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Doutorado em Odontologia
Área de concentração em Periodontia

CRISTIANE GONÇALVES

NOVOS PATÓGENOS ASSOCIADOS ÀS DOENÇAS PERIODONTAIS: UMA REVISÃO SISTEMÁTICA

Guarulhos
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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, intitulada "NOVOS PATÓGENOS ASSOCIADOS ÀS DOENÇAS PERIODONTAIS: UMA REVISÃO SISTEMÁTICA" em sessão pública realizada em 29 de Outubro de 2014 considerou a candidata Cristiane Gonçalves aprovada.

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Guarulhos, 29 de Outubro de 2014.

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RESUMO

Existem fortes evidências na literatura sobre o papel de certas espécies bacterianas no início e progressão das periodontites. Na ultima década, a introdução de novos métodos de diagnóstico moleculares têm apontado para a existência de novos patógenos periodontais. No entanto, os dados destes estudos nunca foram avaliados em conjunto, o que pode gerar um engano quanto ao verdadeiro papel desses microorganismos na etiologia das doenças periodontais destrutivas. O objetivo desta revisão sistemática foi determinar o peso da evidência científica para a existência de novos patógenos periodontais entre as espécies recentemente identificadas, com base em estudos de "associação". Esta revisão sistemática foi conduzida de acordo com os critérios preconizados pelos protocolos de preparo de revisões sistemáticas PRISMA. As bases de dados MEDLINE, EMBASE e Cochrane foram pesquisadas até Setembro de 2013, para estudos comparando dados de amostras de placa bacteriana subgengival coletadas de indivíduos com periodontite e com saúde periodontal, e que avaliaram pelo menos um microorganismo que não os patógenos periodontais já reconhecidos. De 1.450 artigos identificados, 41 estudos foram elegíveis. Os dados foram extraídos e registrados em formulários predefinidos. Os resultados sugerem que há evidência moderada na literatura para apoiar a associação de 17 espécies ou filotipos dos filos *Bacteroidetes, espiroquetas*, *Firmicutes*, *Synergistetes* e *Proteobacteria* com a periodontite. O filo *Candidatus Saccharibacteria* e o domínio *Archaea* também parecem ter uma associação com esta infