodology of culturomics process, due to the implication of ten growing conditions for each

sample and the small number of patients (N = 20). A larger cohort is needed to increase the quantity of bacterial isolates validating our findings. As well as, antimicrobial susceptibility testing and virulence study were impossible due to the high quantity of spotted colonies (N = 23,616), specific strains with antibiotics resistance implicated in periodontal diseases were missed.

Culturomics associated with metagenomics has broadened the scope of subgingival microbiome analysis and this study might serve as the initial step for the identification of new periodontitis associated species. Our findings would be useful to guide future investigations on the role of these species in periodontal tissue breakdown.

5. Conclusion

The procedure of multidisciplinary approaches described in this manuscript focuses on the involvement of several microorganisms in the occurrence of periodontal diseases. The use of modern technologies, including culturomics coupled with metagenomics and molecular biology, has considerably revolutionized the understanding of aetiology related to the presence of specific microorganism. The complementary role of both culture and metagenomics was meaningful to achieve as much as possible the composition of oral microbiota in healthy and periodontitis groups. The key finding was first, the non-significant association of old periodontopathogens with the onset of periodontitis. Also, this study focuses on other bacterial species associated with the disease. These results may contribute to the future clinical applications, aiming to decipher the link between the potentially associated bacteria, in order to better understand their physiopathological mechanisms and to target treatments.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors contribution statement

BLS and GA designed the study. SB performed the experiments. SB and NC contributed to genomic analysis. SB and NC performed statistical analysis. GA and ET collected the samples. SB, GA and BLS analyzed the data. SB, AA, MH, GA and BLS contributed to manuscript redaction and correction.

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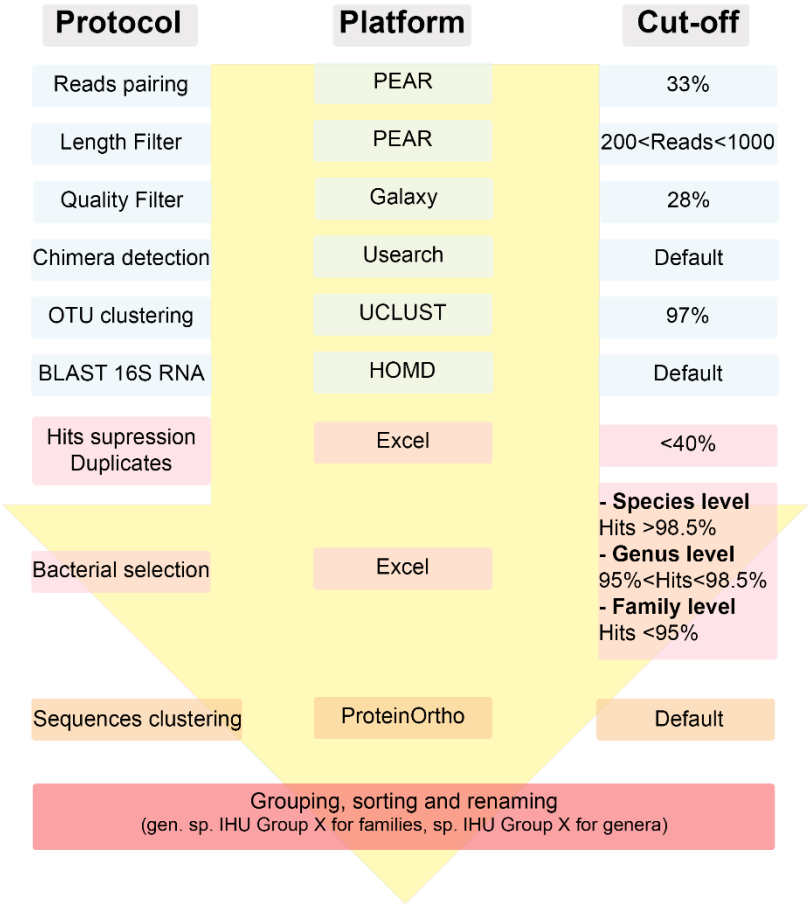
1825

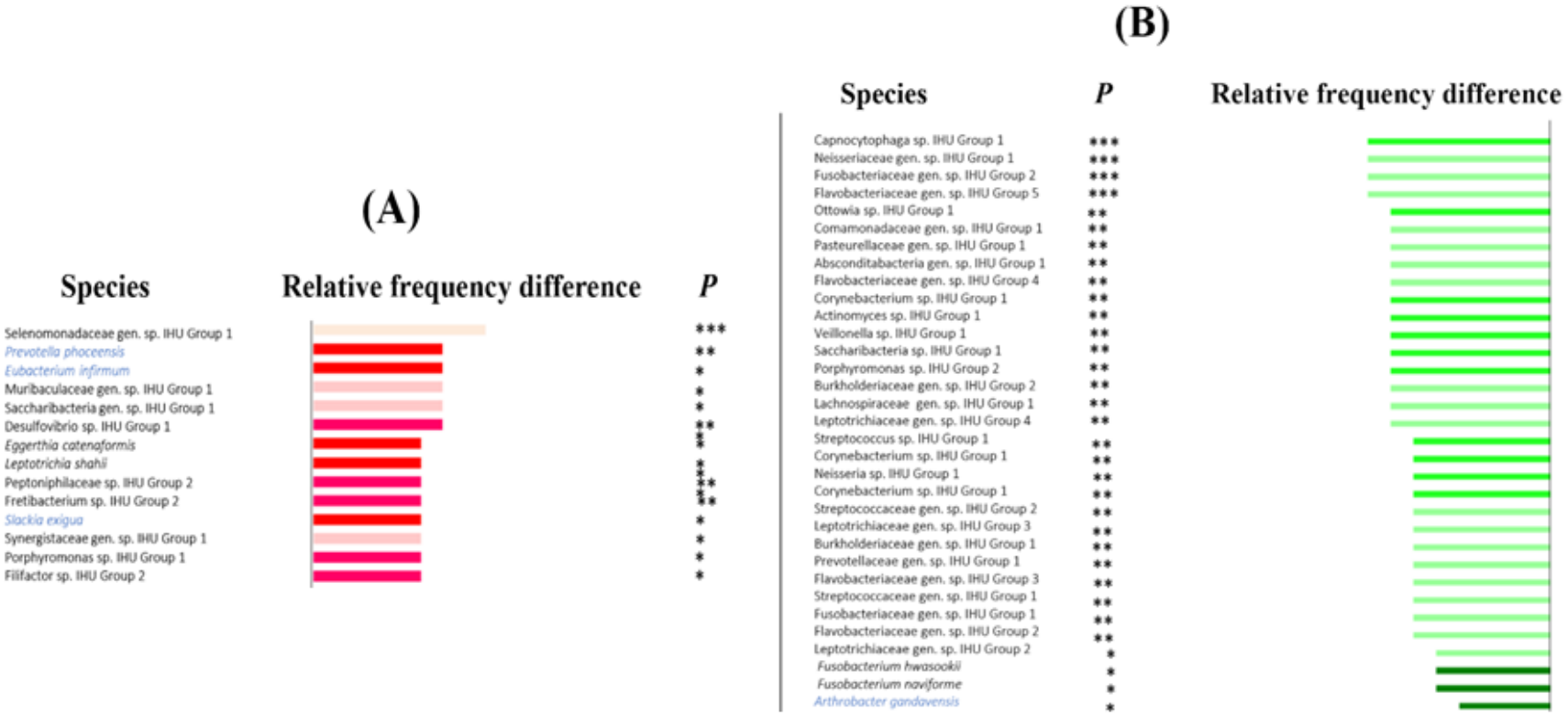


1826

1827 **Scheme 1**: Procedure of metagenomics data analysis

1828

Figure 1: Bacterial species isolated from the 20 samples. Green circles represent the control group, red circles the periodontitis group, orange circles species in common. Species tagged in blue were detected by culturomics, in black by metagenomics and in purple by both techniques.



1833

1834 **Figure 2:** The relative frequency of bacteria identified by culturomics (blue) and metagenomics (black) compared between patients with

1835 periodontitis (A) and controls (B). We used the Fisher exact test, as appropriate. Species with dark red or dark green, BGGOIP and BGGOIC with

1836 medium red or medium green, BFGOIP and BFGOIC in light red or light green. *p< 0.05; **p < 0.001; ***p < 0.0001. Only bacteria with

1837 significative difference are listed. Complete figure is in supplementary data (S5 Figure).

1838

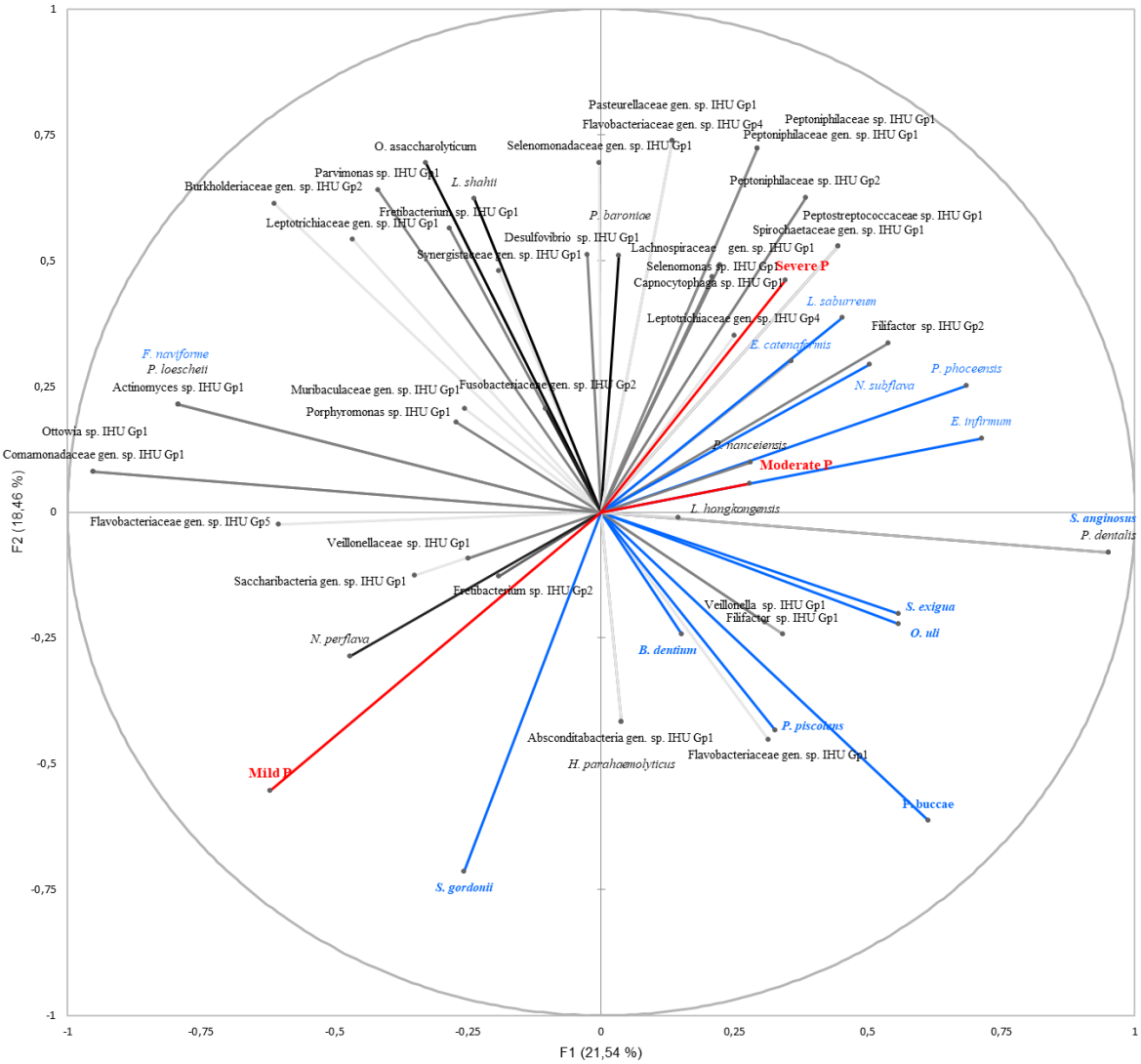
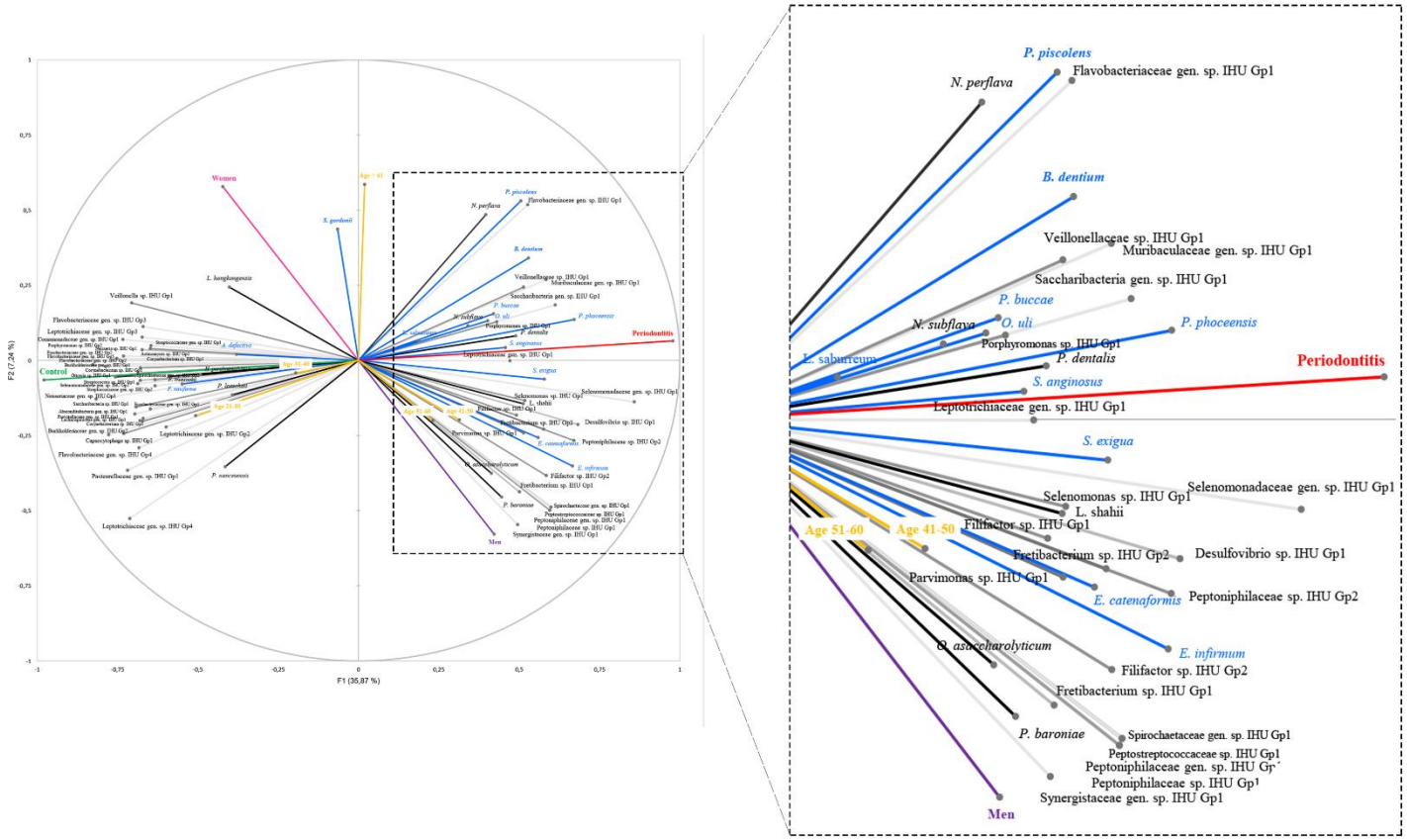


Figure 3: Principal component analysis using XLSTAT performed on species (blue from culturomics and black from metagenomics), BGGOIP and BGGOIC (dark grey), BFGOIP and BFGOIC (light grey) obtained by both techniques and subjects characteristics (sex, age, health status). The first (F1), and second (F2) components accounted for 35.87 and 7.24%, respectively, of the overall variability.



1846

1847 **Figure 4:** Principal component analysis using XLSTAT performed on species (blue from culturomics and black from metagenomics), BGGOIP

1848 and BGGOIC (dark grey), BFGOIP and BFGOIC (light grey) obtained by both techniques and the severity of periodontitis (mild, moderate, severe).

1849 The first (F1), and second (F2) components accounted for 21.54 and 18.46%, respectively, of the overall variability.

		Periodontitis		Controls
Culture	Species	*Bifidobacterium dentium* *Eubacterium infirmum* *Olsenella uli* *Prevotella buccae* *Prevotella phoceensis* *Pyramidobacter piscolens* *Slackia exigua* *Streptococcus anginosus*	Species	*Abiotrophia defectiva*
	Genera	Bifidobacterium Eggerthella Eubacterium Olsenella Pyramidobacter Slackia	Genera	Actinomyces Capnocytophaga Lactobacillus
Metagenomic	Species	*Eggerthia catenaformis* *Leptotrichia shahii* *Neisseria perflava* *Neisseria subflava* *Oribacterium asaccharolyticum* *Prevotella baroniae* *Prevotella dentalis*	Species	*Fusobacterium hwasookii* *Fusobacterium naviforme* *Lachnoanaerobaculum saburreum* *Haemophilus parahaemolyticus* *Leptotrichia hongkongensis* *Prevotella loescheii* *Prevotella nanceiensis* *Streptococcus gordonii*
	BGGOIP	Selenomonas sp. IHU Group 1 Filifactor sp. IHU Group 1 Fretibacterium sp. IHU Group 1 Veillonellaceae sp. IHU Group 1 Parvimonas sp. IHU Group 1 Peptoniphilus sp. IHU Group 1 Porphyromonas sp. IHU Group 1 Filifactor sp. IHU Group 2 Desulfovibrio sp. IHU Group 1 Fretibacterium sp. IHU Group 2	BGGOIC	Corynebacterium sp. IHU Group 1 Neisseria sp. IHU Group 1 Corynebacterium sp. IHU Group 1 Streptococcus sp. IHU Group 1 Porphyromonas sp. IHU Group 2 Saccharibacteria sp. IHU Group 1 Veillonella sp. IHU Group 1 Actinomyces sp. IHU Group 1 Ottowia sp. IHU Group 1 Corynebacterium sp. IHU Group 1 Capnocytophaga sp. IHU Group 1
	BFGOIP	Flavobacteriaceae gen. sp. IHU Group 1 Spirochaetaceae gen. sp. IHU Group 1 Leptotrichiaceae gen. sp. IHU Group 1 Peptoniphilaceae gen. sp. IHU Group 1 Synergistaceae gen. sp. IHU Group 1 Muribaculaceae gen. sp. IHU Group 1 Saccharibacteria gen. sp. IHU Group 1 Selenomonadaceae gen. sp. IHU Group 1 Peptoniphilaceae gen. sp. IHU Group 2	BFGOIC	Flavobacteriaceae gen. sp. IHU Group 2 Leptotrichiaceae gen. sp. IHU Group 2 Fusobacteriaceae gen. sp. IHU Group 1 Streptococcaceae gen. sp. IHU Group 1 Flavobacteriaceae gen. sp. IHU Group 3 Spirochaetaceae gen. sp. IHU Group 2 Selenomonadaceae gen. sp. IHU Group 2 Prevotellaceae gen. sp. IHU Group 1 Burkholderiaceae gen. sp. IHU Group 1

Peptostreptococcaceae gen. sp. IHU Group 1	Leptotrichiaceae gen. sp. IHU Group 3
	Streptococcaceae gen. sp. IHU Group 2
	Leptotrichiaceae gen. sp. IHU Group 4
	Lachnospiraceae gen. sp. IHU Group 1
	Burkholderiaceae gen. sp. IHU Group 2
	Flavobacteriaceae gen. sp. IHU Group 4
	Absconditabacteria gen. sp. IHU Group 1
	Pasteurellaceae gen. sp. IHU Group 1
	Comamonadaceae gen. sp. IHU Group 1
	Flavobacteriaceae gen. sp. IHU Group 5
	Fusobacteriaceae gen. sp. IHU Group 2
	Neisseriaceae gen. sp. IHU Group 1

Table 1. List of different bacteria found by culturomic and metagenomic methods. Bacteria are also classified by taxonomy: species, genera for culture and species, bacterial genera group of interest in periodontitis (BGGOIP) and bacterial family group of interest in periodontitis (BFGOIP), bacterial genera group of interest in controls (BGGOIC) and bacterial family group of interest in controls (BFGOIC) for 16S rRNA metagenomic analysis. Arbitrarily, species and the bacterial groups of interest in periodontitis subjects were defined as those found to be 4 more occurrences than controls. Species and bacterial groups of interest in controls found to be 6 more occurrences than periodontitis subjects.

		Common	
Culture	Species	*Actinobaculum naeslundi*	*Mogibacterium neglectum*
		Actinomyces georgiae	*Neisseria elongata*
		Actinomyces naeslundii	*Neisseria macacae*
		Aggregatibacter segnis	*Neisseria subflava*
		Anaeroglobus germinatus	*Porphyromonas gingivalis*
		Atopobium parvulum	*Prevotella nigrescens*
		Campylobacter concisus	*Pseudoramibacter alactolyticus*
		Campylobacter curvus	*Selenomonas infelix*
		Campylobacter showae	*Shuttleworthia satelles*
		Candida albicans	*Solobacterium moorei*
		Capnocytophaga ochracea	*Staphylococcus epidermidis*
		Capnocytophaga sputigena	*Streptococcus australis*
		Cryptobacterium curtum	*Streptococcus constellatus*
		Enterococcus durans	*Streptococcus cristatus*
		Fusobacterium nucleatum	*Streptococcus gordonii*
		Gemella morbillorum	*Streptococcus intermedius*
		Gemella sanguinis	*Streptococcus massiliensis*
		Kocuria rhizophila	*Streptococcus mitis*
		Lactobacillus rhamnosus	*Streptococcus mutans*
		Megasphaera micronuciformis	*Streptococcus oralis*
		Micrococcus luteus	
	Genera	Anaeroglobus	Rothia
		Cryptobacterium	Selenomonas
		Fusobacterium	Shuttleworthia
		Micrococcus	Solobacterium
		Pseudoramibacter	Staphylococcus
		Actinobaculum	Veillonella
		Aggregatibacter	Gemella
		Candida	Kocuria
		Enterococcus	Megasphaera
		Mogibacterium	Neisseria
		Porphyromonas	Streptococcus
Metagenomic	Species	*Actinomyces georgiae*	*Olsenella uli*
		Actinomyces johnsonii	*Prevotella buccae*
		Actinomyces massiliensis	*Prevotella multiformis*
		Actinomyces naeslundii	*Prevotella nigrescens*
		Actinomyces oris	*Prevotella oulorum*
		Aggregatibacter aphrophilus	*Prevotella shahii*
		Campylobacter curvus	*Rothia aeria*
		Cardiobacterium valvarum	*Selenomonas dianae*
		Dialister micraerophilus	*Selenomonas noxia*

Fusobacterium nucleatum
Granulicatella elegans
Haemophilus pittmaniae
Johnsonella ignava
Lawsonella clevelandensis
Leptotrichia hofstadii
Leptotrichia wadei
Megasphaera micronuciformis
Neisseria mucosa
Oribacterium sinus
Peptidiphaga gingivicola
Porphyromonas gingivalis
Prevotella micans
Prevotella oris
Prevotella pleuritidis
Prevotella saccharolytica
Prevotella veroralis
Pseudoramibacter alactolyticus
Rothia dentocariosa
Selenomonas infelix
Streptococcus intermedius
Streptococcus lactarius
Treponema vincentii
Veillonella atypica
Veillonella denticariosi
Veillonella parvula
Veillonella rogosae
Actinomyces dentalis
Actinomyces gerencseriae
Actinomyces lingnae
Actinomyces oricola
Aggregatibacter actinomycetemcomitans
Atopobium rimae
Campylobacter concisus
Capnocytophaga gingivalis
Capnocytophaga granulosa
Capnocytophaga leadbetteri
Cardiobacterium hominis
Centipeda periodontii
Haemophilus parainfluenzae
Kingella oralis
Lachnoanaerobaculum umeaense
Lautropia mirabilis
Shuttleworthia satelles
Slackia exigua
Stenotrophomonas maltophilia
Stomatobaculum longum
Streptococcus mutans
Streptococcus oralis
Streptococcus vestibularis
Veillonella dispar
Actinomyces odontolyticus
Alloprevotella rava
Alloprevotella tannerae
Bacteroides heparinolyticus
Campylobacter gracilis
Campylobacter showae
Corynebacterium matruchotii
Cutibacterium acnes
Delftia acidovorans
Dialister pneumosintes
Eikenella corrodens
Gemella morbillorum
Lactobacillus gasseri
Mycoplasma salivarium
Parvimonas micra
Porphyromonas endodontalis
Prevotella denticola
Prevotella enoeca
Prevotella fusca
Prevotella histicola
Prevotella marshii
Prevotella salivae
Pseudopropionibacterium propionicum
Rothia mucilaginosa
Scardovia wiggsiae
Selenomonas artemidis
Selenomonas sputigena
Sphingomonas echinoides
Streptococcus anginosus
Streptococcus constellatus
Streptococcus sanguinis
Tannerella forsythia
Treponema lecithinolyticum
Treponema maltophilum
Treponema medium

Mogibacterium neglectum	*Treponema socranskii*
Neisseria elongata	*Saccharibacteria (TM7) [G-1] bacterium*
Neisseria flavescens	

Table 2. List of common species found in equal proportions detected by culturomic and metagenomic methods.

	Patients (/10)	**Controls (/10)**
Methanovibracter sp.	4 (40%)	1 (10%)
TM7	10 (100%)	8 (80%)
Treponema sp.	7 (70%)	7 (70%)
Entamoeba gingivalis	9 (90%)	6 (60%)
Trichomonas tenax	3 (30%)	3 (30%)
Porphyromonas gingivalis	7 (70%)	6 (60%)

Table 3. Results of molecular biology targeted on specific periodontopathogens.

Partie 4

L’approche taxono-génomique dans la description des nouvelles espèces

<u>Publication 4</u>: *Corynebacterium dentalis* sp. nov., a new bacterium isolated from dental plaque of a woman suffering of periodontitis.

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(Accepted in NMNI Journal)

Avant-propos

La taxono-génomique combine la description phénotypique avec l'analyse protéique par MALDI-TOF ainsi l'analyse génomique comparative dans la taxonomie bactérienne dans la description de nouvelles espèces bactériennes isolées. Cette approche est utilisée au sein de notre laboratoire dans de nombreux travaux d'études du microbiote après découverte de nouvelles espèces.

Ce travail décrit et classifie une nouvelle espèce bactérienne isolée à partir de la culturomique réalisée chez un patient de parodontite (Publication 3). Des colonies blanches transparentes ont été cultivées après 24h d'incubation à 37°C dans des conditions aérobies. Le séquençage du gène ARNr 16S et du gène *rpob* ont été effectué après l'échec d'identification par MALDI-TOF MS. Un pourcentage de similarité inférieur à 98.19% avec *Corynebacterium suicordis*, espèce la plus proche phylogénétiquement, confirme le caractère de nouvelle espèce, appartenant à l'ordre des *Corynebacteriales* nommée *Corynebacterium dentalis*.

Les autres espèces bactériennes décrites se trouvent en annexe.

***Corynebacterium dentalis* sp. nov., a new bacterium isolated from dental plaque of a woman suffering of periodontitis**.

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Running title: *Corynebacterium dentalis* sp. nov.

Keywords: Culturomics; taxono-genomics; *Corynebacterium dentalis*; dental plaque.

Abstract:

Strain Marseille-P4122^{T} is a new species from the order *Corynebacteriales* that was isolated from a dental plaque of woman suffering of periodontitis. It is a facultative anaerobic gram-positive rod-shaped bacterium. Strain Marseille-P4122^{T} exhibited a 98.19 % sequence identity with *Corynebacterium suicordis* strain P81/02, the phylogenetically closely related species with standing in nomenclature. The draft genome size of strain Marseille-P4122^{T} is 2.49 Mb with 60.1% of G+C content. We propose that strain Marseille-P4122^{T} (=CSURP4122) is the type strain of the new species *Corynebacterium dentalis* sp. nov.

Introduction

Corynebacterium genus belonging to family *Corynebacteriaceae* was described firstly in 1896 by Lehmann and Neumann [1]. It consists of Gram-positive rods and non-spore forming bacteria with a high DNA G+C content [2]. Several species of this genus are implicated in human and animal diseases while others are members of normal flora on skin and mucous membranes [3-5]. *Corynebacterium diphtheriae* is the major pathogen in human and causes diphtheria worldwide [6]. It is a large genus that regroups currently 132 species with 11 subspecies validly described with standing in nomenclature [7].

Currently it is important to understand the implication of bacterial diversity in normal physiological functions and to the disease [8]. Culturomics is a concept developing different culture conditions in order to enlarge our knowledge of the human microbiota through the discovery of previously uncultured bacteria [9-12]. Once bacterium was isolated, we used a taxono-genomics approach including matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), phylogenetic analysis, main phenotypic description and genome sequencing, to describe it [13-14].

Here we describe *Corynebacterium dentalis* sp. nov., strain Marseille-P4122^{T} (= CSUR P4122), according this taxono-genomics concept.

Isolation and growth conditions

In 2015, we isolated from a dental plaque sample of a woman suffering of periodontitis an unidentified bacterial strain. A screening has been made by MALDI-TOF MS on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [15]. The obtained spectra (Figure 1) were imported into MALDI Biotyper 3.0 software (Bruker Daltonics) and analyzed against the main spectra of the bacteria included in two databases

(Bruker and constantly updated MEPHI databases). The study was validated by the ethics committee of "Institut Fédératif de Recherche IFR48" under number 2016-010. Strain Marseille-P4122^{T} was first isolated in aerobic conditions after the incubation in Culture bottle (bioMérieux, Marcy l'Etoile, France) supplemented with 5mL sheep blood at 37°C medium.

Phenotypic characteristics

After isolation step, the strain Marseille-P4122^{T} was cultured aiming to get pure and isolated colonies on blood agar. The colonies were white and transparent. Bacterial cells were Gram-positive. The sporulation test (10 minutes at 80°C) is negative. Different growth temperatures (20, 28, 32, 37, 45 and 56°C), pH (5, 6, 7, 7.5, 8 and 8.5), NaCl content (5, 10 and 15g) and atmospheres (aerobic, anaerobic and microaerophilic (CampyGEN, Oxoid).) were tested on 5% sheep blood enriched Columbia Agar. Strain Marseille-P4122 ^{T} is as a very-easy-to-cultivate bacterium and grows in all these conditions except at 56°C. API ZYM and API Coryne tests (BioMérieux) were performed in order to determinate specific phenotypic features for strain Marseille-P4122. The results were tabulated in Table 1. Using API 50CH strips (BioMérieux) the carbohydrate metabolism of strain Marseille-P4122 is evaluated according to the manufacturer's instructions (Table 2). Thus, strain Marseille-P4122^{T} has enzymatic activities such as esterase (C4), esterase-lipase (C8), lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, and urease, while only D-fructose and D-trehalose were positive for carbohydrate metabolism. All the other reactions tested were negative. Strain Marseille-P4122^{T} showed catalase-negative and oxidase-negative activities. A comparative study of the biochemical characteristics of this strain with other closely related *Corynebacterium* species is presented in Table 3. For scanning electronic microscopy, a colony was collected from agar and immersed into a 2.5 % glutaraldehyde fixative solution. The slide was gently washed in water, air dried and

examined with a TM4000 microscope. The cells appear to be rod-shaped with a mean length of 1µm and a mean diameter of 0.5µm (Figure 2). Antimicrobial susceptibility testing was done using the E-test strips (BioMérieux) method and obtained data were summarized in Table 4. The major fatty acid found for this strain by far, were Hexadecanoic acid (44 %) and 9-Octadecenoic acid (36 %). Very few other structures were described. No branched fatty acids were detected (Table 5).

Strain identification

The 16S rRNA gene was sequenced in order to classify this bacterium. Amplification was done by using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequencing using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL Genetic Analyzer capillary3500xL sequencer (Thermofisher, Saint-Aubin, France), as previously described [16]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-P4122^{T} exhibited a 98.19 % sequence identity with *Corynebacterium suicordis* strain P81/02 (Genbank accession number NR042151.1), the phylogenetically closest species with standing in nomenclature (Figure 3A). The *rpoB* gene that encodes the β subunit of bacterial RNA polymerase was targeted to discriminate the *Corynebacterium* species [17]. It is shown that *Corynebacterium dentalis* strain Marseille-P4122^{T} is close with strain *Corynebacterium auriscanis* and *Corynebacterium resistens* (Figure 3B). Considering these phylogenetic criteria, we consequently classify this strain as a member of a new species within the genus *Corynebacterium*, family *Corynebacteriaceae*, phylum *Actinobacteria*.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue Kit and then sequenced on the MiSeq technology (Illumina, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired end (Illumina), as previously described [18]. The assembly was performed with a pipeline incorporating different softwares (Velvet [19], Spades [20] and Soap Denovo [21]), and trimmed (MiSeq and Trimmomatic [22] softwares) or untrimmed data (only MiSeq software). GapCloser was used to reduce assembly gaps. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value lower than 25% of the mean depth were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). The genome of *Corynebacterium dentalis* strain Marseille-P4122^{T} is 2,303,041 bp long with a 60.1% G+C content. The degree of genomic similarity of strain Marseille-P4122^{T} with closely related species was estimated using the OrthoANI software [23]. Values among closely related species (Figure 4) ranged from 75.33% between *Corynebacterium gluyciniphilum* and *Corynebacterium terpenotabidum* to 78.14% between *Corynebacterium auriscanis* and *Corynebacterium resistens*. When the isolate was compared to these closely species, values ranged from 67.54 % with *Corynebacterium vitaeruminis* and *Corynebacterium jeikeium* to 78.14 % with *Corynebacterium auriscanis*.

Conclusion

Basing on the results from unique phenotypic characteristics, including API galleries tests, MALDI-TOF spectrum, and phylogenetic and genomic analysis such as 16S rRNA sequence similarity lower than 98.7% and OrthoANI value lower than 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P4122^{T} as the type strain of *Corynebacterium dentalis* sp. nov.

Description of Corynebacterium dentalis sp. nov.

Corynebacterium dentalis (den.ta′lis. N.L. masc. adj. *dentalis* referring to the teeth surrounded by dental plaque from which this strain was isolated). The strain grows easily in varied conditions. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia Agar during less 24 hours. They appear white and transparent. *C. dentalis* is Gram-positive rod-shaped bacterium with a mean length of 1µm and a mean diameter of 0.5µm. Strain Marseille-P4122^{T} produced esterase, lipase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, urease, D-fructose and D-trehalose. But any activities was observed with trypsin, β-galactosidase, α-glucosidase, glycerol, D-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-lactose, D-saccharose, glycogen, D-fucose and D-arabitol. Strain Marseille-P4122^{T} is catalase-negative. It is susceptible to rifampicin, ciprofloxacin, amoxicillin, penicillin G, doxycycline and vancomycin but resistant to erythromycin. The genome size of *Corynebacterium dentalis* strain Marseille-P4122^{T} is about 4.04 Mb long with 60.1 mol% G+C content. The Genbank Accession number for the 16S rRNA gene sequence of strain Marseille-P4122^{T} is LT897837 and for the whole genome shotgun project is OCTS00000000.This strain was isolated from dental plaque of a woman suffering of periodontitis.

Nucleotide sequence accession number. The 16S rRNA gene and genome sequences were deposited in Genbank under accession number LT897837 and OCTS00000000, respectively.

Deposit in culture collections. Strain Marseille-P4122^{T} was deposited in our strain collections under number (= CSURP4122).

Conflict of interest

None to declare

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Table 1: Phenotypic characterization of *Corynebacterium dentalis* sp. nov., based on analytical profile index (API) ZYM and CORYNE tests.

Tests	Characteristics	Results
API ZYM	Alkaline phosphatase	-
	Esterase (C4)	+
	Esterase Lipase (C8)	+
	Lipase (C14)	+
	Leucine arylamidase	-
	Valine arylamidase	+
	Cystine arylamidase	-
	Trypsin	-
	α-chymotrypsin	-
	Acid phosphatase	-
	Naphthol-AS-BI-phosphohydrolase	+
	α-galactosidase	+
	β-galactosidase	-
	β-glucuronidase	-
	α-glucosidase	-
	β-glucosidase	-
	N-acetyl-β-glucosaminidase	-
	α-mannosidase	-
	α-fucosidase	-
	Glycerol	-
API CORYNE	Nitrate reductase	-
	Pyrazinamidase	-
	Pyrrolidonyl Arylamidase	-
	Phosphatase Alcaline	+
	β-glucuronidase	-
	β-galactosidase	-
	α-glucosidase	-
	N-acetyl-β-glucosaminidase	-
	β-glucosidase	-
	Urease	+
	Gelatin	-
	Control	-
	D-glucose	-
	D-ribose	-
	D-xylose	+
	D-mannitol	-
	D-maltose	-
	D-lactose	+
	D-saccharose	-

	Glycogen	+

Table 2: Phenotypic characterization of *Corynebacterium dentalis* sp. nov., based on API 50 CH test.

Tests	Characteristics	Results
50 CH	Erythritol	-
	D-arabinose	-
	L-arabinose	-
	D-ribose	-
	D-xylose	-
	L-xylose	-
	D-Adonitol	-
	Methyl βD-xylopyranoside	-
	D-galactose	-
	D-glucose	-
	D-fructose	+
	D-mannose	-
	L-sorbose	-
	L-rhamnose	+
	Dulcitol	-
	Inositol	-
	D-mannitol	-
	D-sorbitol	-
	Methyl αD-mannopyranoside	-
	Methyl αD-glucopyranoside	-
	N-acetyl-glucosamine	-
	Amygdalin	-
	Arbutin	-
	Esculin ferric citrate	-
	Salicin	-
	D-cellobiose	-
	D-maltose	-
	D-lactose	-
	D-melibiose	-
	D-saccharose	-
	D-trehalose	+
	Inulin	-
	D-melezitose	-
	D-raffinose	-
	Amidon	-
	Glycogen	-
	Xylitol	-
	Gentiobiose	-
	D-turanose	-
	D-xylose	-
	D-tagalose	-
	D-fucose	-
	L-fucose	-
	D-arabitol	-
	L-arabitol	-
	Potassium gluconate	-
	Potassium 2-ketogluconate	-

	Potassium 5-ketogluconate -

Table 3: Comparison of differential characteristics between *Corynebacterium dentalis* sp. nov., and other bacterial species like *Corynebacterium resistens*, *Corynebacterium suicordis*, *Corynebacterium urinapleomorphum*, and *Corynebacterium phoceense.*

Property	*C. dentalis*	*C. resistens*	*C. suicordis*	*C. urinapleomorphum*	*C. phoceense*
Cell diameter (µm)	0.5	NA	NA	0.2	0.5
Oxygen requirement	+	±	±	+	+
Gram stain	+	+	+	+	+
Salt requirement	-	-	-	−	−
Motility	-	-	-	−	−
Endospore formation	-	-	-	−	+
Alkaline phosphatase	-	+	+	+	+
Catalase	-	+	+	+	+
Oxidase	-	-	-	−	−
Nitrate reductase	-	-	-	NA	+
Urease	+	-	+	+	−
β-Galactosidase	-	-	-	−	−
N-acetyl-glucosamine	-	-	-	−	−
Arabinose	-	-	-	−	NA
lipase (C8)	+	+	+	+	+
Pyrrolidonyl arylamidase	-	+	+	−	+
Mannose	-	-	-	−	+
Mannitol	-	-	-	−	−
Sucrose	NA	-	-	NA	−
D-Glucose	-	+	-	−	+
D-Fructose	+	-	-	−	+
D-Maltose	-	-	-	−	+
Source	Human	Human	Pig	Human	Human

Table 4: Sensitivity test to certain antibiotics on the strain Marseille-P4122^{T}

Antibiotics used	MIC (mm)	References values	Interpretations
Rifampicin	0.003	≤0.06 - >0.5	Susceptible
Ciprofloxacin	0.064	<0.06 - >0.5	Susceptible
Daptomycin	0.094	<0.25 - >0.5	Susceptible
Amoxicillin	0.125	≤0.25 - ≥1	Susceptible
Penicillin G	0.19	<0.06 - >0.5	Susceptible
Doxycycline	0.38	≤0.12 - ≥0.5	Susceptible
Vancomycin	0.38	≤2 - >2	Susceptible
Erythromycin	16	≤0.5 - ≥8	Resistant
Imipenem	0.023	≤2 - ≥8	Susceptible
Amikacin	0.5	≤4 - ≥16	Susceptible

Table 5: Fatty acid profiles (%) of *Corynebacterium dentalis* strain Marseille-P4122^{T}.

Fatty acids	Name	Mean relative %*
16:00	Hexadecanoic acid	44.2 ± 1.5
18:1n9	9-Octadecenoic acid	35.6 ± 1.0
18:00	Octadecanoic acid	9.3 ± 0.5
18:2n6	9,12-Octadecadienoic acid	5.9 ± 0.3
17:00	Heptadecanoic acid	4.5 ± 0,3
14:00	Tetradecanoic acid	TR

*****Mean peak area percentage; TR = trace amounts < 1%

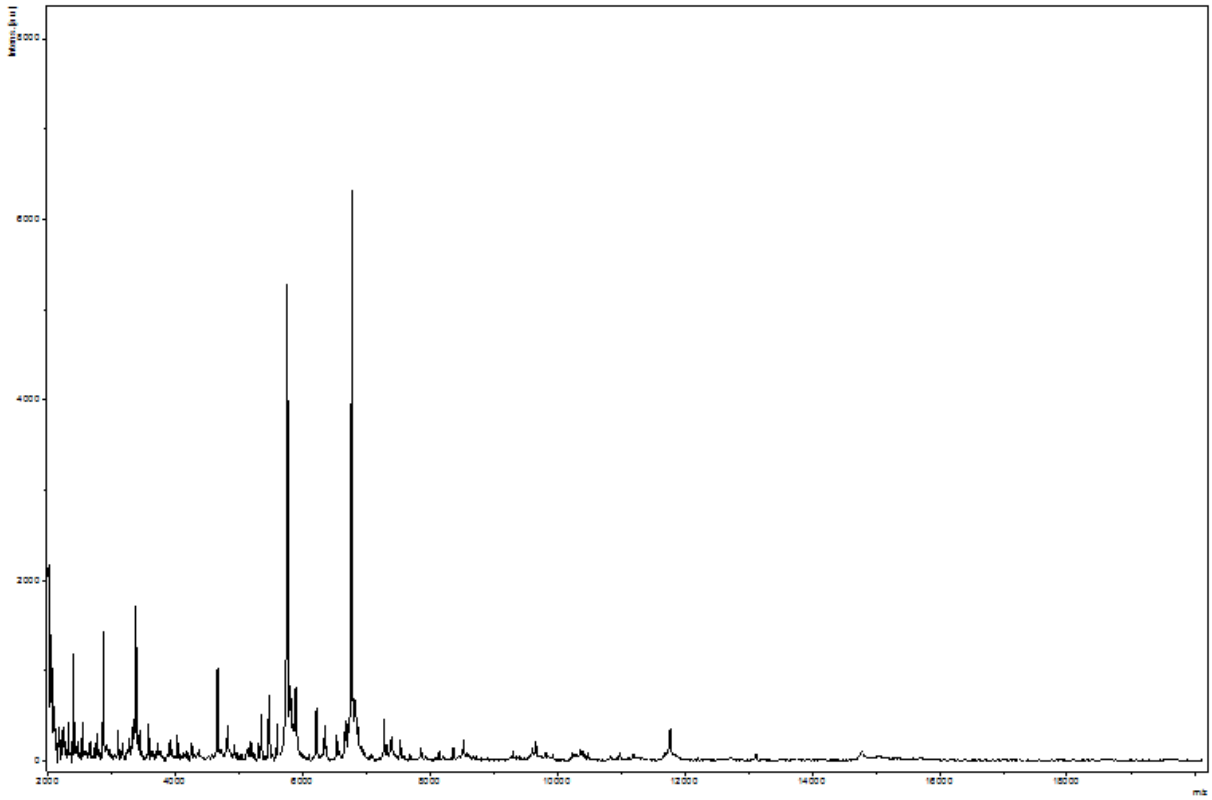


Figure 1: MALDI-TOF MS Reference mass spectrum of *Corynebacterium dentalis* sp. nov., strain Marseille-P4122^{T}. The reference spectrum was generated by comparison of spectra from 12 individual colonies.

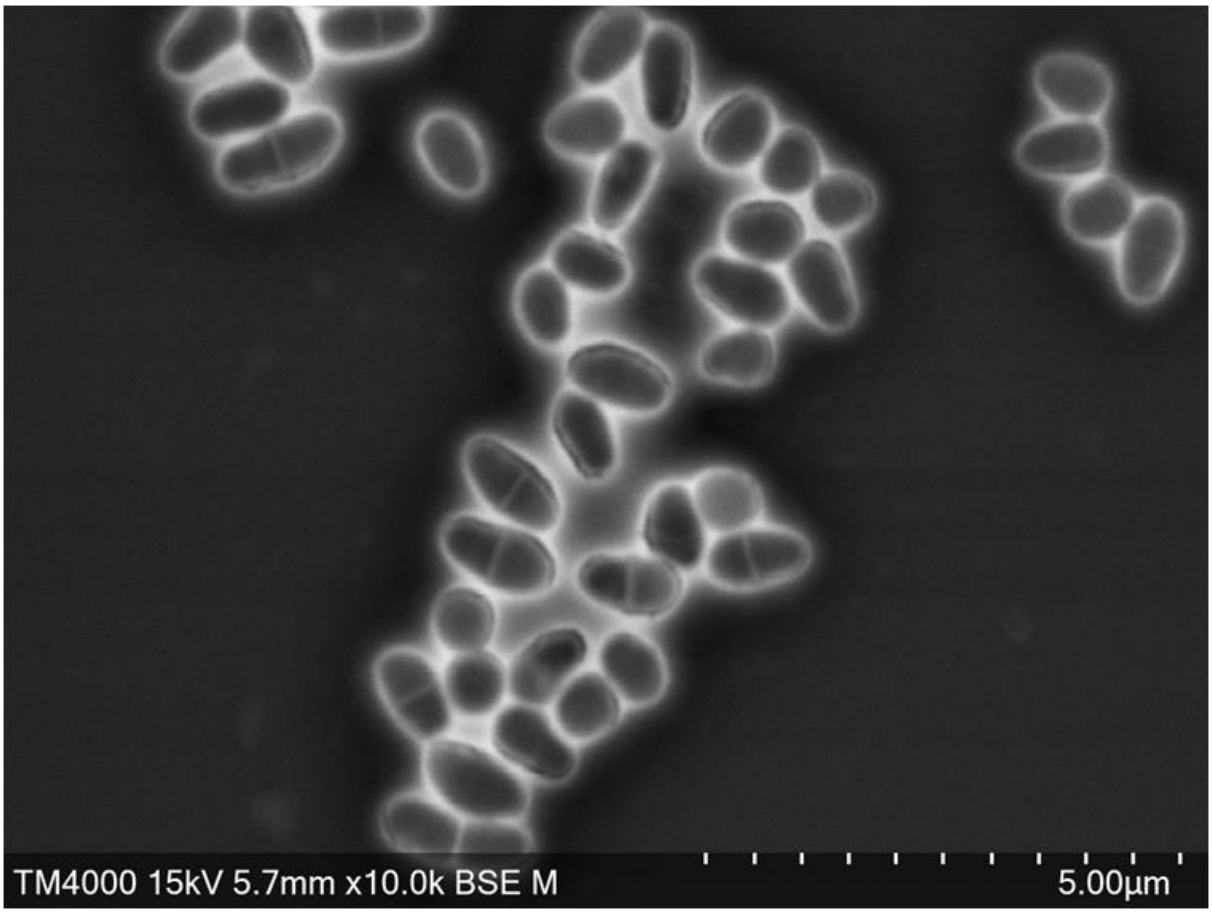
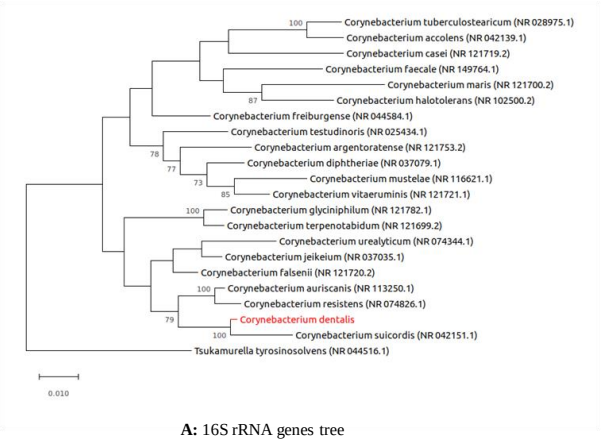
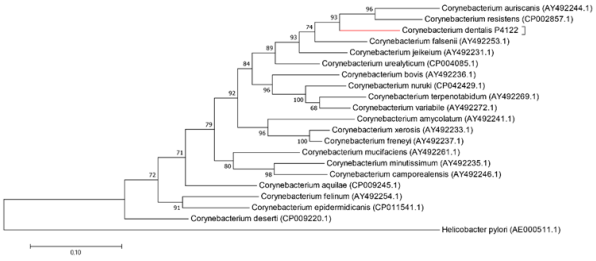


Figure 2: Scanning electron microscopy of stained *Corynebacterium dentalis* sp. nov., (Hitachi TM4000). Scales and acquisition settings are shown on figure.



A: 16S rRNA genes tree



B: *rpoB* rRNA genes tree

Figure 3: Phylogenetic trees highlighting the position of *Corynebacterium dentalis* sp. nov., based on the 16S rRNA gene sequences (**A**) and the *rpoB* gene sequences (**B**) relative to the most closely related type strains within the genus *Corynebacterium*. Genbank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference were obtained using the Maximum likelihood method and the MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 1% nucleotide sequence divergence.

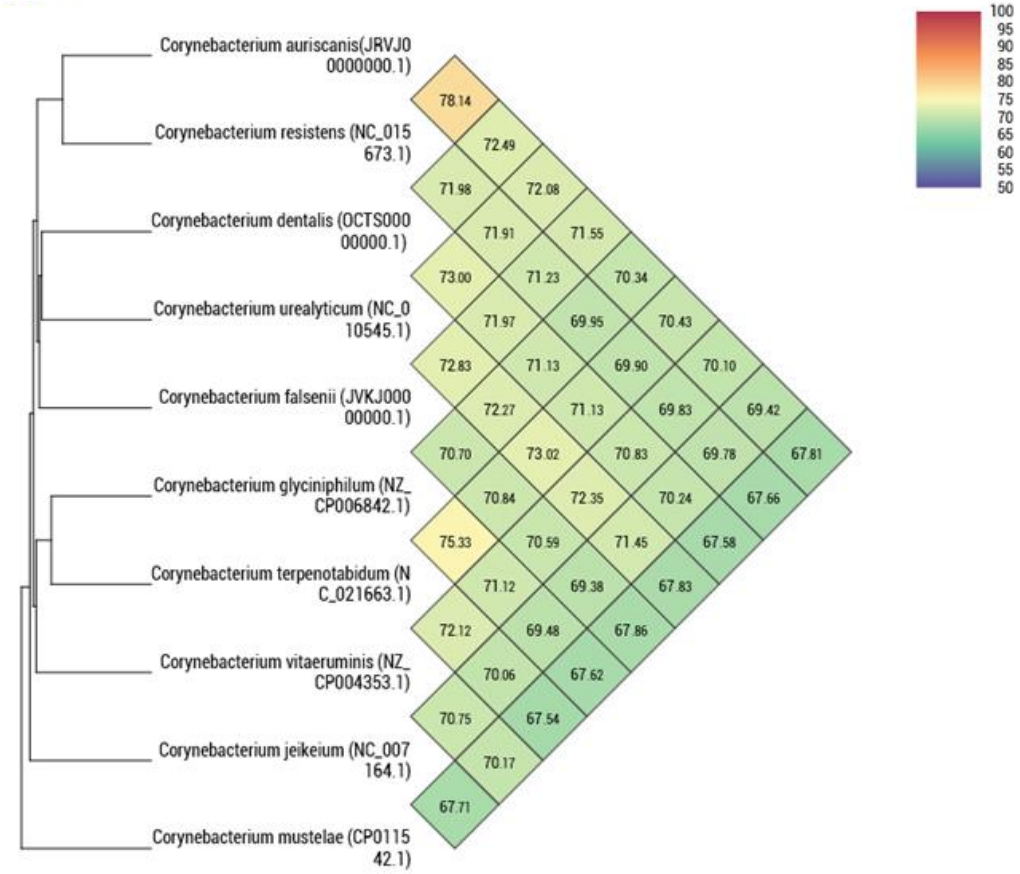


Figure 4: Heatmap generated with OrthoANI values calculated using the OAT software between *Corynebacterium dentalis* sp. nov., and other closely related species with standing in nomenclature.

Conclusion

L'ensemble de travaux décrits de ce manuscrit porte sur l'étude microbiologique de la parodontite se définissant par une dysbiose, modification de la composition bactérienne. L'identification de l'étiologie liée à la présence de pathogènes dans la plaque reste donc nécessaire pour comprendre l'apparition de maladies parodontales. La perturbation de l'écosystème a pu être décrite en combinant différentes technologies telles que des nouvelles méthodes de culture, couplées à de nouveaux outils d'identification moléculaire et accompagnés par d'analyse génomique complémentaire. L'étude de la dysbiose se divise en trois grands axes : l'étude de la relation entre *Trichomonas tenax* et la parodontite ; l'étude multidisciplinaire du microbiote dans la parodontite ; et enfin la taxonomie bactérienne des nouvelles espèces.

Dans un premier temps, la prévalence de *Trichomonas tenax* dans la parodontite a été étudiée par une approche qui associe la culture et la qPCR en temps réel. La revue bibliographie a révélé *T. tenax* comme un potentiel acteur de la parodontite. De nouveaux outils d'identification moléculaire fiables et un protocole de culture efficace ont pu être mis en place pour l'étude des deux cohortes. Par culture, une différence significative a été observée entre les patients et les contrôles à l'inverse de la qPCR qui n'a révélé aucune différence, outil cependant plus sensible à la détection du protiste. En combinant la culture et la biologie moléculaire, nous avons confirmé une prévalence significative du *T. tenax* chez les patients par rapport aux contrôles et sa présence a été également associée à la sévérité de la parodontite. L'analyse du génotypage des 53 isolats de culture avec des clusters significativement constituées de souches issues de patients suggère la présence de clones spécifiques liée à la maladie. Ainsi, la combinaison de plusieurs techniques a suggéré une prévalence élevée de *T. tenax* à la fois chez

les contrôles et les malades, significativement plus élevée dans les stades sévères de la parodontite. Le typage a démontré l'existence de clones spécifiques, ce qui expliquerait ainsi le portage asymptomatique chez les personnes saines n'induisant pas de parodontite. Même si *T. tenax* semble être associée à l'apparition et/ou au développement de maladies parodontales, le rôle physiopathologique des clones virulents de *T. tenax* doit être exploré dans des études supplémentaires.

Dans un second temps, l'approche multidisciplinaire combinant la culturomique, la métagénomique et la biologie moléculaire décrit l'ensemble des microorganismes du microbiote dans la maladie parodontale. Cette étude a été comparé par rapport à un microbiote sain pour identifier de nouveaux acteurs éventuellement impliqués dans le déséquilibre de l'écosystème de la plaque. La complémentarité de la culture et de la génomique était significative pour obtenir une description exhaustive du profil microbien des deux cohortes étudiées. Au total, 308 espèces bactériennes ont été décrites. D'une part, cette étude s'appuie sur une analyse différente des résultats obtenus par métagénomique révèle de nouvelles espèces, genres et familles potentiellement associées à la maladie. D'autre part, certains micro-organismes identifiés lors du travail bibliographique de la revue comme étant impliqués dans le développement de la maladie, à l'instar du complexe rouge ont été analysé par génomique ainsi que par détection directe en biologie moléculaire. Ces techniques ont démontré l'association non significative de ces parodontopathogènes avec l'apparition d'une parodontite. Ce travail ouvre ainsi de nouvelles perspectives en termes de prédéfinition du microbiote et d'études complémentaire sur la participation en association de plusieurs microorganismes à la survenue de maladies parodontales.

Dans un dernier temps, les bactéries isolées par techniques de culturomique dans différents projets de description de microbiote lors de dysbioses ont été étudiées par l'approche taxono-génomique. Cette stratégie récente utilisée depuis 2014 a permis à la fois de classifier

les nouvelles espèces par la taxonomie et de les comparer avec des espèces proches par la génomique, mais aussi par les caractéristiques phénotypiques couplées à l'analyse protéique.

En conclusion, les nouveaux outils d'analyse utilisés au cours de cette ont permis d'identifier de nouveaux acteurs potentiellement impliqués dans la pathogénèse ou dans les mécanismes de dysbiose. Cette étude microbiologie des parodontopathies offre de nouvelles perspectives en microbiologie en termes d'études futures sur d'éventuels associations entre microorganismes de la plaque. Mais aussi sur des applications cliniques et de nouvelles stratégies thérapeutiques en ciblant les nouveaux potentiels pathogènes identifiés impliqués dans la parodontite par antibiothérapie ou par vaccination.

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ANNEXES

<u>Publication 5</u>: *Collinsella provencensis* sp. nov., a new species identified from healthy human gut microbiota.

Sarah Benabdelkader, Sabrina Naud, Amaël Fadlane , Niokhor Dione, Cheikh Ibrahima Lo, Gerard Aboudharam, Florence Fenollar and Bernard La Scola

(Submitted in NMNI Journal)

Avant-propos

Dans ce travail, une nouvelle espèce est décrite et classifiée isolée sur des selles lors d'un travail de culturomique réalisée chez un volontaire sain. Des petites colonies transparentes ont été cultivées après 72h d'incubation à 37°C dans des conditions d'anaérobie. Le séquençage du gène ARNr 16S a été effectué après l'échec d'identification par MALDI-TOF MS. Un pourcentage de similarité inférieur à 96.31% avec *Collinsella intestinalis*, espèce la plus proche phylogénétiquement, confirme le caractère de nouvelle espèce, appartenant à l'ordre des *Coriobacteriales* nommée *Collinsella provencensis.*

***Collinsella provencensis* sp. nov., a new species identified from healthy human gut microbiota.**

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Running title: *Collinsella provencensis* sp. nov.

Keywords: *Collinsella provencensis* sp. nov., culturomics, human gut microbiota, new bacteria, new species

Abstract:

Strain Marseille-P3740^{T} is a new species from the order *Coriobacteriales,* that was isolated from healthy French volunteer. It is an anaerobic gram-positive rod-shaped bacterium. Strain Marseille-P3740^{T} exhibited a 96.31 % sequence identity with *Collinsella intestinalis* strain JCM 10643, the phylogenetically closely related species with standing in nomenclature. The draft genome size of strain Marseille-P3740^{T} is 1.74 Mb with 59.1% of G+C content. We propose that strain Marseille-P3470^{T} (=CSURP3740) is the type strain of the new species *Collinsella provencensis* sp. nov.

Introduction

Currently it is important to understand the implication of bacterial diversity in normal physiological functions and to the disease [1]. Culturomics is a concept developing different culture conditions in order to enlarge our knowledge of the human microbiota through the discovery of previously uncultured bacteria [2-5]. Once bacterium was isolated, we used a taxono-genomics approach including matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), phylogenetic analysis, main phenotypic description and genome sequencing, to describe it [6-7].

Here we describe *Collinsella provencensis* sp. nov., strain Marseille-P3740^{T} (= CSUR P3740), according this taxono-genomics concept.

Isolation and growth conditions

We isolated from a fresh stool of a 32-year old man volunteer living in France, an unidentified bacterial strain. A screening has been made by MALDI-TOF MS on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [6]. The obtained spectra (Figure 1) were imported into MALDI Biotyper 3.0 software (Bruker Daltonics) and analyzed against the main spectra of the bacteria included in two databases (Bruker and constantly updated MEPHI databases). The study was validated by the ethics committee of "Institut Fédératif de Recherche IFR48" under number 2016-010. Strain Marseille-P3740^{T} was first isolated after 3-days of preincubation in an anaerobic blood culture bottle (Becton-Dickinson Diagnostics, Le Pont-de-Claix, France) supplemented with 5% sheep blood and rumen fluid at 37°C.

Phenotypic characteristics

After isolation step, the strain Marseille-P3740^{T} was cultured aiming to get pure and isolated colonies on blood agar. The colonies were small and transparent. Bacterial cells were Gram-positive. The sporulation test (10 minutes at 80°C) is negative. Different growth temperatures

(20, 28, 32, 37, 45 and 56°C), pH (5, 6, 7, 7.5, 8 and 8.5), NaCl content (5, 10 and 15g) and atmospheres (aerobic, anaerobic and microaerophilic (CampyGEN, Oxoid).) were tested on 5% sheep blood enriched Columbia Agar. Strain Marseille-P3740 ^{T} is as a very-hard-to-cultivate bacterium and grows only at 37°C in anaerobic conditions at pH 7. API ZYM (BioMérieux) was performed in order to determinate specific phenotypic features for strain Marseille-P3740. The results were tabulated in Table 1. Using API 50CH strips (BioMérieux) the carbohydrate metabolism of strain Marseille-P3740 is evaluated according to the manufacturer's instructions (Table 2). Thus, strain Marseille-P3740^{T} has enzymatic activities such as acid phosphatase, naphthol-AS-BI-phosphohydrolase, and alkaline phosphatase while only D-trehalose was positive for carbohydrate metabolism. All the other reactions tested were negative. Strain Marseille-P3740^{T} showed catalase-negative and oxidase-negative activities. A comparative study of the biochemical characteristics of this strain with other closely related *Collinsella* species is presented in Table 3. For scanning electronic microscopy, a colony was collected from agar and immersed into a 2.5 % glutaraldehyde fixative solution. The slide was gently washed in water, air dried and examined with a TM4000 microscope. The cells appear to be rod-shaped with a mean length of 1µm and a mean diameter of 0.5µm (Figure 2). The major fatty acid found for this strain by far, were Hexadecanoic acid (41 %) and 9-Octadecenoic acid (23 %). Very few other structures were described. No branched fatty acids were detected. Minor amounts of other unsaturated and saturated fatty acids were also described, including an unsaturated C20 structure. (Table 4).

Strain identification

The 16S rRNA gene was sequenced in order to classify this bacterium. Amplification was done by using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequencing using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL Genetic Analyzer capillary3500xL sequencer (Thermofisher, Saint-Aubin, France), as previously described [8].

The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-P3740^T exhibited a 96.31 % sequence identity with *Collinsella intestinalis* strain JCM 10643 (Genbank accession number NR_113165.1), the phylogenetically closest species with standing in nomenclature (Figure 3A). It is shown that *Collinsella provencensis* strain Marseille-P3740^T is close with strain *Collinsella intestinalis* and *Collinsella stercoris* (Figure 3). Considering these phylogenetic criteria, we consequently classify this strain as a member of a new species within the genus *Collinsella*, family *Coriobacteriaceae* phylum *Actinobacteria*.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue Kit and then sequenced on the MiSeq technology (Illumina, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired end (Illumina), as previously described [9]. The assembly was performed with a pipeline incorporating different softwares (Velvet [10], Spades [11] and Soap Denovo [12]), and trimmed (MiSeq and Trimmomatic [13] softwares) or untrimmed data (only MiSeq software). GapCloser was used to reduce assembly gaps. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value lower than 25% of the mean depth were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). The genome of *Collinsella provencensis* strain Marseille-P3740^T is 1,737,922 bp long with a 58.2% G+C content. The degree of genomic similarity of strain Marseille-P3740^T with closely related species was estimated using the OrthoANI software [14]. Values among closely related species (Figure 4) ranged from 78.63% between *Collinsella stercoris* and *Collinsella phocaeensis* to 81.70% between *Collinsella intestinalis* and *Collinsella stercoris*. When the isolate was compared to these closely species, values ranged from 73.42 % with *Collinsella bouchesdurhonensis* and *Corynebacterium intestinalis* to 81.70 % with *Corynebacterium stercoris*.

Conclusion

Basing on the results from unique phenotypic characteristics, including API galleries tests, MALDI-TOF spectrum, and phylogenetic and genomic analysis such as 16S rRNA sequence similarity lower than 96% and OrthoANI value lower than 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P3740^T as the type strain of *Collinsella provencensis* sp. nov.

Description of *Collinsella provencensis* sp. nov.

Collinsella provencensis (pro.ven.cen'cis, N.L. fem. adj. provencensis, pertaining to Provence, the region of France where the type strain was isolated). The strain grows hardly in varied conditions. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia Agar during after 3 days. They appear small and transparent. *C. provencensis* is Gram-positive rod-shaped bacterium with a mean length of 1µm and a mean diameter of 0.5µm. Strain Marseille-P3740^T produced alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and D-trehalose. But any activities was observed with trypsin, β-galactosidase, α-glucosidase, glycerol, D-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-lactose, D-saccharose, glycogen, D-fucose and D-arabitol. Strain Marseille-P3740^T is catalase-negative and oxidase-negative. The genome size of *Collinsella provencensis* strain Marseille-P3740^T is about 1.74 Mb long with 58.1 mol% G+C content. The Genbank Accession number for the 16S rRNA gene sequence of strain Marseille-P3740^T is LT722680 and for the whole genome shotgun project is FZRI00000000.This strain was isolated from fresh stool of a healthy French men.

Nucleotide sequence accession number. The 16S rRNA gene and genome sequences were deposited in Genbank under accession number LT722680 and FZRI00000000, respectively.

Deposit in culture collections. Strain Marseille-P3740^T was deposited in our strain collections under number (= CSURP3740).

Conflict of interest

None to declare

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13. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30:2114–20. doi:10.1093/bioinformatics/btu170.
14. Lee I, Ouk Kim Y, Park SC, Chun J. OrthoANI: An improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol 2016; 66:1100–3. doi:10.1099/ijsem.0.000760.

Table 1: Phenotypic characterization of *Collinsella provencensis* sp. nov., based on analytical profile index (API) ZYM tests.

Tests	Characteristics	Results
API ZYM	Alkaline phosphatase	+
	Esterase (C4)	-
	Esterase Lipase (C8)	-
	Lipase (C14)	-
	Leucine arylamidase	-
	Valine arylamidase	-
	Cystine arylamidase	-
	Trypsin	-
	α-chymotrypsin	-
	Acid phosphatase	+
	Naphthol-AS-BI-phosphohydrolase	+
	α-galactosidase	-
	β-galactosidase	-
	β-glucuronidase	-
	α-glucosidase	-
	β-glucosidase	-
	N-acetyl-β-glucosaminidase	-
	α-mannosidase	-
	α-fucosidase	-
	Glycerol	-

Table 2: Phenotypic characterization of *Collinsella provencensis* sp. nov., based on API 50 CH test.

Tests	Characteristics	Results
50 CH	Erythritol	-
	D-arabinose	-
	L-arabinose	-
	D-ribose	-
	D-xylose	-
	L-xylose	-
	D-Adonitol	-
	Methyl βD-xylopyranoside	-
	D-galactose	-
	D-glucose	-
	D-fructose	-
	D-mannose	-
	L-sorbose	-
	L-rhamnose	-
	Dulcitol	-
	Inositol	-
	D-mannitol	-
	D-sorbitol	-
	Methyl αD-mannopyranoside	-
	Methyl αD-glucopyranoside	-
	N-acetyl-glucosamine	-
	Amygdalin	-
	Arbutin	-
	Esculin ferric citrate	-
	Salicin	-
	D-cellobiose	-
	D-maltose	-
	D-lactose	-
	D-melibiose	-
	D-saccharose	-
	D-trehalose	+
	Inulin	-
	D-melezitose	-
	D-raffinose	-
	Amidon	-
	Glycogen	-
	Xylitol	-
	Gentiobiose	-
	D-turanose	-
	D-xylose	-

	D-tagalose	-
	D-fucose	-
	L-fucose	-
	D-arabitol	-
	L-arabitol	-
	Potassium gluconate	-
	Potassium 2-ketogluconate	-
	Potassium 5-ketogluconate	-

Table 4: Fatty acid profiles (%) of *Collinsella provencensis* strain Marseille-P3740^{T}.

Fatty acids	Name	Mean relative %*
16:0	Hexadecanoic acid	41.0 ± 1.9
18:1n9	9-Octadecenoic acid	23.6 ± 0.5
18:2n6	9,12-Octadecadienoic acid	15.8 ± 0.8
18:0	Octadecanoic acid	11.9 ± 0.8
20 :4n6	5,8,11,14-Eicosatetraenoic acid	1.9 ± 0.7
14:0	Tetradecanoic acid	1.6 ± 0.1
17:0	Heptadecanoic acid	1.4 ± 0.3
16:1n7	9-Hexadecenoic acid	1.2 ± 0.2
12:0	Dodecanoic acid	TR
15:0	Pentadecanoic acid	TR
17:1n8	9-Heptadecenoic acid	TR

*****Mean peak area percentage; TR = trace amounts < 1

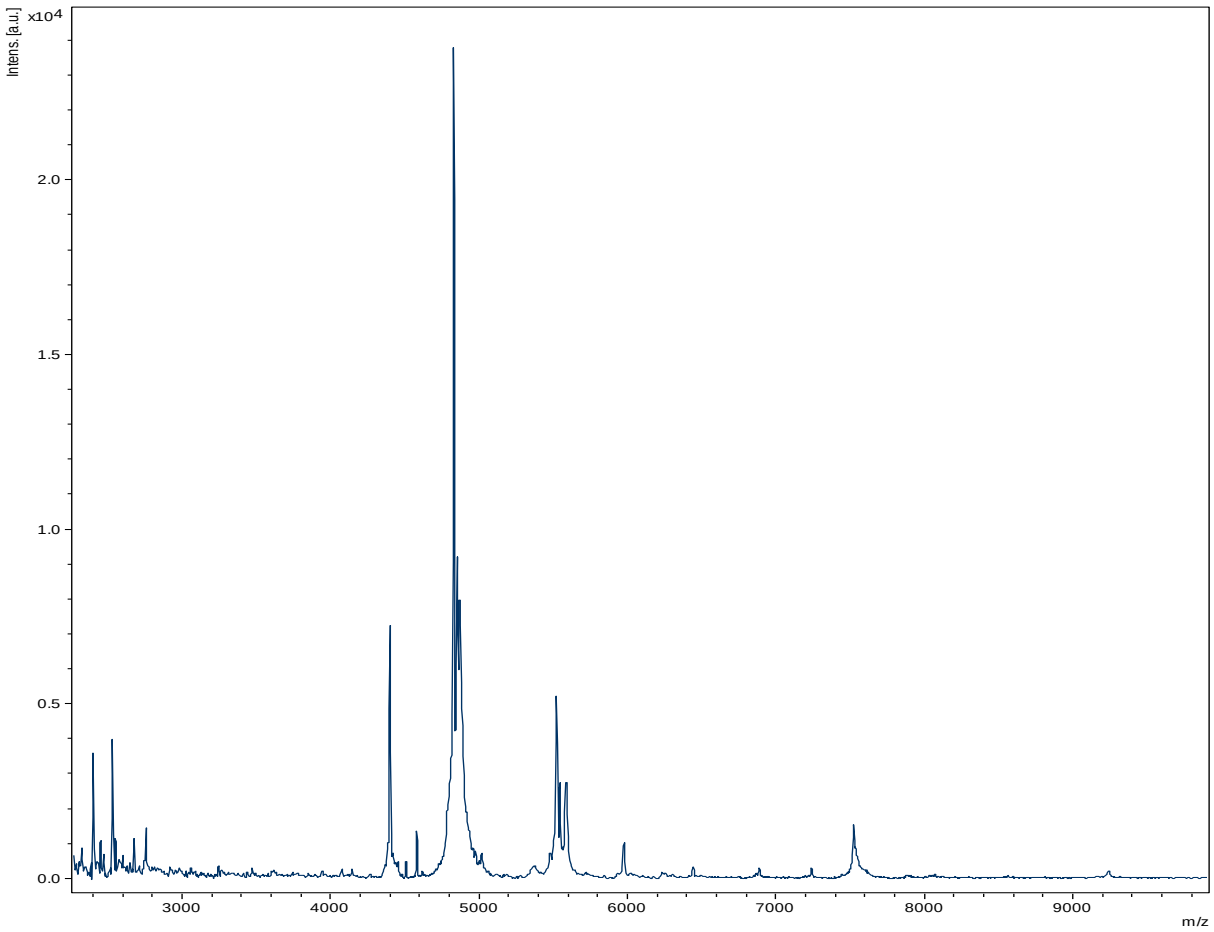

Figure 1: MALDI-TOF MS Reference mass spectrum of *Collinsella provencensis* sp. nov., strain Marseille-P3740^{T}. The reference spectrum was generated by comparison of spectra from 12 individual colonies.

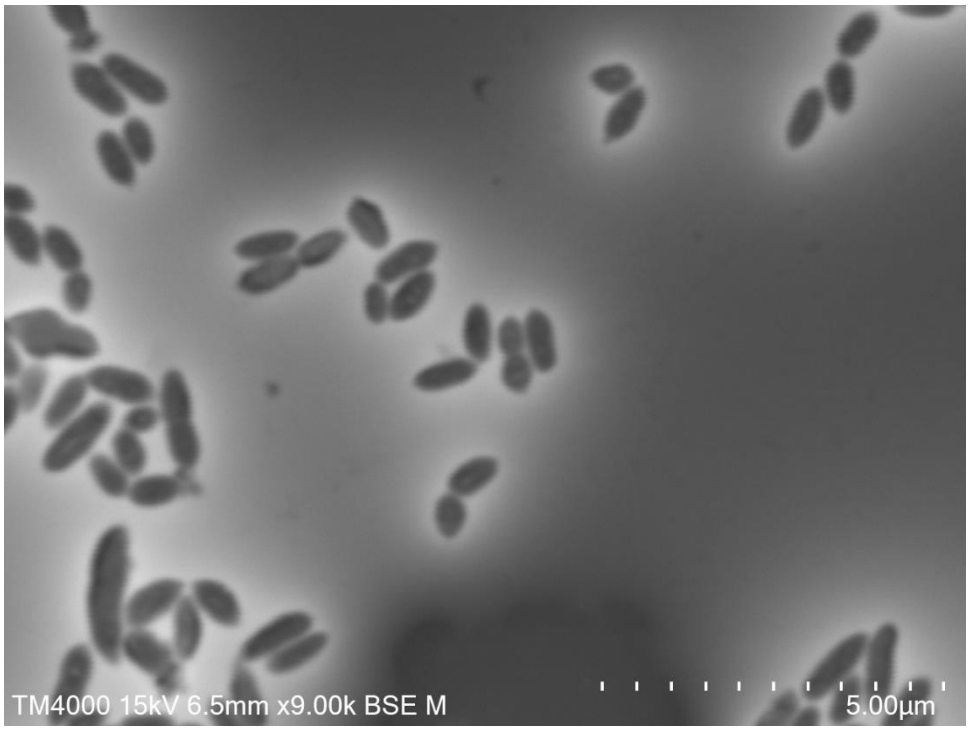

Figure 2: Scanning electron microscopy of stained *Collinsella provencensis* sp. nov., (Hitachi TM4000). Scales and acquisition settings are shown on figure.

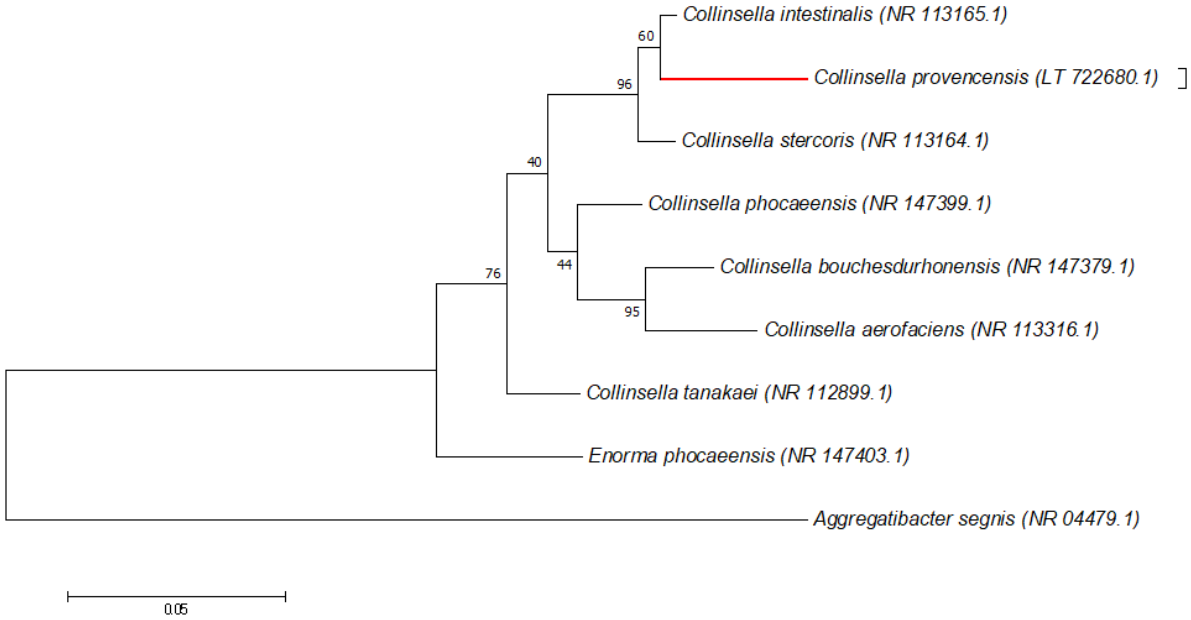

Figure 3: Phylogenetic trees highlighting the position of *Collinsella provencensis* sp. nov., based on the 16S rRNA gene sequences relative to the most closely related type strains within the genus *Collinsella*. Genbank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference were obtained using the Maximum likelihood method and the MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 1% nucleotide sequence divergence.

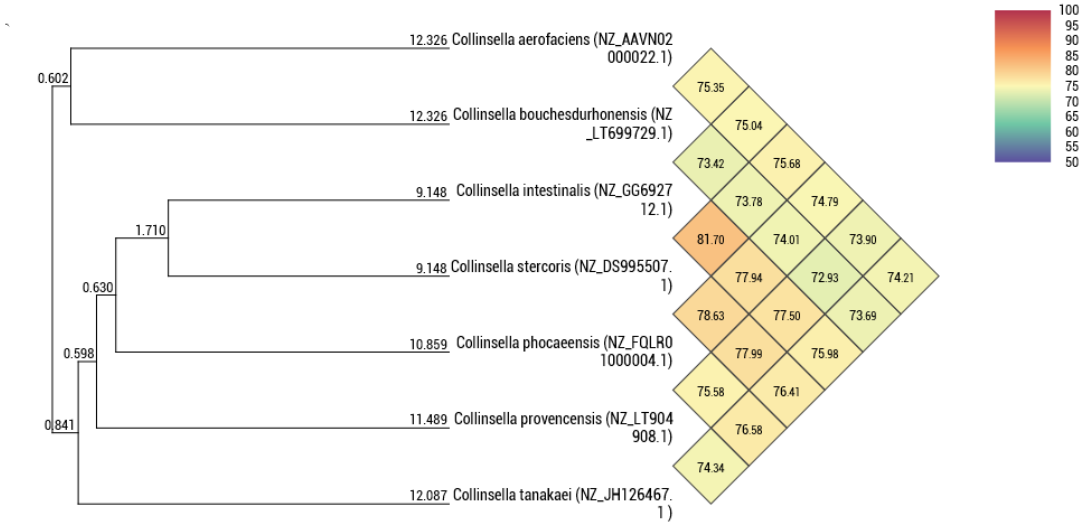

Figure 4: Heatmap generated with OrthoANI values calculated using the OAT software between *Collinsella provencensis* sp. nov., and other closely related species with standing in nomenclature.

Publication 6: *Parabacteroides pacaensis* sp. nov. and *Parabacteroides provencensis sp. nov*., two new species identified from human gut microbiota.

Sarah Benabdelkader, Sabrina Naud, Amaël Fadlane, Sory Ibrahima, Cheikh Ibrahima LO, Gerard Aboudharam, Florence Fenollar and Bernard La Scola

(Submitted in NMNI Journal)

Avant-propos

Dans ce travail, deux nouvelles espèces ont été décrites phénotypiquement et classifiées isolée à partir de selles dans le cadre de l'étude par culturomique réalisée chez un volontaire sain. Des petites colonies beiges et lisses ont été cultivées après 5 jours d'incubation à 37°C dans des conditions d'anaérobie. Le séquençage du gène ARNr 16S a été effectué après l'échec d'identification par MALDI-TOF MS. Les pourcentages de similarité inférieurs à 95.45% avec *Parabacteroides goldsteinii* et 95.45% avec *Parabacteroides merdae*, espèces les plus proches phylogénétiquement, confirme le caractère de nouvelles espèces, appartenant à l'ordre des *Bacteroidales* nommées respectivement *Parabacteroides pacaensis* et *Parabacteroides provencensis.*

***Parabacteroides pacaensis* sp. nov., and *Parabacteroides provencensis* sp. nov., two new species identified from human gut microbiota.**

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Running title: *Parabacteroides pacaensis* sp. nov., *Parabacteroides provencensis* sp. nov.

Keywords: P*abacteroides pacaensis* sp. nov., *Parabacteroides provencensis* sp. nov., culturomics, human gut microbiota, new bacteria.

Abstract:

Strains Marseille-P4001 and Marseille-P3668 are new species from the order *Bacteroidales* isolated from healthy French volunteers. They are anaerobic Gram-negative rod-shaped-bacteria. They exhibited 92.68 and 96.68% 16S rRNA sequence identities with *Parabacteroides gordonii* strain MS-1 and *Parabacteroides chinchillae* JCM 17104 respectively, the phylogenetically closest species. Their respective draft genome measured 5.23Mb and 3.73Mb with 39.2 and 40.8 mol% of G+C content. Using taxonogenomics method, we propose here brief description of *Parabacteroides pacaensis* sp. nov., strain Marseille-P4001^{T} and *Parabacteroides provencensis* sp. nov., strain Marseille-P3668^{T} as new bacterial species.

Introduction

Currently it is important to understand the implication of bacterial diversity in normal physiological functions and to the disease [1]. Culturomics is a concept developing different culture conditions in order to enlarge our knowledge of the human microbiota through the discovery of previously uncultured bacteria [2-5]. Once bacterium was isolated, we used a taxono-genomics approach including matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), phylogenetic analysis, main phenotypic description and genome sequencing, to describe it [6-7].

Here we describe *Parabacteroides pacaensis* sp. nov., strain Marseille-P4001^T (= CSUR P4001), and *Parabacteroides provencensis* sp. nov., strain Marseille-P3668^T (= CSUR P3668), according this taxono-genomics concept.

Isolation and growth conditions

We isolated two unidentified bacterial strains from the fresh stools of two volunteers living in France. A screening has been made by MALDI-TOF MS on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [8]. The obtained spectra (**Figure 1**) were imported into MALDI Biotyper 3.0 software (Bruker Daltonics) and analyzed against the main spectra of the bacteria included in two databases (Bruker and constantly updated URMS databases). The study was validated by the ethics committee of "Institut Fédératif de Recherche IFR48" under number 2016-010. Strains Marseille-P4001^T and Marseille-P3668^{T}were first isolated after 7-days of preincubation in an anaerobic blood culture bottle (Becton-Dickinson Diagnostics, Le Pont-de-Claix, France) supplemented with 5% sheep blood at 37°C.

Phenotypic characteristics

After isolation step, the strain Marseille-P4001^T and strain Marseille-P3668^T were cultured aiming to get pure and isolated colonies on blood agar. The colonies of Marseille-P4001 and

Marseille-P3668 had almost the same morphological aspect, namely beige, small and smooth. Bacterial cells were Gram-negative for both strains. The sporulation test (10 minutes at 80°C) is negative. Different growth temperatures (20, 28, 32, 37, 45 and 56°C), pH (5, 6, 7, 7.5, 8 and 8.5), and atmospheres (aerobic, anaerobic and microaerophilic (CampyGEN, Oxoid).) were tested on 5% sheep blood enriched Columbia agar. Strain Marseille-P4001^{T} grows at 28 and 37°C in anaerobic conditions at pH 7. Strain Marseille-P3668^{T} grows from 28 to 45°C (optimally at 37°C) at pH ranging from 6 to 8.5 (optimally at pH 7) in anaerobic conditions. API ZYM (bioMérieux) was performed in order to determinate specific enzymatic properties for both strains. The results were tabulated in **Table 1**. Using API 50 CH strips (bioMérieux) the carbohydrate metabolism of both strains is evaluated according to the manufacturer's instructions (**Table 2**). Thus for strain Marseille-P4001^{T} the following positive reactions were noted: esterase (C4), leucine arylamidase, α-galactosidase, β-galactosidase, N-acetyl-β-glycosaminidase, alkaline phosphatase, esculin ferric citrate, D-melezitose, D-saccharose, D-mannitol, methyl-αD-glucopyranoside and glycogen. All the other reactions tested were negative. Also, strain Marseille-P3668^{T} had positive reactions for alkaline phosphatase, leucine arylamidase, α-galactosidase, β-galactosidase, Naphthol-AS-BI-phosphohydrolase, phosphatase acid, N-acetyl-β-glycosaminidase, α-fucosidase, esculin ferric citrate and dulcitol. All the other reactions tested were negative. Strain Marseille-P4001^{T} and strain Marseille-P3668^{T} showed catalase-positive and oxidase-negative activities. A comparative study of the biochemical characteristics of those strains with other closely related *Parabacteroides* species is presented in **Table 3**. For scanning electronic microscopy, a colony was collected from agar and immersed into a 2.5 % glutaraldehyde fixative solution for each strain. The slide was gently washed in water, air dried and examined with a TM4000 microscope. The cells of strain Marseille-P4001 appear to be rod-shaped with a mean length of 1.5 μm and a mean diameter of

0.5 μm. The cells of strain Marseille-P3668 are rod-shaped with a mean length of 2 μm and a mean diameter of 0.7 μm (**Figure 2**).

Strains identification

The 16S rRNA gene was sequenced in order to classify those bacteria. Amplification was done by using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequencing using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL Genetic Analyzer capillary3500xL sequencer (Thermofisher, Saint-Aubin, France), as previously described [9]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-P4001^T exhibited a 92.68 % sequence identity with *Parabacteroides gordonii* strain MS-1 (Genbank accession number NR112835.1) and strain Marseille-P3668^T exhibited a 96.68 % sequence identity with *Parabacteroides chinchillae* JCM 17104 (Genbank accession number NR113208.1), the phylogenetically closest species with standing in nomenclature (**Figure 3**). Considering these phylogenetic values lower than the thresholds fixed to delineate new bacterial taxon [10-11], we consequently classify these strains as members within the genus *Parabacteroides* belonging to family *Tannerellaceae*.

Genome sequencing

Genomic DNA were extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue Kit and then sequenced on the MiSeq technology (Illumina, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired end (Illumina), as previously described [12]. The assembly was performed with a pipeline incorporating different softwares (Velvet [13], Spades [14] and Soap Denovo [15]) and trimmed (MiSeq and Trimmomatic [16] softwares) or untrimmed data (only MiSeq software). GapCloser software [17] was used to reduce assembly gaps. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value lower than 25% of the mean depth were removed. The best assembly was selected

by using different criteria (number of scaffolds, N50, number of N). The genome of *Parabacteroides pacaensis* strain Marseille-P4001^T is 5,238,628 bp long with a 39.21 mol% G+C content. Thus, the genome of *Parabacteroides provencensis* strain Marseille-P3668^T is 3,732,078 bp long with a 40.8 mol% G+C content. The degree of genomic similarity of strain Marseille-P4001^T and Marseille-P3668^T with closest species was estimated using the OrthoANI software [14]. Values among closely related species (**Figure 4**) ranged from 78.31% between *Parabacteroides chinchillae* and *Parabacteroides provencensis* to 82.18% between *Parabacteroides goldsteinii* and *Parabacteroides gordonii*. 71.26% of similarity is shared between *P. provencensis* and *P. pacaensis.*

Conclusion

Basing on the results from unique phenotypic characteristics, including API galleries tests, MALDI-TOF spectrum, and phylogenetic and genomic analysis such as 16S rRNA sequence similarity lower than 95% and OrthoANI value lower than 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P4001^T and strain Marseille-P3668^T as type strain of *Parabacteroides pacaensis* sp. nov and *Parabacteroides provencensis* sp. nov., respectively.

Description of *Parabacteroides pacaensis* sp. nov.

Parabacteroides pacaensis (pa.ca'en.sis N.L. masc. adj. pacaensis, derived from the abbreviation PACA, for the region of Provence Alpes Côte d'Azur, where the strain was first isolated). The strain grows in varied conditions. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia agar during after 3 days in anaerobic atmosphere. They appear smooth and small. *P. pacaensis* is Gram-negative rod-shaped bacterium with a mean length of 1.4 μm and a mean diameter of 0.5 μm. Strain Marseille-P4001^T produced esterase (C4), leucine arylamidase, α and β galactosidase, N-acetyl-β-glycosaminidase, and alkaline phosphatase and metabolize esculin ferric citrate, D-melezitose, D-saccharose, D-

mannitol, methyl-αD-glucopyranoside and glycogen. But any activities were observed with trypsin, α-glucosidase, glycerol, D-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-lactose, D-fucose and D-arabitol. Strain Marseille-P4001^T is catalase-positive and oxidase-negative. The genome size of *Parabacteroides pacaensis* sp. nov., strain Marseille-P4001^T is about 5.24 Mb long with 39.2 mol% G+C content. The Genbank accession number for the 16S rRNA gene sequence of strain Marseille-P4001^T is LT985457 and for the whole genome shotgun project is OLMS00000000.This strain was isolated from fresh stool of a healthy French volunteer.

Description of *Parabacteroides provencensis* sp. nov.

Parabacteroides pacaensis (pro.ven.cen'cis, N.L. fem. adj. provencensis, pertaining to Provence, the region of France where the type strain was isolated). The strain grows in varied conditions. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia Agar during after 3 days in anaerobia. They appear smooth and small. *P. pacaensis* is Gram-negative rod-shaped bacterium with a mean length of 2µm and a mean diameter of 0.7µm. Strain Marseille-P3668^T produced alkaline phosphatase, leucine arylamidase, α and β galactosidase, Naphthol-AS-BI-phosphohydrolase, acid phosphatase, N-acetyl-β-glycosaminidase, and α-fucosidase and metabolize only esculin ferric citrate and Dulcitol. But any activities were observed with trypsin, α-glucosidase, glycerol, D-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-lactose, D-fucose and D-arabitol. Strain Marseille-P3668^T is catalase-positive and oxidase-negative. The genome size of *Parabacteroides provencensis* strain Marseille-P3668^T is about 3.73 Mb long with 40.8 mol% G+C content. The Genbank accession number for the 16S rRNA gene sequence of strain Marseille-P3668^T is LT722681 and for the whole genome shotgun project is FYCK00000000.This strain was isolated from fresh stool of a healthy French volunteer.

Nucleotide sequence accession number. The 16S rRNA gene and genome sequences were deposited in Genbank under accession number LT985457 and OLMS00000000, respectively for Strain Marseille-P4001^{T} and under accession number LT722681 and FYCK00000000, respectively for Strain Marseille-P3668^{T}.

Deposit in culture collections. Strain Marseille-P4001^{T} was deposited in our strain collections under number (= CSUR P4001) and Strain Marseille-P3668^{T} under number (= CSUR P3668).

Conflict of interest

None to declare

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Table 1: Phenotypic characterization of *Parabacteroides pacaensis* strain Marseille-P4001^T sp. nov. and *Parabacteroides provencensis* sp. nov strain Marseille-P3668^T, based on analytical profile index (API) ZYM tests.

Tests	Characteristics	P4001^T	P3668^T
API ZYM	Alkaline phosphatase	+	+
	Esterase (C4)	+	-
	Esterase Lipase (C8)	-	-
	Lipase (C14)	-	-
	Leucine arylamidase	+	+
	Valine arylamidase	-	-
	Cystine arylamidase	-	-
	Trypsin	-	-
	α-chymotrypsin	-	-
	Acid phosphatase	-	+
	Naphthol-AS-BI-phosphohydrolase	-	+
	α-galactosidase	+	+
	β-galactosidase	+	+
	β-glucuronidase	-	-
	α-glucosidase	-	-
	β-glucosidase	-	-
	N-acetyl-β-glucosaminidase	+	+
	α-mannosidase	-	-
	α-fucosidase	-	+
	Glycerol	-	-

Table 2: Phenotypic characterization of *Parabacteroides pacaensis* strain Marseille-P4001^T sp. nov. and *Parabacteroides provencensis* sp. nov strain Marseille-P3668^T, based on API 50 CH test.

Tests	Characteristics	P4001^T	P3668^T
50 CH	Erythritol	-	-
	D-arabinose	-	-
	L-arabinose	-	-
	D-ribose	-	-
	D-xylose	-	-
	L-xylose	-	-
	D-Adonitol	-	-
	Methyl βD-xylopyranoside	-	-
	D-galactose	-	-
	D-glucose	-	-
	D-fructose	-	-
	D-mannose	-	-
	L-sorbose	-	-
	L-rhamnose	-	-
	Dulcitol	-	+
	Inositol	-	-
	D-mannitol	+	-
	D-sorbitol	-	-
	Methyl αD-mannopyranoside	-	-
	Methyl αD-glucopyranoside	+	-
	N-acetyl-glucosamine	-	-
	Amygdalin	-	-
	Arbutin	-	-
	Esculin ferric citrate	+	+
	Salicin	-	-
	D-cellobiose	-	-
	D-maltose	-	-
	D-lactose	-	-
	D-melibiose	-	-
	D-saccharose	+	-
	D-trehalose	-	-
	Inulin	-	-
	D-melezitose	+	-
	D-raffinose	-	-
	Amidon	+	-
	Glycogen	-	-
	Xylitol	-	-
	Gentiobiose	-	-

D-turanose	-	-
D-xylose	-	-
D-tagalose	-	-
D-fucose	-	-
L-fucose	-	-
D-arabitol	-	-
L-arabitol	-	-
Potassium gluconate	-	-
Potassium 2-ketogluconate	-	-
Potassium 5-ketogluconate	-	-

Table 3: Comparison of differential characteristics of *Parabacteroides pacaensis* sp. nov., *Parabacteroides provencensis* sp. nov., *Parabacteroides timonensis* and *Parabacteroides chartae.*

Property	*P. pacaensis*	*P. provencensis*	*P. timonensis*	*P. chartae*
Cell diameter (μm)	0.5	0.7	0.5	0.7-1
Oxygen requirement	-	-	-	-
Gram stain	-	-	-	-
Salt requirement	-	-	-	-
Motility	-	-	-	-
Endospore formation	-	-	-	-
Alkaline phosphatase	+	+	+	+
Catalase	+	+	+	-
Oxidase	-	-	-	NA
Urease	-	-	-	-
β-Galactosidase	+	+	+	+
N-acetyl-glucosamine	-	+	+	+
Arabinose	-	-	+	+
Lipase (C8)	+	-	+	+
Mannose	-	-	+	+
Mannitol	+	-	+	-
Sucrose	+	-	+	+
D-Glucose	-	-	+	+
D-Fructose	-	-	+	-
D-Maltose	-	-	+	+
Source	Human	Human	Human	Environment

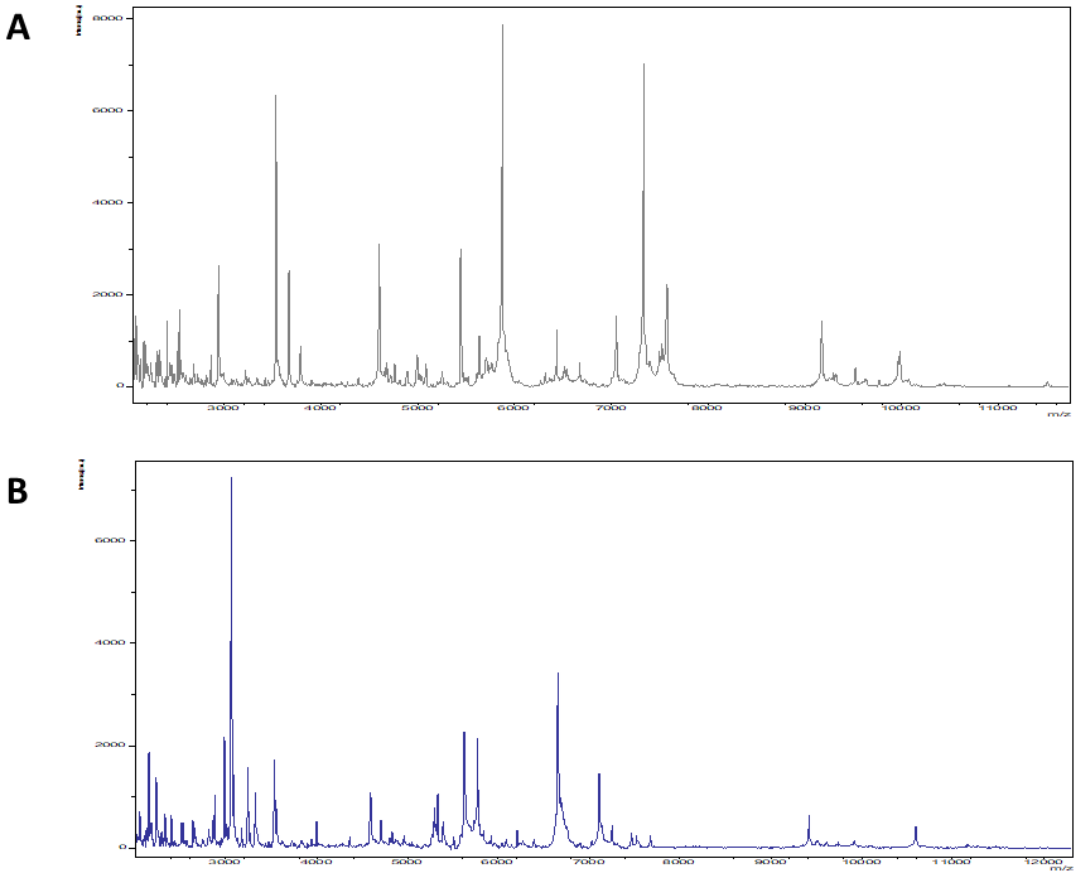

Figure 1: MALDI-TOF MS Reference mass spectrum of *Parabacteroides pacaensis* sp. nov. strain Marseille-P4001^{T} (**A**) and *Parabacteroides provencensis* sp. nov., strain Marseille-P3668^{T} (**B**). The reference spectrum was generated by comparison of spectra from 12 individual colonies.

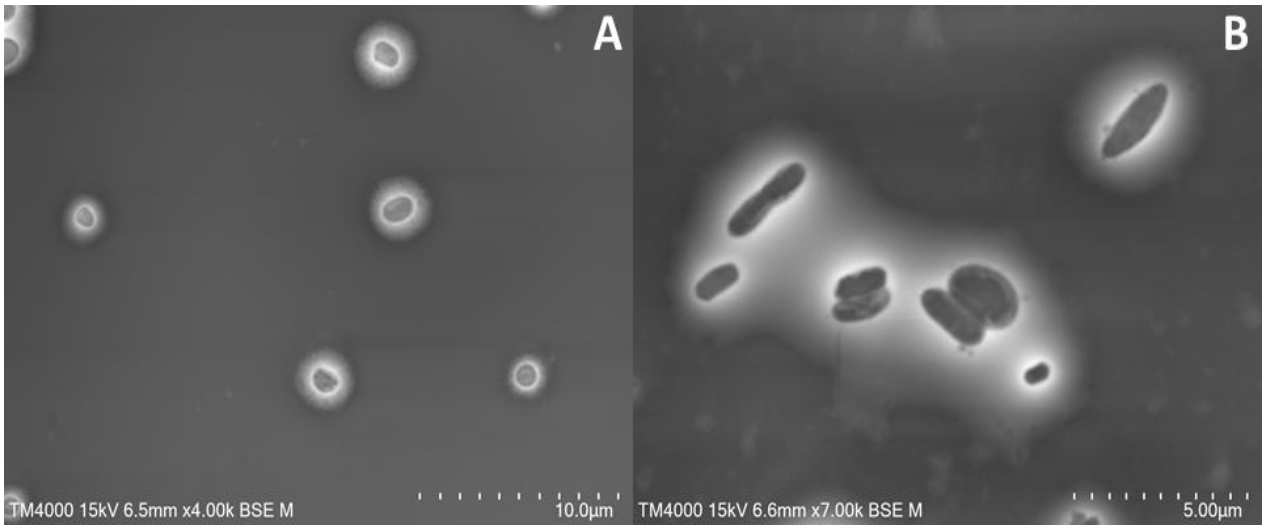

Figure 2: Scanning electron microscopy of stained *Parabacteroides pacaensis* sp. nov., strain Marseille-P4001^{T} (**A**) and *Parabacteroides provencensis* sp. nov., strain Marseille-P3668^{T} (**B**) (Hitachi TM4000). Scales and acquisition settings are shown on figure.

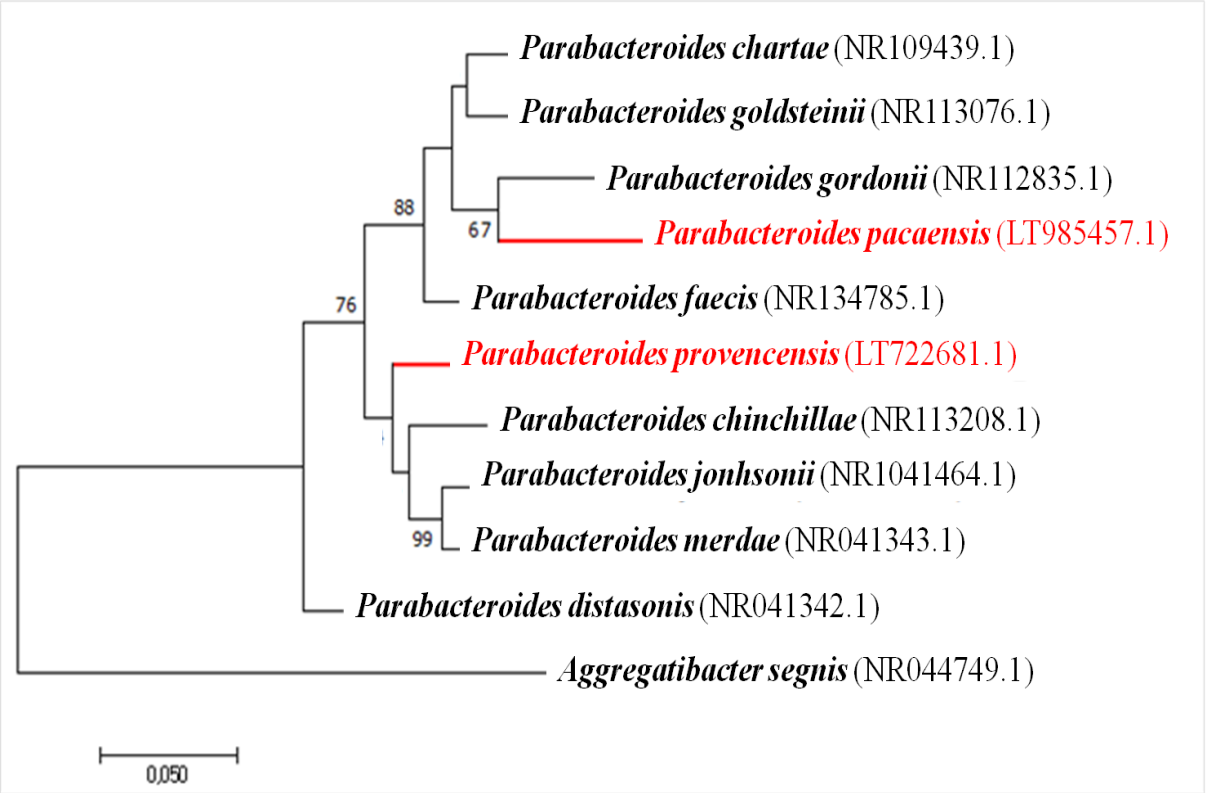

Figure 3: Phylogenetic trees highlighting the position of *Parabacteroides pacaensis* sp. nov., and *Parabacteroides provencensis* sp. nov., based on the 16S rRNA gene sequences relative to the most closely related type strains within the genus *Parabacteroides* Genbank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference were obtained using the Maximum likelihood method and the MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 5% nucleotide sequence divergence.

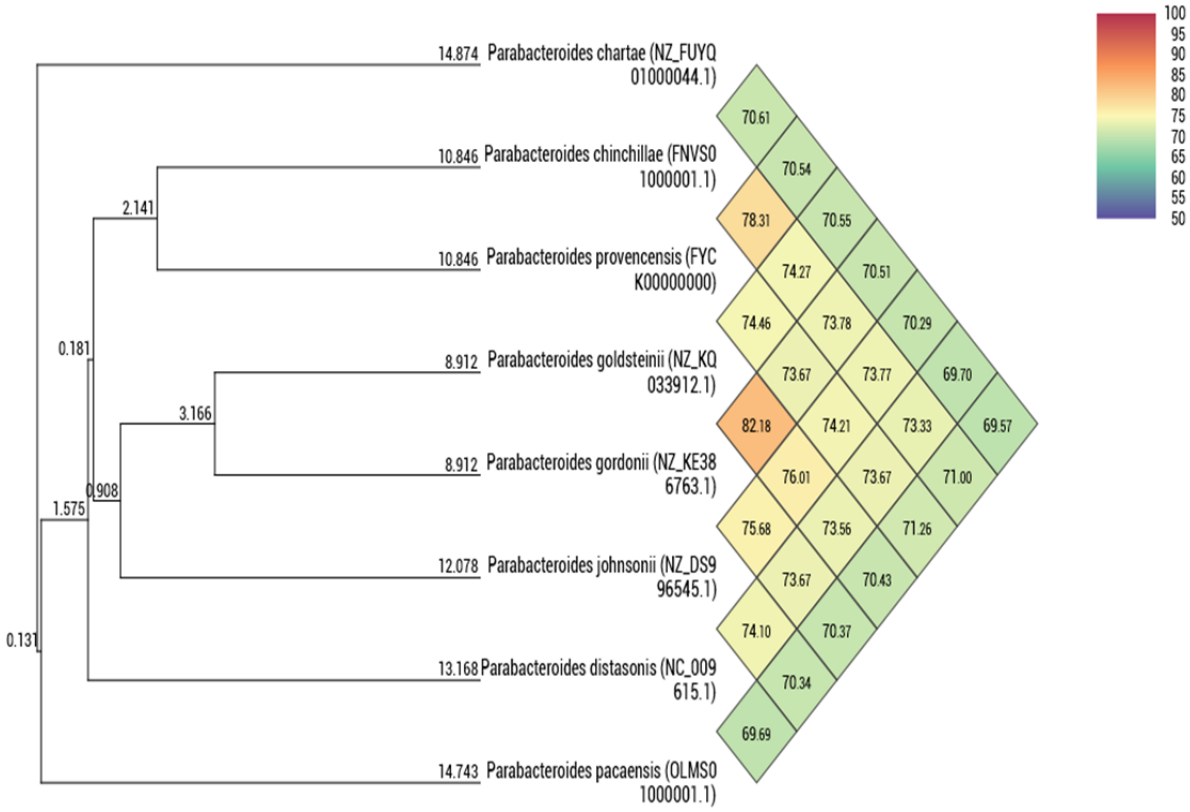

Figure 4: Heatmap generated with OrthoANI values calculated using the OAT software between *Parabacteroides pacaensis* sp. nov., and *Parabacteroides provencensis* sp. nov., and other closely related species with standing in nomenclature.

Publication 7: *Peptoniphilus colimassliensis* sp. nov. and *Peptoniphilus duodeni sp. nov* two new species identified from human gut microbiota.

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(Submitted in NMNI Journal)

Avant-propos

Dans ce dernier travail, deux nouvelles espèces ont été décrites phénotypiquement et classifiées isolée à partir de selles dans le cadre de l'étude par culturomique réalisée chez un volontaire sain. Des petites colonies circulaires ont été cultivées après 7 jours d'incubation à 37°C dans des conditions d'anaérobie. Le séquençage du gène ARNr 16S a été effectué après l'échec d'identification par MALDI-TOF MS. Les pourcentages de similarité inférieurs à 95.72% avec *Peptoniphilus coxii* et 92.64% avec *Peptoniphilus assacharolyticus*, espèces les plus proches phylogénétiquement, confirme le caractère de nouvelles espèces, appartenant à l'ordre des Tissierellales nommées respectivement *Peptoniphilus colimassiliensis* et *Peptoniphilus duodeni*.

***Peptoniphilus colimassliensis* sp. nov. and *Peptoniphilus duodeni sp. nov* two new species identified from human gut microbiota.**

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Running title: *Peptoniphilus colimassiliensis* sp. nov., *Peptoniphilus duodeni* sp. nov.

Keywords: *Peptoniphilus colimassiliensis* sp. nov., *Peptoniphilus duodeni* sp. nov., culturomics, human gut microbiota, new bacteria, new species

Abstract:

Strains Marseille-P3761^{T} and Strain Marseille-P2932 are new species from the order *Tissierellales,* that were isolated from healthy volunteers. Those are anaerobic gram-negative rod-shaped bacteria. Strain Marseille-P3761^{T} exhibited a 95.72 % sequence identity with *Peptoniphilus coxii* strain RMA 16757 and Strain Marseille-P2932^{T} exhibited a 92.64 % sequence identity with *Peptoniphilus assacharolyticus* JCM 1765, the phylogenetically closely related species with standing in nomenclature. The draft genome size of strain Marseille-P3761^{T} is 1.99 Mb long with 48.6 mol% G+C content and 1.53 Mb with 30.9% of G+C content for strain Marseille-P2932^{T}. We propose strains Marseille-P3761^{T} (=CSURP3761) and Marseille- P2932^{T} (=CSURP2932) are the type strain of the new species respectively, *Peptoniphilus colimassiliensis* sp. nov and *Peptoniphilus duodeni sp. nov*.

Introduction

Currently it is important to understand the implication of bacterial diversity in normal physiological functions and to the disease [1]. Culturomics is a concept developing different culture conditions in order to enlarge our knowledge of the human microbiota through the discovery of previously uncultured bacteria [2-5]. Once bacterium was isolated, we used a taxono-genomics approach including matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), phylogenetic analysis, main phenotypic description and genome sequencing, to describe it [6-7].

Here we describe *Peptoniphilus colimassiliensis sp. nov* strain Marseille- P3761^{T} (=CSURP3761), *and Peptoniphilus duodeni sp. nov.*, strain Marseille-P2932^{T} (= CSUR P2932), according this taxono-genomics concept.

Isolation and growth conditions

We isolated from fresh stools of two volunteers living in France, two unidentified bacterial strains. A screening has been made by MALDI-TOF MS on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [6]. The obtained spectra (Figure 1 A, B) were imported into MALDI Biotyper 3.0 software (Bruker Daltonics) and analyzed against the main spectra of the bacteria included in two databases (Bruker and constantly updated MEPHI databases). The study was validated by the ethics committee of "Institut Fédératif de Recherche IFR48" under number 2016-010. Strain Marseille-P3761^{T} and Marseille-P2932^{T}were first isolated after 7-days of preincubation in an anaerobic blood culture bottle (Becton-Dickinson Diagnostics, Le Pont-de-Claix, France) supplemented with 5% sheep blood at 37°C.

Phenotypic characteristics

After isolation step, the strain Marseille-P3761^T and strain Marseille-P2932^T were cultured aiming to get pure and isolated colonies on blood agar. The colonies of Marseille-P3761^T were grey and circular, as well for strain Marseille- P2932^T. Bacterial cells were Gram-positive for both strains. The sporulation test (10 minutes at 80°C) is negative. Different growth temperatures (20, 28, 32, 37, 45 and 56°C), pH (5, 6, 7, 7.5, 8 and 8.5), NaCl content (5, 10 and 15g) and atmospheres (aerobic, anaerobic and microaerophilic (CampyGEN, Oxoid).) were tested on 5% sheep blood enriched Columbia Agar. Strain Marseille-P3761^T grows only at 37°C in anaerobic conditions at pH 6-8.5 (optimally at pH7). Strain Marseille-P2932^T grows at 28-37°C (optimally at 37°C) at pH 6.5 in anaerobic conditions. API ZYM (BioMérieux) was performed in order to determinate specific phenotypic features for both strains. The results were tabulated in Table 1. Using API 50CH strips (BioMérieux) the carbohydrate metabolism of both strains is evaluated according to the manufacturer's instructions (Table 2). Thus, strain Marseille-P3761^T metabolizes D-ribose, D-galactose, D-glucose, D-fructose, D-mannitol, D-maltose, D-lactose, sucrose and D-trehalose. All the other reactions tested were negative. Also, strain Marseille-P2932^T has enzymatic activities such as acid phosphatase, leucine arylamidase and Naphthol-AS-BI-phosphohydrolase while glycerol D-mannose, D-melezitose, amidon and gentiobiose were positive for carbohydrate metabolism. All the other reactions tested were negative. Strain Marseille-P3761^T and strain Marseille- P2932^T showed catalase-negative and oxidase-negative activities. A comparative study of the biochemical characteristics of those strains with other closely related *Peptoniphilus* species is presented in Table 3. For scanning electronic microscopy, a colony was collected from agar and immersed into a 2.5 % glutaraldehyde fixative solution for each strain. The slide was gently washed in water, air dried and examined with a TM4000 microscope. The cells for strain Marseille- P3761^T appear to be rod-shaped with a mean length of 1.2µm and a mean diameter of 0.5µm. The cells for strain Marseille- P2932^T are

rod-shaped with a mean length of 1 μm and a mean diameter of 0.7μm (Figure 2). The major fatty acids for strain-Marseille- P3761^T were Hexadecanoic acid (49 %), 9-Octadecenoic acid (2%) and 3-methyl-Butanoic acid (14 %). A high quantity of 3-methyl-Butanoic acid (short chain fatty acid) was detected. Minor amounts of unsaturated and other saturated structures were also detected. (Table 4.1). The analysis of fatty acids of strain Marseille- P2932^T indicates an unusual profile. The top three most abundant fatty acids (> 10 %) were saturated structures: 14:0 (44 %), 16:0 (28 %) and 12:0 (11%). Unsaturated fatty acids were described at lower abundancies (< 6 %) (Table 4.2).

Strain identification

The 16S rRNA gene was sequenced in order to classify those bacteria. Amplification was done by using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequencing using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL Genetic Analyzer capillary3500xL sequencer (Thermofisher, Saint-Aubin, France), as previously described [8]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-P3761^T exhibited a 95.72% sequence identity with *Peptoniphilus coxii* strain RMA 16757 (Genbank accession number NR_117556.1) and Strain Marseille-P2932^T exhibited a 92.64 % sequence identity with *Peptoniphilus asaccharolyticus* strain JCM 1765 (Genbank accession number NR_113382.1), the phylogenetically closest species with standing in nomenclature (Figure 3). It is shown that *Peptoniphilus colimassiliensis* strain Marseille-P3761^T is close with *Peptoniphilus coxii* strain and *Peptoniphilus ivorii* strain (Figure 3) and *Peptoniphilus duodeni* strain Marseille-P2932^T with strain *Peptoniphilus obesi* and *Peptoniphilus catoniae*. Considering these phylogenetic criteria, we consequently classify this strain as a member of a new species within the genus *Peptoniphilus*, family *Peptoniphilaceae* phylum *Firmicutes*.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue Kit and then sequenced on the MiSeq technology (Illumina, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired end (Illumina), as previously described [9]. The assembly was performed with a pipeline incorporating different softwares (Velvet [10], Spades [11] and Soap Denovo [12]) and trimmed (MiSeq and Trimmomatic [13] softwares) or untrimmed data (only MiSeq software). GapCloser was used to reduce assembly gaps. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value lower than 25% of the mean depth were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). The genome of *Peptoniphilus colimassiliensis* strain Marseille-P3761^{T} is 1,986,843 bp long with a 48.6% G+C content. Thus, the genome of *Peptoniphilus duodeni* strain Marseille-P2932^{T} is 1,534,044 bp long with a 30.9% G+C content. The degree of genomic similarity of strain Marseille-P3761^{T} and Marseille-P2932^{T} with closely related species was estimated using the OrthoANI software [14]. Values among closely related species (Figure 4) ranged from 67.62% between *Peptoniphilus timonensis and Peptoniphilus colimassiliensis* to 75.44% between *Peptoniphilus colimassiliensis* and *Peptoniphilus coxii*. When the isolates were compared to these closely species, values ranged from 63.06 % with *Peptoniphilus timonensis* and *Peptoniphilus ivorii* to 75.44 % with *Peptoniphilus colimassiliensis* and *Peptoniphilus coxii*.

Conclusion

Basing on the results from unique phenotypic characteristics, including API galleries tests, MALDI-TOF spectrum, and phylogenetic and genomic analysis such as 16S rRNA sequence similarity lower than 95% and OrthoANI value lower than 95% with the phylogenetically

closest species with standing in nomenclature, we formally proposed strain Marseille-P3761^T and strain Marseille-P2932^T as respectively the type strain of *Peptoniphilus colimassiliensis* sp. nov and *Peptoniphilus duodeni sp. nov.*

Description of *Peptoniphilus colimassiliensis* sp. nov.

Peptoniphilus colimassiliensis (rom latin ko:li, the genitive case of colon, meaning "belonging to the colon" and mas.si.li.en'sis. L. masc. adj. massiliensis, of Massilia, the Latin name of Marseille where strain was isolated). The strain grows at 37°C in anaerobia only. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia Agar during after 3 days in anaerobia. They appear grey and circular. *P. colimassiliensis* is Gram-positive rod-shaped bacterium with a mean length of 1.2µm and a mean diameter of 0.5µm. Strain Marseille-P3761^T metabolizes D-ribose, D-galactose, D-glucose, D-fructose, D-mannitol, D-maltose, D-lactose, sucrose and D-trehalose. But any activities were observed with trypsin, α-glucosidase, glycerol, D-arabinose, D-ribose, D-xylose, D-mannose, L-rhamnose, D-fucose and D-arabitol. Strain Marseille-P3761^T is catalase-negative and oxidase-negative. The genome size of *P. colimasssiliensis* is about 1.99 Mb long with 48.6 mol% G+C content. The Genbank Accession number for the 16S rRNA gene sequence of strain Marseille-P3761^T is LT972121 and for the whole genome shotgun project is OPYI00000000.This strain was isolated from fresh stool of a healthy French volunteer.

Description of *Peptoniphilus duodeni* sp. nov.

Peptoniphilus duodeni (duode'ni, N.L. mas. adj. duodeni in the colon where the type strain was isolated). The strain grows in varied conditions. Optimum growth of colonies was obtained at 37°C on 5% sheep blood enriched Columbia Agar during after 3 days in anaerobia. They appear grey and circular. *P. duodeni* is Gram-positive rod-shaped bacterium

with a mean length of 1µm and a mean diameter of 0.7µm. Strain Marseille-P2932^{T} produced acid phosphatase, leucine arylamidase and Naphthol-AS-BI-phosphohydrolase and metabolizes glycerol D-mannose, D-melezitose, amidon and gentiobiose. But any activities were observed with trypsin, α-glucosidase, D-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, L-rhamnose, D-lactose, D-fucose and D-arabitol. Strain Marseille-P2932^{T} is catalase-negative and oxidase-negative. The genome size of *P. duodeni* is about 1.53Mb long with 30.9 mol% G+C content. The Genbank Accession number for the 16S rRNA gene sequence of strain Marseille-P3668^{T} is LT576413 and for the whole genome shotgun project is XX. This strain was isolated from fresh stool of a healthy French volunteer.

Nucleotide sequence accession number. The 16S rRNA gene and genome sequences were deposited in Genbank under accession number LT972121 and OPYI00000000, respectively for Strain Marseille-P3761^{T} and under accession number LT576413 and XX, respectively for Strain Marseille-P2932^{T}.

Deposit in culture collections. Strain Marseille-P3761^{T} was deposited in our strain collections under number (= CSURP3761) and Strain Marseille-P2932^{T} under number (= CSURP2932).

Conflict of interest

None to declare

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Table 1: Phenotypic characterization of *Peptoniphilus colimassiliensis* strain Marseille-P3761^{T} sp. nov. and *Peptoniphilus duodeni* sp. nov strain Marseille-P2932^{T}, based on analytical profile index (API) ZYM tests.

Tests	Characteristics	P3761^{T}	P2932^{T}
API ZYM	Alkaline phosphatase	-	-
	Esterase (C4)	-	-
	Esterase Lipase (C8)	-	-
	Lipase (C14)	-	-
	Leucine arylamidase	-	+
	Valine arylamidase	-	-
	Cystine arylamidase	-	-
	Trypsin	-	-
	α-chymotrypsin	-	-
	Acid phosphatase	-	+
	Naphthol-AS-BI-phosphohydrolase	-	+
	α-galactosidase	-	-
	β-galactosidase	-	-
	β-glucuronidase	-	-
	α-glucosidase	-	-
	β-glucosidase	-	-
	N-acetyl-β-glucosaminidase	-	-
	α-mannosidase	-	-
	α-fucosidase	-	-
	Glycerol	-	+

Table 2: Phenotypic characterization of *Peptoniphilus colimassiliensis* strain Marseille-P3761^T sp. nov. and *Peptoniphilus duodeni* sp. nov strain Marseille-P2932^T, based on API 50 CH test.

Tests	Characteristics	P3761^T	P2932^T
50 CH	Erythritol	-	-
	D-arabinose	-	-
	L-arabinose	-	-
	D-ribose	+	-
	D-xylose	-	-
	L-xylose	-	-
	D-Adonitol	-	-
	Methyl βD-xylopyranoside	-	-
	D-galactose	+	-
	D-glucose	+	-
	D-fructose	+	-
	D-mannose	-	+
	L-sorbose	-	-
	L-rhamnose	-	-
	Dulcitol	-	-
	Inositol	-	+
	D-mannitol	+	-
	D-sorbitol	-	-
	Methyl αD-mannopyranoside	-	-
	Methyl αD-glucopyranoside	-	-
	N-acetyl-glucosamine	-	-
	Amygdalin	-	-
	Arbutin	-	-
	Esculin ferric citrate	-	-
	Salicin	-	-
	D-cellobiose	-	-
	D-maltose	+	-
	D-lactose	+	-
	D-melibiose	-	-
	D-saccharose	+	-
	D-trehalose	+	-
	Inulin	-	-
	D-melezitose	-	+
	D-raffinose	-	-
	Amidon	-	+
	Glycogen	-	-
	Xylitol	-	-
	Gentiobiose	-	+
	D-turanose	-	-
	D-xylose	-	-
	D-tagalose	-	-
	D-fucose	-	-
	L-fucose	-	-
	D-arabitol	-	-
	L-arabitol	-	-

	Potassium gluconate	-	-
	Potassium 2-ketogluconate	-	-
	Potassium 5-ketogluconate	-	-

Table 3: Comparison of differential characteristics between *Peptoniphilus colimassiliensis* sp. nov., *Peptoniphilus duodeni* sp. nov., and other bacterial species like *and Peptoniphilus coxii* and *Peptoniphilus obesi.*

Property	*P. colimassiliensis*	*P. duodeni*	*P. coxii*	*P. obesi*
Cell diameter (µm)	0.5	0.7	0.7	0.7-0.93
Oxygen requirement	-	-	-	-
Gram stain	+	+	+	+
Salt requirement	-	-	-	-
Motility	-	-	-	-
Endospore formation	-	-	-	-
Alkaline phosphatase	-	-	-	-
Catalase	-	-	-	-
Oxidase	-	-	-	-
β-Galactosidase	-	-	-	-
N-acetyl-glucosamine	-	-	-	-
Arabinose	-	-	-	-
Lipase (C8)	-	-	NA	-
Mannose	-	+	+	+
Mannitol	+	-	NA	NA
Sucrose	+	-	NA	-
D-Glucose	+	-	NA	NA
D-Fructose	+	-	NA	NA
D-Maltose	+	-	NA	NA
Source	Human	Human	Human	Human

Table 4.1: Fatty acid profiles (%) of *Peptoniphilus colimassiliensis* strain Marseille-P3761^{T}.

Fatty acids	Name	Mean relative %*
16:0	Hexadecanoic acid	49.4 ± 1.4
18:1n9	9-Octadecenoic acid	19.6 ± 1.5
5:0 iso	3-methyl-Butanoic acid	13.5 ± 0.6
14:0	Tetradecanoic acid	7.2 ± 0.5
18:2n6	9,12-Octadecadienoic acid	5.7 ± 0.2
10:0	Decanoic acid	1.6 ± 0.1
18:0	Octadecanoic acid	1.4 ± 0.3
12:0	Dodecanoic acid	TR
15:0	Pentadecanoic acid	TR

*****Mean peak area percentage; TR = trace amounts < 1

Table 4.2: Fatty acid profiles (%) of *Peptoniphilus duodeni* strain Marseille-P2932^{T}.

Fatty acids	Name	Mean relative %*
14:0	Tetradecanoic acid	44.3 ± 0.7
16:0	Hexadecanoic acid	27.7 ± 0.3
12:0	Dodecanoic acid	10.9 ± 0.3
18:1n9	9-Octadecenoic acid	5.2 ± 0.2
18:0	Octadecanoic acid	3.5 ± 0.3
15:0	Pentadecanoic acid	2.1 ± 0.4
18:2n6	9,12-Octadecadienoic acid	1.9 ± 0.1
10:0	Decanoic acid	1.0 ± 0.1
13:0	Tridecanoic acid	TR
18:1n7	11-Octadecenoic acid	TR
16:1n7	9-Hexadecenoic acid	TR
13:0 iso	11-methyl-Dodecanoic acid	TR
17:0	Heptadecanoic acid	TR
18:1n6	12-Octadecenoic acid	TR
14:1**	Tetradecenoic acid	TR
11:0	Undecanoic acid	TR

*****Mean peak area percentage; TR = trace amounts < 1; **double bond position was not defined

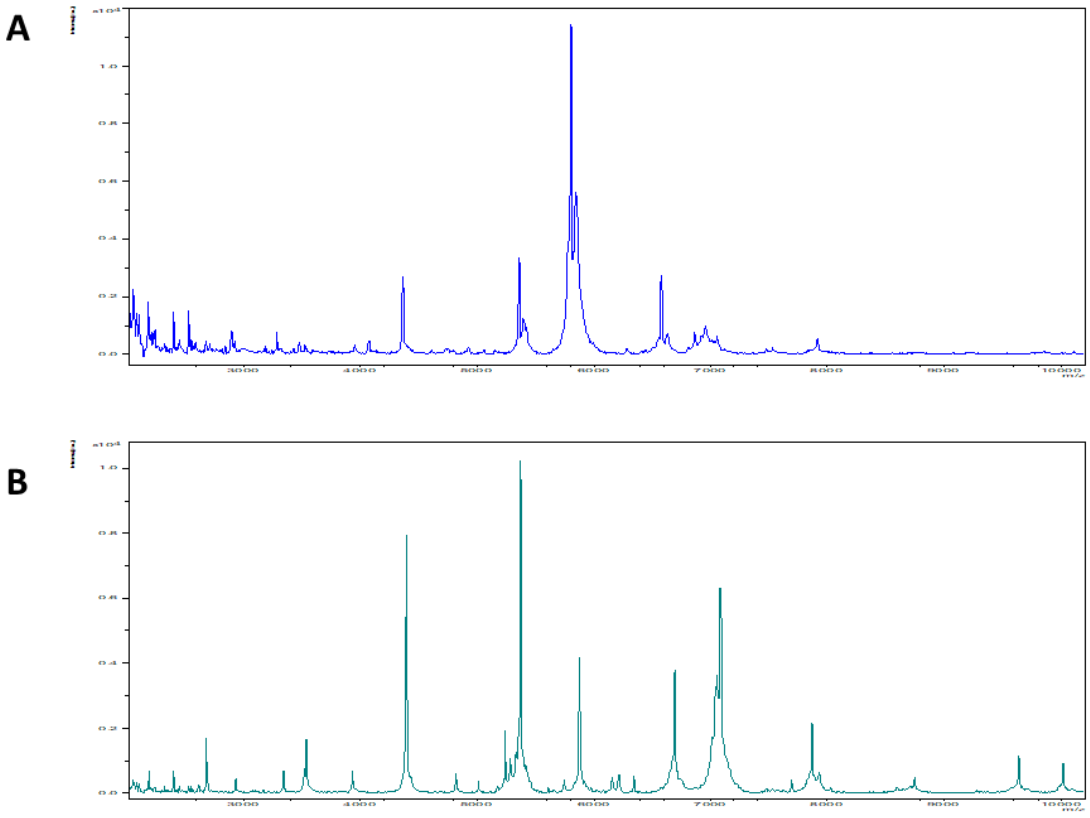


Figure 1: MALDI-TOF MS Reference mass spectrum of *Peptoniphilus colimassiliensis* sp. nov., strain Marseille-P3761^{T}(**A**)and *Peptoniphilus duodeni* sp. nov., strain Marseille-P2932^{T}(**B**). The reference spectrum was generated by comparison of spectra from 12 individual colonies.

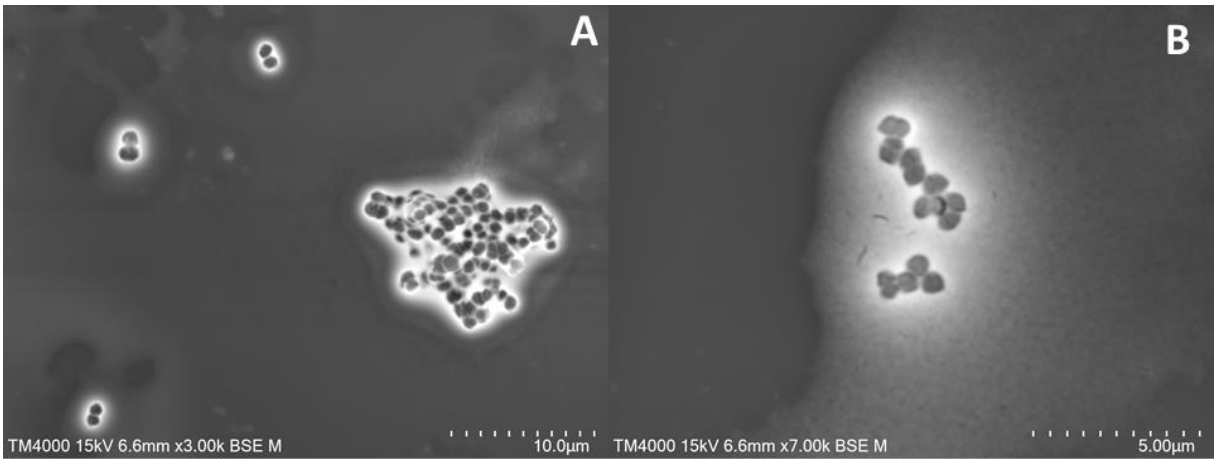


Figure 2: Scanning electron microscopy of stained *Peptoniphilus colimassiliensis* sp. nov., strain Marseille-P3761^{T}(**A**) and *Peptoniphilus duodeni* sp. nov., strain Marseille-P2932^{T}(**B**) (Hitachi TM4000). Scales and acquisition settings are shown on figure.

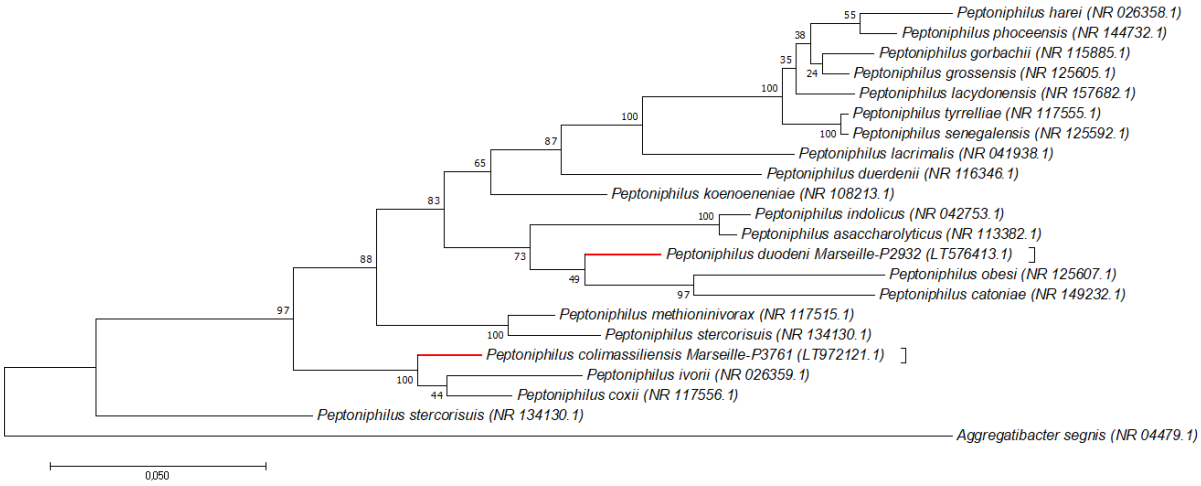


Figure 3: Phylogenetic trees highlighting the position of *Peptoniphilus colimassiliensis sp. nov. and Peptonophilus duodeni* sp. nov., based on the 16S rRNA gene sequences relative to the most closely related type strains within the genus *Peptoniphilus*. Genbank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference were obtained using the Maximum likelihood method and the MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 1% nucleotide sequence divergence.

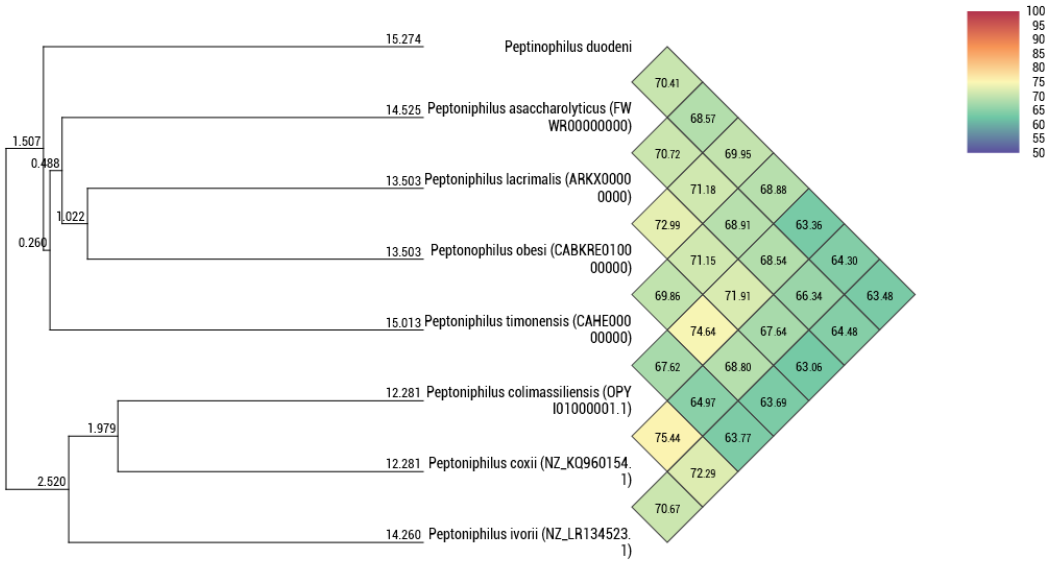
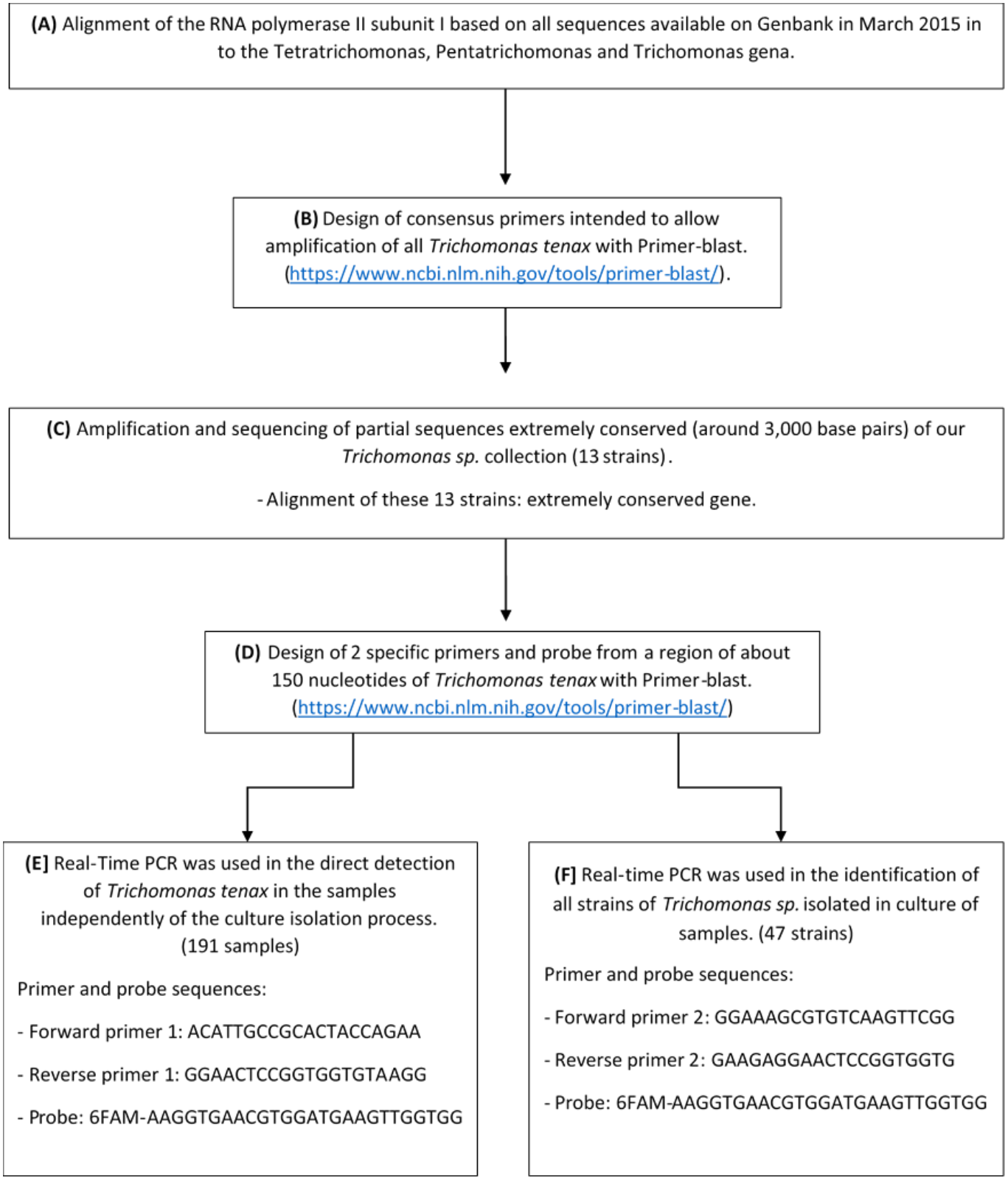


Figure 4: Heatmap generated with OrthoANI values calculated using the OAT software between *Peptoniphilus colimassiliensis sp. nov.* and *Peptoniphilus duodeni* sp. nov., and other closely related species with standing in nomenclature.

Annexe de la Publication n°2

Supporting information

Supplementary Scheme 1. Quantitative Real-Time PCR (q-RT PCR) specific for *Trichomonas tenax*



S1 Table : The table below presents the classification of each patient. The numbers correspond to a patient and preserve anonymity. The discontinuity of the numbering is linked to the change of operator in the laboratory. In black: patients with periodontitis. - In bold: the patient controls.

PATIENTS				**Classification**	**Smoking**
Number	*Name*	*Sexe*	*Age*	*M : Mild, Mo : Moderate, S : Severe*	
8	H. H	F	38	S	Yes
9	V. H	F	56	S	No
10	**G. J**	F	29	-	No
11	**N. H**	M	37	-	No
12	**S. M**	M	22	-	Yes
13	S. L	F	56	S	No
14	E. P	M	55	S	Yes
17	D. J-L	M	60	Mo	No
18	Z. P-W	M	44	Mo	No
19	F. G	M	57	S	No
20	**N. N**	M	32	-	No
22	**K. M**	F	61	-	Yes
23	H. S	F	45	S	No
24	**P. L-P**	M	42	-	No

25	M. C	F	67	S	No
27	**N. J**	F	32	-	Yes
29	V. M-D	F	65	M	No
30	R. A	M	59	S	Yes
31	**T. M**	F	25	-	Yes
34	J. C	F	63	Mo	No
35	**C. C**	F	24	-	No
36	M. M	F	50	S	No
37	**F. D**	M	73	-	No
38	E. A	M	61	Mo	Yes
39	C. J	F	66	S	No
40	**F. M-L**	F	50	-	No
41	G. B	M	47	S	No
42	J. C	F	77	S	No
43	**P. J-L**	M	63	-	No
44	M. V	M	67	S	Yes
45	C. C	F	45	Mo	No
46	S. M	M	61	S	Yes
47	C. O	M	19	M	Yes
48	P. L	M	44	M	Yes
50	**C. A**	M	64	-	Yes
52	**B. M**	F	50	-	Yes
53	G. R	M	51	S	Yes
54	B. B	M	56	S	Yes

55	**H. M**	F	21	-	No
56	**N. M**	M	50	-	Yes
57	**G. R**	M	33	-	No
58	**B. M**	M	21	-	Yes
59	**P. J**	M	23	-	Yes
60	**N. O**	M	24	-	No
61	C. C	M	47	M	No
62	Q. G	M	70	Mo	No
63	G. S	M	35	S	No
64	M. J-L	M	67	Mo	No
65	T. L	M	69	S	No
66	C. V	F	45	S	No
100	B. B	M	54	S	Yes
101	**S. K**	M	21	-	No
102	A. S	M	52	Mo	Yes
103	**T. S**	F	30	-	No
104	G. M-J	F	51	S	No
105	C. A	F	76	Mo	No
106	G. H	M	19	M	No
107	D. E	F	55	M	Yes
108	N. M	M	44	M	No
109	E. S	F	42	Mo	No
110	H. M	M	75	S	No
111	**C. Y**	F	87	-	No

112	D. M	F	61	Mo	Yes
113	A. A	M	30	S	Yes
114	D. C	M	58	S	No
115	B. J	M	41	Mo	Yes
116	N. M	F	66	S	No
117	B. C	M	46	S	No
118	R. N	F	35	Mo	Yes
119	**K. M**	M	45	-	No
120	S. C	F	29	S	Yes
121	B. C	F	57	S	Yes
122	B. G	M	72	S	Yes
123	B. R	M	83	M	No
124	G. I	F	56	S	No
125	B. C	F	52	S	No
126	N. L	M	36	S	No
127	**A. E**	F	22	-	Yes
128	T. M	M	65	S	No
129	**F. S**	M	28	-	No
130	D. M	F	52	S	No
131	A. J-J	M	68	S	No
132	G. L	M	45	Mo	No
133	C. A	M	70	S	No
134	R. J	F	64	S	Yes
135	G. M	F	75	S	Yes

136	**B. J**	M	81	-	No
137	M. A-K	M	53	Mo	No
138	P. M-T	F	59	S	No
139	B. G	M	68	S	No
140	G. A	M	74	S	No
141	C. B	F	56	S	Yes
142	F. D	M	68	M	No
143	**D. S**	M	24	-	No
144	B. A	F	67	Mo	No
145	T. C	F	55	Mo	No
146	A. M	F	45	S	Yes
147	B. A	F	65	Mo	No
148	M. P	M	43	S	Yes
149	A. R	F	71	S	No
150	E. T	F	40	S	Yes
151	A. A	F	54	S	Yes
152	S. P	M	53	S	Yes
153	B. F	F	43	S	Yes
200	B. A	M	61	S	No
201	C. L	F	45	S	No
202	C. C	F	51	S	Yes
203	S. A	F	56	Mo	No
204	L. M	M	42	Mo	Yes
205	F. P	M	66	S	No

206	D. S	F	49	M	No
207	**P. J**	F	44	-	No
208	C. N	F	55	M	No
209	M. G	F	68	M	No
210	R. P	M	89	Mo	No
211	F. S	F	63	S	Yes
212	K. G	F	48	Mo	No
213	D. L	M	64	S	No
214	R. F	M	35	S	No
215	F. D	F	66	Mo	No
216	F. M-J	F	64	Mo	No
217	D. Y	F	54	Mo	Yes
220	T. P	M	50	S	Yes
221	L. J-P	M	50	S	Yes
222	K. C	F	45	S	No
223	**B. R**	F	27	-	No
224	**B. M**	F	30	-	Yes
225	D. A	M	57	M	Yes
227	P. O-M	M	55	S	Yes
228	**A. S**	M	18	-	No
229	**D. Y**	M	35	-	Yes
230	C. C	F	65	Mo	No
231	S. J-L	M	40	Mo	Yes
232	**D. A**	M	41	-	No

233	**E. J-J**	M	45	-	No
234	**P. E**	F	34	-	No
235	**B. N**	F	76	-	No
236	**T. J**	M	59	-	Yes
237	**Z. E**	F	60	-	No
238	**G. L**	F	31	-	No
239	**B. G**	M	58	-	Yes
240	**Q. T**	F	76	-	No
241	**F. P**	F	40	-	Yes
242	**C. P**	F	55	-	No
243	**P. D**	F	52	-	No
244	**L. A**	F	35	-	No
245	**E. S**	M	20	-	Yes
246	**S. F**	M	42	-	No
247	**K. M**	F	75	-	No
248	**L. C**	F	67	-	Yes
249	**D. J**	M	62	-	No
250	**D. O**	F	33	-	No
251	B. M	M	71	M	No
252	**D. M**	M	28	-	No
253	D. A-M	F	66	M	No
255	**L. F**	M	29	-	Yes
256	**F. R**	F	65	-	No
257	**M. M**	F	67	-	No

258	**B. H**	M	22	-	Yes
259	**T. R-M**	F	49	-	Yes
260	**S. M**	M	25	-	No
261	**C. S**	F	45	-	No
262	**H. O**	M	37	-	No
263	**M. J**	M	24	-	Yes
264	**M. G**	M	68	-	No
265	**D. N**	F	54	-	Yes
266	**D. M**	F	28	-	No
267	**N. G**	M	28	-	No
268	**L. Y**	M	63	-	No
269	**L. L**	F	42	-	No
270	**P. J**	M	34	-	No
271	**M. V**	M	26	-	Yes
272	**K. C**	M	26	-	No
273	**B. A**	F	31	-	Yes
274	**M. I**	M	18	-	Yes
275	**T. H**	M	57	-	No
276	C. M	F	72	M	No
277	**G. A-T**	F	20	-	No
278	**S. R**	F	33	-	No
279	**L. M**	F	23	-	No
280	G. L	M	43	M	Yes
281	**A. S**	F	51	-	No

282	**D. C**	F	34	-	Yes
283	**A. I**	F	22	-	No
284	**A. H**	F	27	-	No
285	**N. T**	M	50	-	No
286	**D. M**	F	27	-	No
287	P. R	M	56	M	No
288	**T. H**	F	33	-	No
289	**C. G**	F	63	-	Yes
290	B. S	F	36	M	No

S2 Table: Sequence types of *Trichomonas tenax* based on single-nucleotide polymorphism against the *Trichomonas tenax* reference strain. In bold: the number of sequences types.

	ALTS	**DMRP**	**SHMT**	**M6PI**	**GAT1**	**HIST**	**CYS**	**ST**
Trichomonas_tenax_NIH4	ID	ID	ID	ID	ID	ID	ID	**0**
249	0	0	0	0	0	1	0	**1**
37	0	0	0	0	0	2	0	**2**
56	0	0	0	0	2	1	0	**3**
48	0	0	0	1	1	0	0	**4**
229	0	0	0	1	1	0	0	**4**
213	0	0	0	1	2	0	0	**5**
200	0	0	0	3	0	1	0	**6**
141	0	0	0	5	0	0	0	**7**
216	0	0	2	0	0	1	0	**8**
207	0	0	2	0	1	0	0	**9**
282	0	0	2	0	1	1	0	**10**
241	0	0	2	3	2	3	0	**11**
150	0	0	2	6	0	3	0	**12**
242	0	1	0	1	0	0	0	**13**
30	0	1	0	1	1	0	0	**14**
290	0	1	0	1	1	0	0	**14**
205	0	1	0	1	1	1	0	**15**
264	0	1	0	3	1	3	0	**16**
253	0	1	0	3	2	4	0	**17**
64	0	1	0	3	3	4	0	**18**
112	0	1	0	4	0	1	0	**19**
204	0	1	2	0	2	4	0	**20**
212	0	1	2	1	0	0	0	**21**
53	0	1	2	1	0	1	0	**22**

220	0	1	2	1	0	1	0	**22**
104	0	3	3	0	0	5	0	**23**
17	1	0	0	2	0	1	0	**24**
13	1	1	1	1	0	0	0	**25**
120	1	1	2	1	1	0	0	**26**
115	1	1	2	4	0	1	0	**27**
31	2	0	0	0	0	1	0	**28**
34	2	0	0	0	0	1	0	**28**
100	2	0	0	0	1	5	0	**29**
203	2	0	0	1	1	3	0	**30**
285	2	0	0	3	0	3	0	**31**
153	2	0	0	7	0	8	0	**32**
146	2	0	4	5	0	6	0	**33**
52	2	1	0	1	0	0	0	**34**
261	2	1	0	3	0	0	0	**35**
63	2	1	0	3	0	4	0	**36**
139	2	1	2	1	0	0	0	**37**
101	2	2	0	0	1	5	0	**38**
151	2	4	2	6	0	7	0	**39**
221	2	5	0	1	0	0	0	**40**
211	3	1	2	3	0	1	0	**41**
217	4	1	2	0	2	0	0	**42**
246	5	0	0	0	0	3	0	**43**

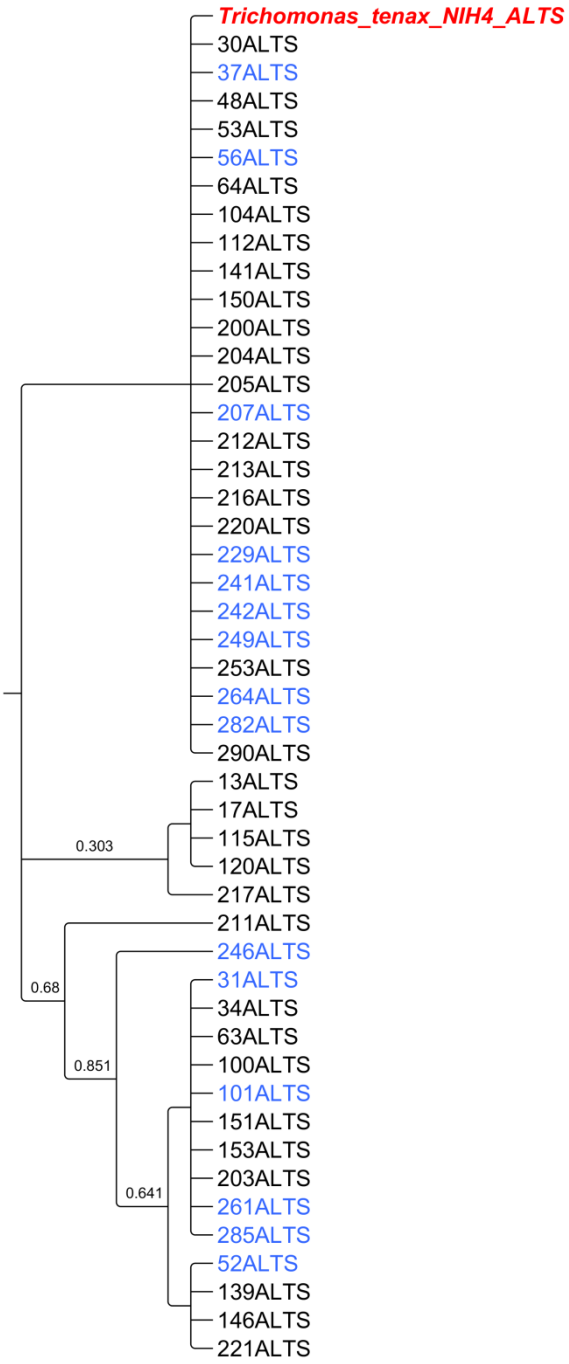
S3 Table: The table below presents the cycle threshold obtained in q-PCR for the *RPB1*gene and the albumin gene for the standardization. In black: periodontitis patients. In bold: controls.

Samples	**Culture**	***RPB1*** **(Ct1)**	**Albumine (Ct2)**	**Ratio (Ct1/Ct2)**
8	Pos	36,74	27,73	1,32
9	Pos	35,35	27,6	1,28
13	Pos	34,43	25,32	1,36
14	*Neg*	32,08	31,49	1,02
17	Pos	34,38	28,98	1,19
22	Pos	34,65	29,05	1,19
24	*Neg*	35	24,88	1,41
31	Pos	34,48	29,46	1,17
34	Pos	36,7	29	1,27
37	Pos	31,93	25,37	1,26
44	*Neg*	37,4	25,76	1,45
47	*Neg*	34,38	28,6	1,20
48	Pos	35,72	25,54	1,40
52	Pos	32,83	26,7	1,23
53	Pos	32,12	25,12	1,28
54	Pos	30,63	23,16	1,32
56	Pos	35,14	28,36	1,24
61	*Neg*	32,04	26,07	1,23
63	Pos	35,03	25,57	1,37
64	Pos	34,67	26,3	1,32

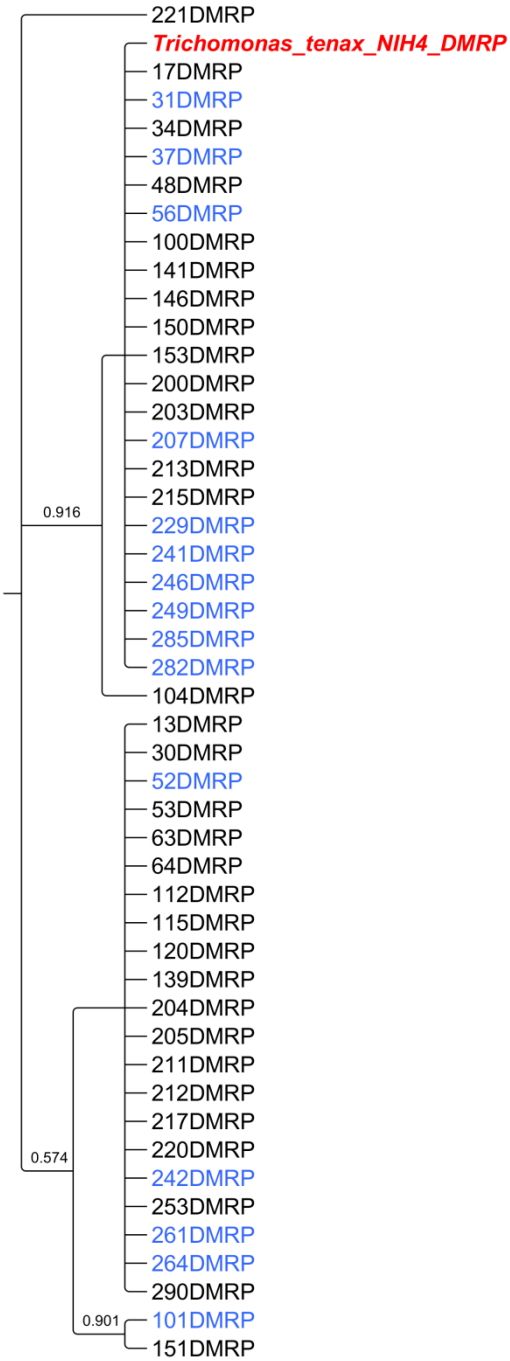
100	Pos	36,51	27,4	1,33
101	Pos	36,56	28,54	1,28
104	Pos	33,34	28,9	1,15
109	*Neg*	35,91	29,88	1,20
112	Pos	30,31	26,56	1,14
115	Pos	30,41	27,61	1,10
120	Pos	31,76	28,31	1,12
131	*Neg*	35,44	31,67	1,12
139	Pos	37,12	32,11	1,16
146	Pos	33,58	30,29	1,11
150	Pos	35,11	26,91	1,30
153	Pos	38,2	28,44	1,34
200	Pos	35,13	25,68	1,37
203	Pos	37,85	27,33	1,38
204	Pos	39	29,18	1,34
205	Pos	36,98	25,52	1,45
207	Pos	35,36	27,8	1,27
211	Pos	35,93	28,26	1,27
212	Pos	34,68	27,31	1,27
220	Pos	38,21	29,96	1,28
221	Pos	38,45	30,05	1,28
229	Pos	37,73	38,46	0,98
231	*Neg*	37,02	28,68	1,29
241	Pos	33,23	29,05	1,14

242	Pos	37,2	30,08	1,24
246	Pos	34,42	27,06	1,27
249	Pos	35,11	30,03	1,17
253	Pos	37,26	30,8	1,21
259	*Neg*	38,02	24,37	1,56
261	Pos	37,87	25,38	1,49
264	Pos	38,91	25,8	1,51
265	*Neg*	37,92	26,07	1,45
279	*Neg*	38	25,29	1,50
280	*Neg*	38,97	26,02	1,50
282	Pos	33,69	24,24	1,39
284	*Neg*	37,92	24,05	1,58
285	Pos	34,14	26,02	1,31
286	*Neg*	37,63	24,09	1,56
288	*Neg*	38,81	24,47	1,59
290	Pos	34,33	23,61	1,45

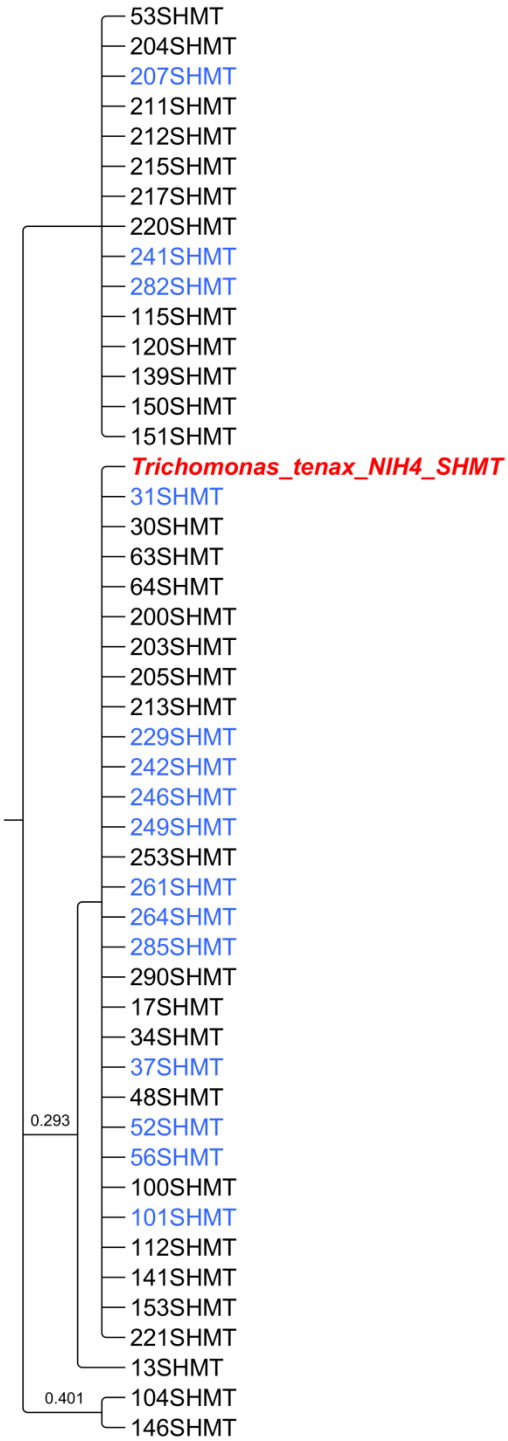
Supplementary Figure 1 *Trichomonas tenax* phylogenetic analysis based on Alanyl tRNA synthetase (ALTS) gene. In black: patients with periodontitis. In blue: the patient controls.



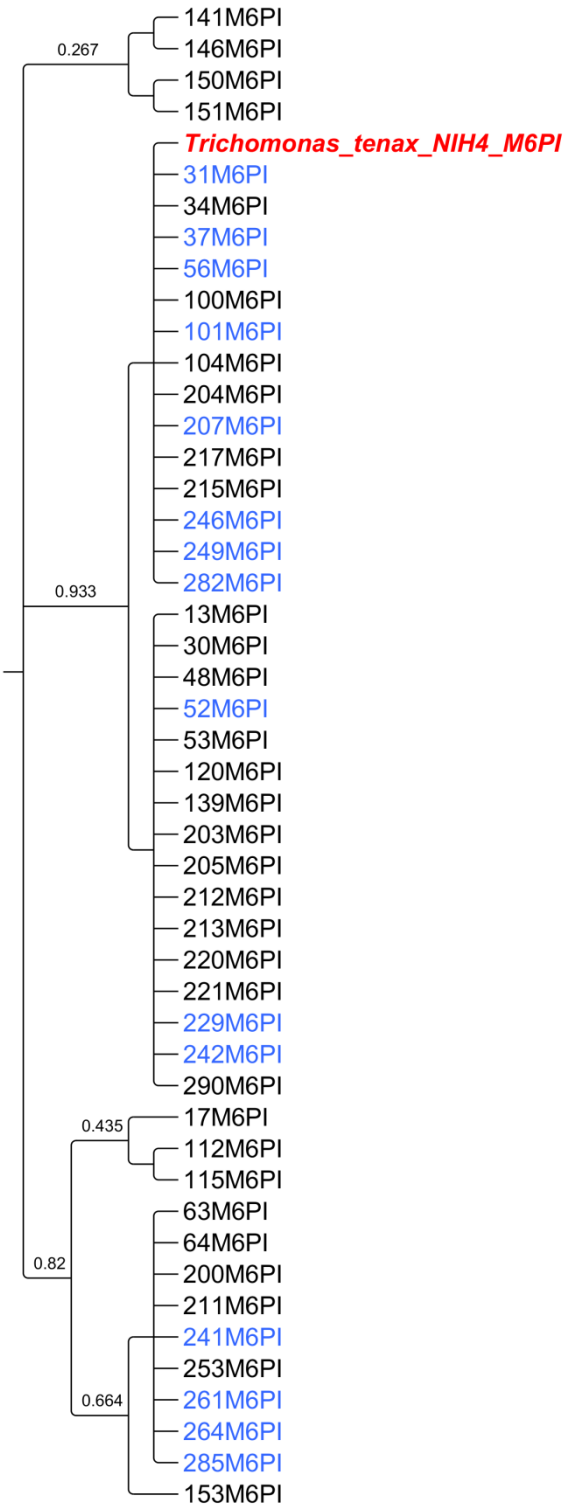
Supplementary Figure 2 *Trichomonas tenax* phylogenetic analysis based on DNA mismatch repair protein (DMRP) gene. In black: patients with periodontitis. In blue: the patient controls.



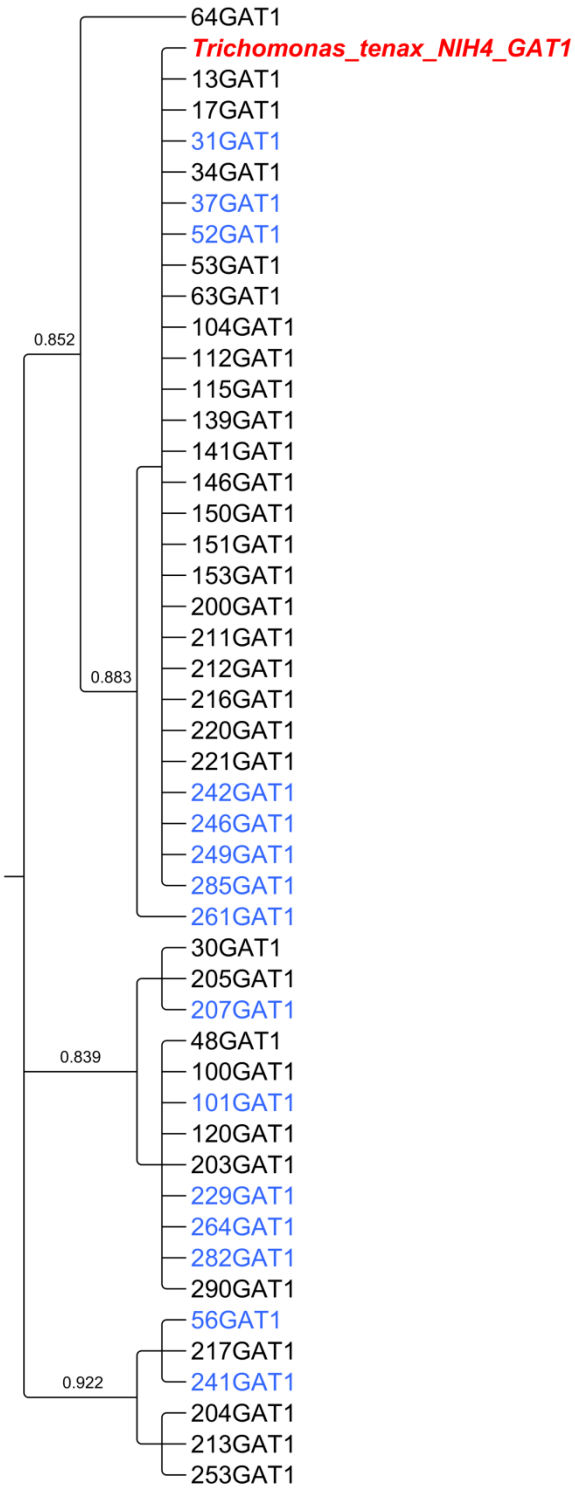
Supplementary Figure 3 *Trichomonas tenax* phylogenetic analysis based on Serine hydromethyltransferase (SHMT) gene. In black: patients with periodontitis. In blue: the patient controls.



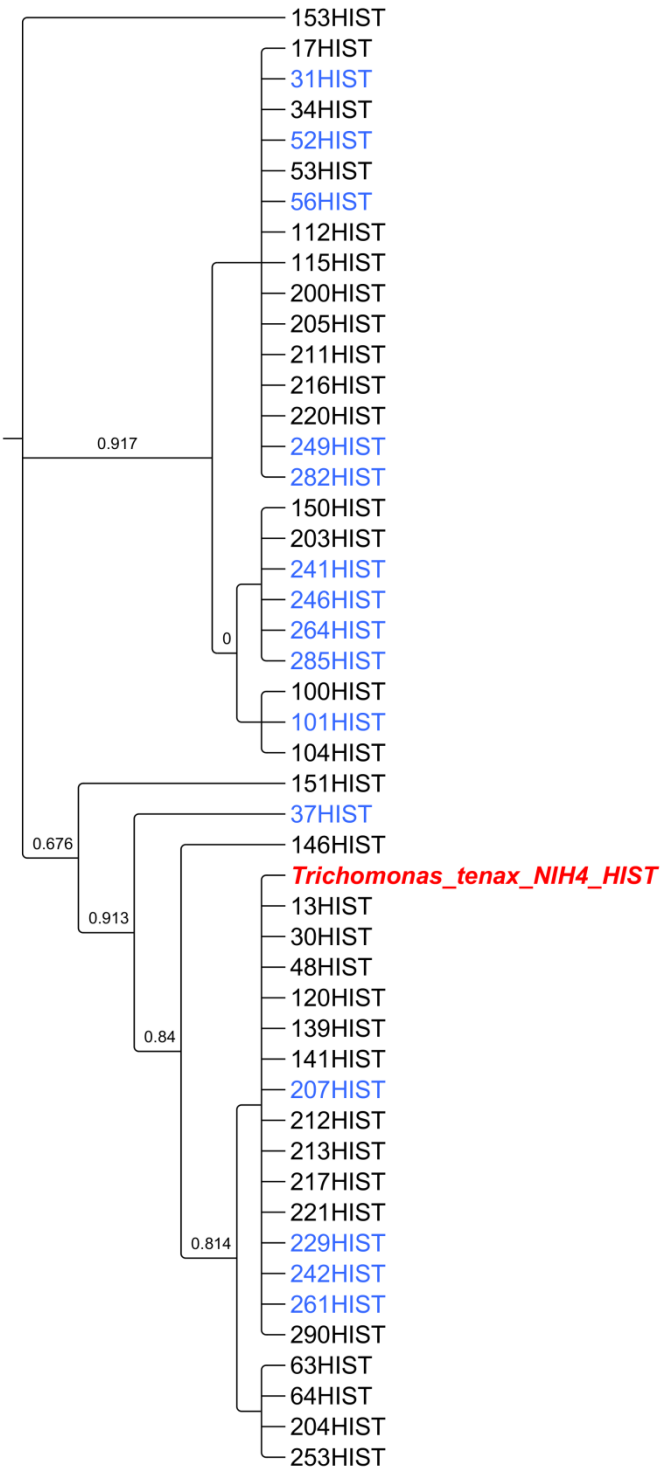
Supplementary Figure 4. *Trichomonas tenax* phylogenetic analysis based on Mannose -6-phosphate isomerase (M6PI) gene. In black: patients with periodontitis. In blue: the patient controls



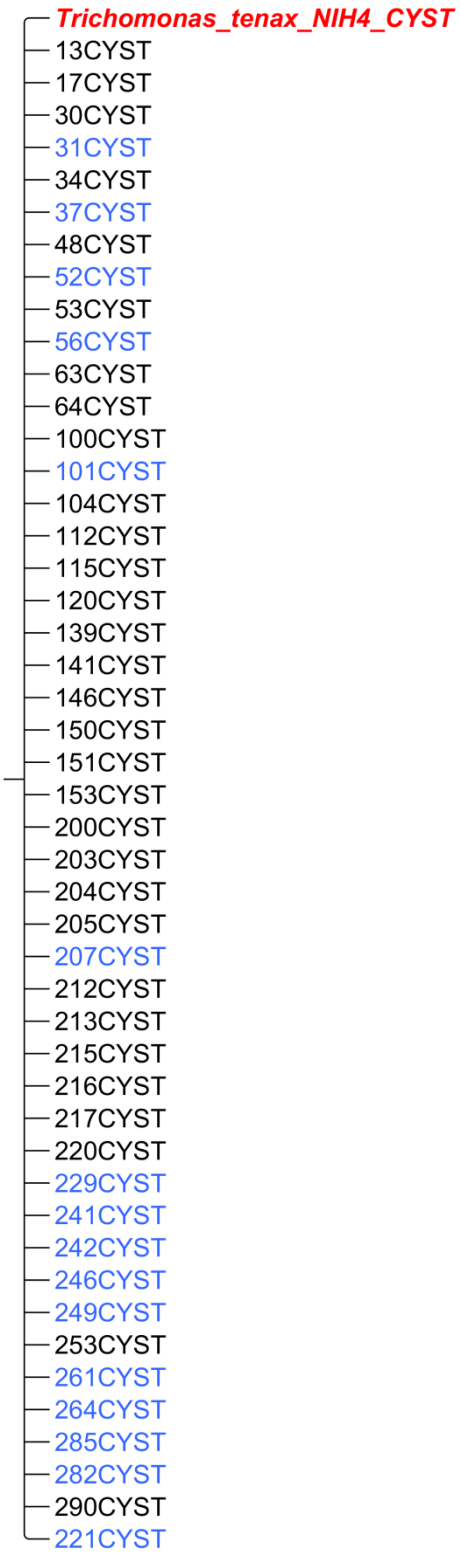
Supplementary Figure 5. *Trichomonas tenax* phylogenetic analysis based on Glutamine amidotransferase class-I (GAT1) gene. In black: patients with periodontitis. In blue: the patient controls.



Supplementary Figure 6. *Trichomonas tenax* phylogenetic analysis based on Histidyl tRNA synthetase (HIST) gene. In black: patients with periodontitis. In blue: the patient controls.



Supplementary Figure 7. *Trichomonas tenax* phylogenetic analysis based on Cysteinyl tRNA synthetase (CYST) gene. In black: patients with periodontitis. In blue: the patient controls.



Annexe de la Publication n°3

S1 Table : The table below presents the classification of each patient. The numbers correspond to a patient and preserve anonymity. In black: patients with periodontitis. - In bold: the patient controls

PATIENTS			**Classification**
Number	*Sex*	*Age*	*M : Mild, Mo : Moderate, S : Severe*
P1	M	59	S
P2	W	67	Mo
P3	M	52	Mi
P4	M	43	Mo
P5	W	41	S
P6	M	59	S
P7	M	57	S
P8	W	71	Mi
P9	W	49	S
P10	M	64	Mi
C1	W	26	-
C2	W	47	-
C3	W	25	-
C4	W	27	-
C5	M	26	-
C6	W	66	-
C7	W	64	-
C8	W	33	-
C9	W	58	-
C10	M	46	-

S2 Table : Primers used targeted on species related to periodontitis.

Species	Genes	Forward	Reverse	Sequence length (bp)	Article on pubmed
Methanovibracter sp.	16S rRNA	5'- TCCAGGCCCTACGGG-3'	5'-YCCGGCGTTGAMTCCAATT-3'	-	Grine et al.(2018)
CPR (TM7)	16S rRNA	5'-GACCTGAGATCATCCCCTCCTTCC-3'	5'-AYTGGGCGTAAAGAGTTCC-3'	597	Takenaka et al. (2018), Sizova et al. (2015)
Treponema sp.	16S rRNA	5'- AGAGTTTGATCMTGGCTCAG-3'	5'-GTTACGACTTCACCCTCCT-3'	-	Huo et al. (2017)
Entamoeba gingivalis	18S rRNA	5'-TACCATACAAGGAATAGCTTTGTGAATAA-3'	5'- ACAATTGTAAATTTGTTCTTTTTCT-3'	135	Trim et al. (2011)
Trichomonas tenax	Rpb1	5'-ACATTGCCGCACTACCAGAA-3'	5'-GGAACTCCGGTGGTGTAAGG-3'	830	Benabdelkader et al (2018)
Porphyromonas gingivalis	rpob	5'-GGAAGAGAAGACCGTAGCACAAGGA-3'	5'-GAGTAGGCGAAACGTCCATCAGGTC-3'	143	Park et al. (2011)

S3 Table : Number of reads obtained in analysis after 16S rRNA pyrosequencing by Miseq Technlogy

Sample	Reads	After assembly	Length	Quality Filtered	Unique Seq.	Non Chimerique.	OTUs	Blast	Species	Genus	Family
Patient 1	35 752	35 181	210-491	26 270	15 597	15 314	2 052	2 044	108	72	1 054
Patient 2	40 123	39 467	203-490	28 587	18 535	18 399	2 411	2 407	100	64	1 149
Patient 3	35 540	35 035	211-485	25 010	14 624	14 433	2 056	2 054	80	54	1 148
Patient 4	36 611	36 037	200-488	26 542	18 651	18 632	2 623	2 621	95	64	1 281
Patient 5	133 678	131 950	200-491	95 855	47 881	43 584	5 043	5 019	103	68	2 777
Patient 6	105 598	103 957	226-490	77 982	41 963	39 448	4 550	4 537	101	69	2 387
Patient 7	107 073	105 357	200-490	77 962	37 554	33 158	3 333	3 325	99	55	1 834
Patient 8	51 581	50 602	200-485	35 025	17 479	15 837	1 278	1 272	69	39	725
Patient 9	127 056	125 268	200-491	93 641	45 696	41 156	4 100	4 091	110	64	2 263
Patient 10	141 146	138 947	200-491	101 065	46 536	40 515	4 376	4 335	91	71	2 718
Control 1	122 385	118 799	200-491	85 133	44 499	42 277	4 417	4 221	116	44	2 318
Control 2	112 045	110 383	203-492	82 469	45 306	43 460	5 744	5 721	95	68	3 131
Control 3	123 432	121 744	200-492	86 441	46 582	43 758	4 914	4 894	77	56	2 843
Control 4	105 555	104 011	200-492	76 848	47 720	46 342	6 018	6 008	125	47	3 065
Control 5	98 643	97 139	200-491	71 760	38 765	37 178	4 829	4 808	101	60	2 817
Control 6	62 868	61 501	200-487	41 349	24 693	23 584	2 626	2 610	87	57	1 575
Control 7	105 942	104 224	200-490	76 903	39 555	36 639	4 326	4 315	96	59	2 635
Control 8	124 994	123 085	200-491	89 438	48 455	46 188	6 079	6 063	132	74	3 283
Control 9	109 952	108 027	200-492	75 696	36 064	32 935	2 984	2 958	11	43	2 231
Control 10	111 271	109 544	200-491	82 067	43 992	41 820	5 228	5 218	116	64	2 885

S1 Figure. List of species obtained by culturomic method.

(A)

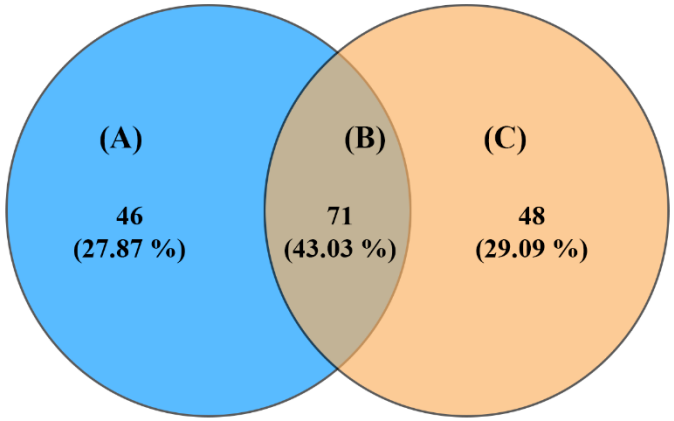
Periodontitis
Actinomyces europaeus
Actinomyces grossensis
Actinomyces mediterraneense
Bacillus pumilus
Bacteriodetes pyogenes
Bacteroides heparinolyticus
Bifidobacterium dentium
Bifidobacterium longum
Bilophila wadsworthia
Brachybacterium rhamnosum
Campylobacter gracilis
Citrobacter freundii
Corynebacterium dentalis
Dialister invisus
Eggerthella lenta
Eggerthella timonensis
Eggerthia catenaformis
Fusonucleatum naviforme
Gemella haemolysans
Hafnia alvei
Jeddahella massiliensis
Klebsiella oxytoca
Lactobacillus mucosae
Lactobacillus reuteri
Mogibacterium timidum
Neisseria bacilliformis
Neisseria oralis
Olsenella phoceensis
Olsenella scatoligenes
Olsenella timonensis
Pediococcus pentosaceus
Prevotella dentalis
Prevotella heparinolytica
Prevotella intermedia
Prevotella marshii
Prevotella oris
Propionibacterium acidifacien
Propionibacterium lymphophilum
Propionobacterium propionilum
Pyramidobacter piscolens
Staphyloccocus aureus
Staphylococcus pasteuri
Stenotrophomonas maltophila
Streptococcus dentisani
Streptococcus pneumoniae
Streptococcus vestibularis

(B)

Common bacterial species
Actinobaculum naeslundi
Actinomyces georgiae
Actinomyces naeslundii
Actinomyces odontolyticus
Actinomyces oris
Aggregatibacter segnis
Anaeroglobus germinatus
Atopobium parvulum
Atopobium rimae
Campylobacter concisus
Campylobacter curvus
Campylobacter showae
Candida albicans
Capnocytophaga gingivalis
Capnocytophaga ochracea
Capnocytophaga sputigena
Cryptobacterium curtum
Dialister pneumosintes
Eikenella corrodens
Enterococcus durans
Enterococcus faecalis
Enterococcus faecium
Eubacterium infirmum
Fusobacterium nucleatum
Gemella morbillorum
Gemella sanguinis
Granulicatella adiacens
Haemophilus parainfluenzae
Kocuria rhizophila
Lactobacillus paracasei
Lactobacillus rhamnosus
Megasphaera micronuciformis
Micrococcus luteus
Mogibacterium neglectum
Neisseria bacilliformis
Neisseria elongata
Neisseria flavescens
Neisseria macacae
Neisseria subflava
Olsenella uli
Parvimonas micra
Porphyromonas gingivalis
Prevotella buccae
Prevotella nigrescens
Prevotella oralis
Prevotella phoceensis
Propionibacterium acnes
Pseudoramibacter alactolyticus
Rothia aeria
Rothia dentocariosa
Ruminococcus merdae
Selenomonas infelix
Shuttleworthia satelles
Slackia exigua
Solobacterium moorei
Staphylococcus epidermidis
Streptococcus anginosus
Streptococcus australis
Streptococcus constellatus
Streptococcus cristatus
Streptococcus gordonii
Streptococcus intermedius
Streptococcus massiliensis
Streptococcus mitis
Streptococcus mutans
Streptococcus oralis
Streptococcus parasanguinis
Streptococcus salivarius
Streptococcus sanguinis
Veillonella atypica
Veillonella parvula

(C)

Controls
Abiotrophia defectiva
Actinomyces denticolens
Actinomyces johnsonii
Actinomyces meyeri
Actinomyces oralis
Actinomyces timonensis
Aggregatibacter aphrophilus
Alloscardovia omnicolens
Arthrobacter gandavensis
Bacillus licheniformis
Bacillus sinesaloumensis
Brachymonas massiliensis
Brevibacterium pityocampae
Campylobacter rectus
Carnobacterium divergens
Citrobacter koseri
Collinsella massiliensis
Corynebacterium durum
Dialister propionicifaciens
Drancourtella massiliensis
Enterobacter asburiae
Enterobacter cloacae
Escherichia coli
Klebsiella pneumoniae
Lachnoanaerobaculum saburreum
Lactobacillus agilis
Lactobacillus delbrueckii
Lactobacillus fermentum
Lactobacillus lactis
Leptotrichia goodfellowii
Leptotrichia hofstadii
Leptotrichia wadei
Marseillibacter massiliensis
Microbacterium liquefaciens
Microbacterium maritypicum
Microbacterium oxydans
Neisseria cinerea
Pantoea calida
Peptostreptococcus stomatis
Prevotella denticola
Prevotella melaninogenica
Propionimicrobium lymphophilum
Propionobacterium granulosum
Pseudopropionibacterium propionicum
Rothia mucilaginosa
Serratia marcescens
Staphylococcus capitis
Vagococcus fluviales
Yersinia intermedia



S2 Figure. List of species detected by metagenomic method.

(A)

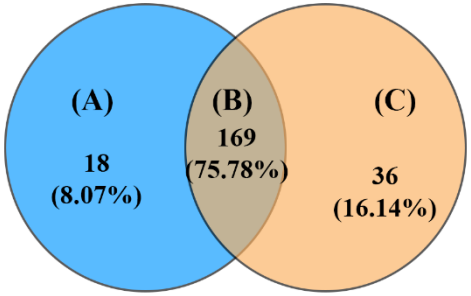
Periodontitis
Actinomyces meyeri
Aggregatibacter paraphrophilus
Bacteroides zoogleoformans
Catonella morbi
Corynebacterium tuberculostearicum
Gemella haemolysans
Jonquetella anthropi
Klebsiella aerogenes
Lactobacillus oris
Lactobacillus salivarius
Mogibacterium timidum
Moraxella osloensis
Ochrobactrum anthropi
Oribacterium parvum
Parascardovia denticolens
Porphyromonas catoniae
Porphyromonas uenonis
Staphylococcus warneri

(C)

Controls
Acidipropionibacterium acidifaciens
Actinomyces graevenitzii
Actinomyces timonensis
Bacteroidales bacterium
Bifidobacterium longum
Capnocytophaga ochracea
Corynebacterium amycolatum
Corynebacterium bovis
Desulfomicrobium orale
Enterococcus durans
Fusobacterium hwasookii
Gemella bergeri
Gracilibacteria bacterium
Haemophilus haemolyticus
Kocuria palustris
Lachnoanaerobaculum saburreum
Lachnospiraceae bacterium
Lactobacillus johnsonii
Lactobacillus vaginalis
Lactococcus lactis
Microbacterium flavescens
Neisseria cinerea
Neisseria lactamica
Neisseria polysaccharea
Paracoccus yeei
Porphyromonas asaccharolytica
Prevotella aurantiaca
Prevotella multisaccharivorax
Ruminococcaceae bacterium
Streptococcus australis
Streptococcus parasanguinis
Streptococcus sobrinus
Treponema amylovorum
Treponema pectinovorum
Variovorax paradoxus
Veillonellaceae bacterium

(B)

Common bacterial species	Common bacterial species	Common bacterial species	Common bacterial species
Abiotrophia defectiva	*Granulicatella elegans*	*Prevotella oris*	*Lachnoanaerobaculum orale*
Acinetobacter johnsonii	*Haemophilus parahaemolyticus*	*Prevotella oulorum*	*Mycoplasma lipophilum*
Actinomyces cardiffensis	*Haemophilus parainfluenzae*	*Prevotella pallens*	
Actinomyces dentalis	*Haemophilus pittmaniae*	*Prevotella pleuritidis*	
Actinomyces georgiae	*Johnsonella ignava*	*Prevotella saccharolytica*	
Actinomyces gerencseriae	*Kingella denitrificans*	*Prevotella salivae*	
Actinomyces israelii	*Kingella oralis*	*Prevotella scopos*	
Actinomyces johnsonii	*Lachnoanaerobaculum umeaense*	*Prevotella shahii*	
Actinomyces lingnae	*Lactobacillus gasseri*	*Prevotella veroralis*	
Actinomyces massiliensis	*Lactobacillus reuteri*	*Pseudomonas pseudoalcaligenes*	
Actinomyces naeslundii	*Lautropia mirabilis*	*Pseudopropionibacterium propionicum*	
Actinomyces odontolyticus	*Lawsonella clevelandensis*	*Pseudoramibacter alactolyticus*	
Actinomyces oricola	*Leptotrichia buccalis*	*Pyramidobacter piscolens*	
Actinomyces oris	*Leptotrichia goodfellowii*	*Rothia aeria*	
Aggregatibacter actinomycetemcomitans	*Leptotrichia hofstadii*	*Rothia dentocariosa*	
Aggregatibacter aphrophilus	*Leptotrichia hongkongensis*	*Rothia mucilaginosa*	
Agrobacterium tumefaciens	*Leptotrichia shahii*	*Scardovia wiggsiae*	
Alloprevotella rava	*Leptotrichia wadei*	*Selenomonas artemidis*	
Alloprevotella tannerae	*Megasphaera micronuciformis*	*Selenomonas dianae*	
Anaeroglobus geminatus	*Micrococcus luteus*	*Selenomonas flueggei*	
Atopobium parvulum	*Mogibacterium neglectum*	*Selenomonas infelix*	
Atopobium rimae	*Mycoplasma faucium*	*Selenomonas noxia*	
Bacteroides heparinolyticus	*Mycoplasma salivarium*	*Selenomonas sputigena*	
Bifidobacterium dentium	*Neisseria bacilliformis*	*Shuttleworthia satelles*	
Bulleidia extructa	*Neisseria elongata*	*Slackia exigua*	
Campylobacter concisus	*Neisseria flavescens*	*Solobacterium moorei*	
Campylobacter curvus	*Neisseria mucosa*	*Sphingomonas echinoides*	
Campylobacter gracilis	*Neisseria oralis*	*Stenotrophomonas maltophilia*	
Campylobacter showae	*Neisseria perflava*	*Stomatobaculum longum*	
Capnocytophaga gingivalis	*Neisseria subflava*	*Streptococcus anginosus*	
Capnocytophaga granulosa	*Olsenella uli*	*Streptococcus constellatus*	
Capnocytophaga haemolytica	*Oribacterium asaccharolyticum*	*Streptococcus cristatus*	
Capnocytophaga leadbetteri	*Oribacterium sinus*	*Streptococcus gordonii*	
Capnocytophaga sputigena	*Parvimonas micra*	*Streptococcus intermedius*	
Cardiobacterium hominis	*Peptidiphaga gingivicola*	*Streptococcus lactarius*	
Cardiobacterium valvarum	*Peptostreptococcus stomatis*	*Streptococcus mutans*	
Centipeda periodontii	*Porphyromonas endodontalis*	*Streptococcus oralis*	
Corynebacterium durum	*Porphyromonas gingivalis*	*Streptococcus sanguinis*	
Corynebacterium matruchotii	*Porphyromonas pasteri*	*Streptococcus sinensis*	
Cryptobacterium curtum	*Prevotella baroniae*	*Streptococcus vestibularis*	
Cutibacterium acnes	*Prevotella buccae*	*Tannerella forsythia*	
Delftia acidovorans	*Prevotella dentalis*	*Treponema denticola*	
Dialister invisus	*Prevotella denticola*	*Treponema lecithinolyticum*	
Dialister micraerophilus	*Prevotella enoeca*	*Treponema maltophilum*	
Dialister pneumosintes	*Prevotella fusca*	*Treponema medium*	
Eggerthia catenaformis	*Prevotella histicola*	*Treponema parvum*	
Eikenella corrodens	*Prevotella intermedia*	*Treponema socranskii*	
Escherichia coli	*Prevotella loescheii*	*Treponema vincentii*	
Filifactor alocis	*Prevotella maculosa*	*Veillonella atypica*	
Fretibacterium fastidiosum	*Prevotella marshii*	*Veillonella denticariosi*	
Fusobacterium naviforme	*Prevotella melaninogenica*	*Veillonella dispar*	
Fusobacterium nucleatum	*Prevotella micans*	*Veillonella parvula*	
Fusobacterium periodonticum	*Prevotella multiformis*	*Veillonella rogosae*	
Gemella morbillorum	*Prevotella nanceiensis*		
Gemella sanguinis	*Prevotella nigrescens*	*SR1*	
Granulicatella adiacens	*Prevotella oralis*	*TM7*	



S2 Figure. List of species obtained by both methods combined.

(A)

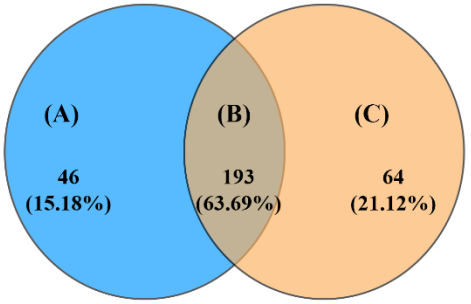
Periodontitis
Actinomyces europaeus
Actinomyces grossensis
Actinomyces mediterraneense
Bacillus pumilus
Bacteriodetes pyogenes
Bilophila wadsworthia
Brachybacterium rhamnosum
Citrobacter freundii
Corynebacterium dentalis
Eggertha lenta
Eggerthella timonensis
Fusonucleatum naviforme
Gemella haemolysans
Hafnia alvei
Jeddahella massiliensis
Klebsiella oxytoca
Lactobacillus mucosae
Mogibacterium timidum
Olsenella phoceensis
Olsenella scatoligenes
Olsenella timonensis
Pediococcus pentosaceus
Prevotella heparinolytica
Propionibacterium acidifacien
Propionibacterium lymphophilum
Propionobacterium propionilum
Staphyloccocus aureus
Staphylococcus pasteuri
Stenotrophomonas maltophila
Streptococcus dentisani
Streptococcus pneumoniae
Aggregatibacter paraphrophilus
Bacteroides zoogleoformans
Catonella morbi
Corynebacterium tuberculostearicum
Jonquetella anthropi
Klebsiella aerogenes
Lactobacillus oris
Lactobacillus salivarius
Moraxella osloensis
Ochrobactrum anthropi
Oribacterium parvum
Parascardovia denticolens
Porphyromonas catoniae
Porphyromonas uenonis
Staphylococcus warneri

(B)

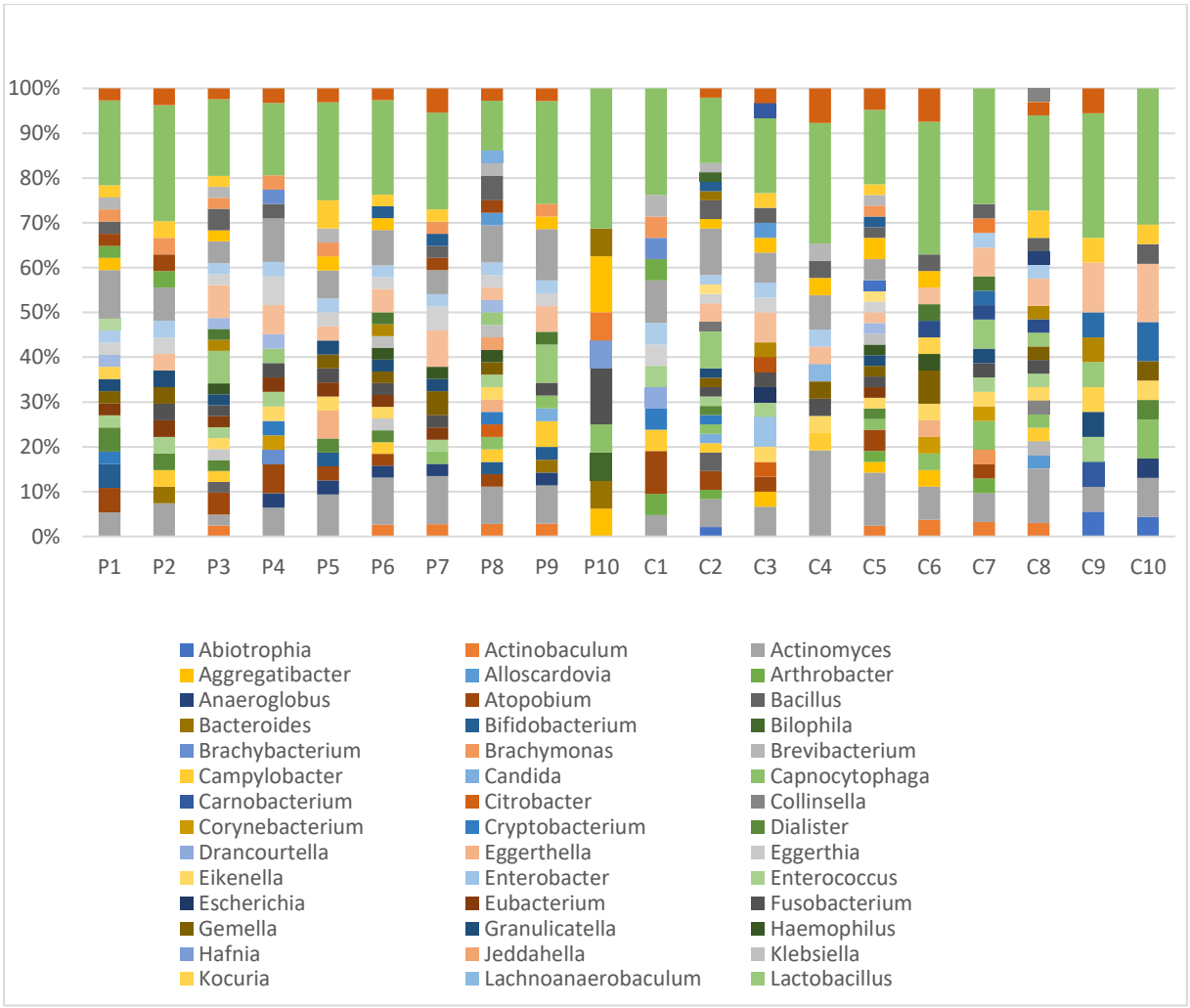
Common bacterial species	Common bacterial species	Common bacterial species
Abiotrophia defectiva	*Solobacterium moorei*	*Mycoplasma salivarium*
Actinobaculum naeslundi	*Staphylococcus epidermidis*	*Neisseria bacilliformis*
Actinomyces georgiae	*Streptococcus anginosus*	*Neisseria mucosa*
Actinomyces johnsonii	*Streptococcus australis*	*Neisseria oralis*
Actinomyces meyeri	*Streptococcus constellatus*	*Neisseria perflava*
Actinomyces naeslundii	*Streptococcus cristatus*	*Oribacterium asaccharolyticum*
Actinomyces odontolyticus	*Streptococcus gordonii*	*Oribacterium sinus*
Actinomyces oris	*Streptococcus intermedius*	*Peptidiphaga gingivicola*
Aggregatibacter aphrophilus	*Streptococcus massiliensis*	*Porphyromonas endodontalis*
Aggregatibacter segnis	*Streptococcus mitis*	*Porphyromonas pasteri*
Lachnoanaerobaculum orale	*Streptococcus mutans*	*Prevotella baroniae*
Atopobium parvulum	*Streptococcus oralis*	*Prevotella dentalis*
Atopobium rimae	*Streptococcus parasanguinis*	*Prevotella enoeca*
Campylobacter concisus	*Streptococcus salivarius*	*Prevotella fusca*
Campylobacter curvus	*Streptococcus sanguinis*	*Prevotella histicola*
Campylobacter showae	*Veillonella atypica*	*Prevotella intermedia*
Candida albicans	*Veillonella parvula*	*Prevotella loescheii*
Capnocytophaga gingivalis	*Acinetobacter johnsonii*	*Prevotella maculosa*
Capnocytophaga ochracea	*Actinomyces cardiffensis*	*Prevotella marshii*
Capnocytophaga sputigena	*Actinomyces dentalis*	*Prevotella micans*
Corynebacterium durum	*Actinomyces gerencseriae*	*Prevotella multiformis*
Cryptobacterium curtum	*Actinomyces israelii*	*Prevotella nanceiensis*
Dialister pneumosintes	*Actinomyces lingnae*	*Prevotella oris*
Eikenella corrodens	*Actinomyces massiliensis*	*Prevotella oulorum*
Enterococcus durans	*Actinomyces oricola*	*Prevotella pallens*
Enterococcus faecalis	*Aggregatibacter actinomycetemcomitans*	*Prevotella pleuritidis*
Enterococcus faecium	*Agrobacterium tumefaciens*	*Prevotella saccharolytica*
Escherichia coli	*Alloprevotella rava*	*Prevotella salivae*
Eubacterium infirmum	*Alloprevotella tannerae*	*Prevotella scopos*
Fusobacterium nucleatum	*Anaeroglobus geminatus*	*Prevotella shahii*
Gemella morbillorum	*Bacteroides heparinolyticus*	*Prevotella veroralis*
Gemella sanguinis	*Bifidobacterium dentium*	*Pseudomonas pseudoalcaligenes*
Granulicatella adiacens	*Bifidobacterium longum*	*Pyramidobacter piscolens*
Haemophilus parainfluenzae	*Bulleidia extructa*	*Scardovia wiggsiae*
Kocuria rhizophila	*Campylobacter gracilis*	*Selenomonas artemidis*
Lactobacillus paracasei	*Capnocytophaga granulosa*	*Selenomonas dianae*
Lactobacillus rhamnosus	*Capnocytophaga haemolytica*	*Selenomonas flueggei*
Leptotrichia goodfellowii	*Capnocytophaga leadbetteri*	*Selenomonas noxia*
Leptotrichia hofstadii	*Cardiobacterium hominis*	*Selenomonas sputigena*
Leptotrichia wadei	*Cardiobacterium valvarum*	*Sphingomonas echinoides*
Megasphaera micronuciformis	*Centipeda periodontii*	*Stenotrophomonas maltophilia*
Micrococcus luteus	*Corynebacterium matruchotii*	*Stomatobaculum longum*
Mogibacterium neglectum	*Cutibacterium acnes*	*Streptococcus lactarius*
Neisseria elongata	*Delftia acidovorans*	*Streptococcus sinensis*
Neisseria flavescens	*Dialister invisus*	*Streptococcus vestibularis*
Neisseria macacae	*Dialister micraerophilus*	*Tannerella forsythia*
Neisseria subflava	*Eggerthia catenaformis*	*Treponema denticola*
Olsenella uli	*Filifactor alocis*	*Treponema lecithinolyticum*
Parvimonas micra	*Fretibacterium fastidiosum*	*Treponema maltophilum*
Peptostreptococcus stomatis	*Fusobacterium naviforme*	*Treponema medium*
Porphyromonas gingivalis	*Fusobacterium periodonticum*	*Treponema parvum*
Prevotella buccae	*Granulicatella elegans*	*Treponema socranskii*
Prevotella denticola	*Haemophilus parahaemolyticus*	*Treponema vincentii*
Prevotella melaninogenica	*Haemophilus pittmaniae*	*Veillonella denticariosi*
Prevotella nigrescens	*Johnsonella ignava*	*Veillonella dispar*
Prevotella oralis	*Kingella denitrificans*	*Veillonella rogosae*
Prevotella phoceensis	*Kingella oralis*	*SR1*
Propionibacterium acnes	*Lachnoanaerobaculum umeaense*	*TM7*
Pseudopropionibacterium propionicum	*Lactobacillus gasseri*	*Mycoplasma lipophilum*
Pseudoramibacter alactolyticus	*Lactobacillus reuteri*	*Nesseria baciliformis*
Rothia aeria	*Lautropia mirabilis*	*Ruminococus merdae*
Rothia dentocariosa	*Lawsonella clevelandensis*	
Rothia mucilaginosa	*Leptotrichia buccalis*	
Selenomonas infelix	*Leptotrichia hongkongensis*	
Shuttleworthia satelles	*Leptotrichia shahii*	
Slackia exigua	*Mycoplasma faucium*	

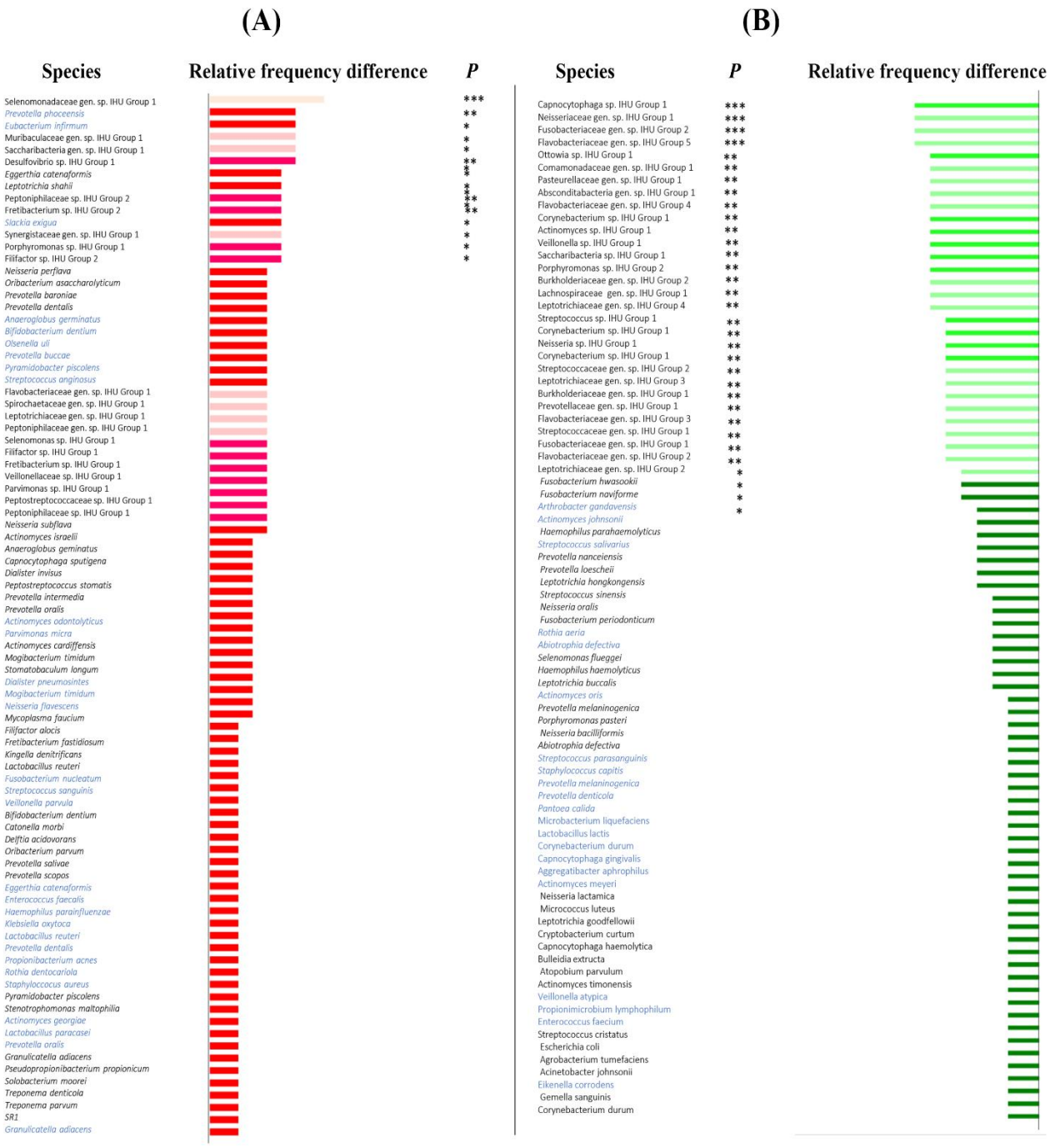
(C)

Controls
Actinomyces denticolens
Actinomyces oralis
Actinomyces timonensis
Alloscardovia omnicolens
Arthrobacter gandavensis
Bacillus licheniformis
Bacillus sinesaloumensis
Brachymonas massiliensis
Brevibacterium pityocampae
Campylobacter rectus
Carnobacterium divergens
Citrobacter koseri
Collinsella massiliensis
Dialister propionicifaciens
Drancourtella massiliensis
Eggerthella lenta
Enterobacter asburiae
Enterobacter cloacae
Klebsiella pneumoniae
Lachnoanaerobaculum saburreum
Lactobacillus agilis
Lactobacillus delbrueckii
Lactobacillus fermentum
Lactobacillus lactis
Marseillibacter massiliensis
Microbacterium liquefaciens
Microbacterium maritypicum
Microbacterium oxydans
Neisseria cinerea
Pantoea calida
Propionimicrobium lymphophilum
Propionobacterium granulosum
Serratia marcescens
Staphylococcus capitis
Vagococcus fluviales
Yersinia intermedia
Acidipropionibacterium acidifaciens
Actinomyces graevenitzii
Bacteroidales bacterium
Corynebacterium amycolatum
Corynebacterium bovis
Desulfomicrobium orale
Fusobacterium hwasookii
Gemella bergeri
Gracilibacteria bacterium
Haemophilus haemolyticus
Kocuria palustris
Lachnospiraceae bacterium
Lactobacillus johnsonii
Lactobacillus vaginalis
Lactococcus lactis
Microbacterium flavescens
Neisseria lactamica
Neisseria polysaccharea
Paracoccus yeei
Porphyromonas asaccharolytica
Prevotella aurantiaca
Prevotella multisaccharivorax
Ruminococcaceae bacterium
Streptococcus sobrinus
Treponema amylovorum
Treponema pectinovorum
Variovorax paradoxus
Veillonellaceae bacterium



S4 Figure. Analysis of oral flora by genus level using culturomics approch





S5 Figure: The relative frequency of bacteria identified by culturomics (blue) and metagenomics (black) compared between patients with periodontitis (A) and controls (B). We used the Fisher exact test, as appropriate. Species with dark red or dark green, BGGOIP and BGGOIC with medium red or medium green, BFGOIP and BFGOIC in light red or light green. *p< 0.05; **p < 0.001; ***p < 0.0001 (complete figure).

« Ce n'est pas la fin,

Ce n'est même pas le commencement de la fin,

Mais, c'est peut-être la fin du commencement. »

Winston Churchill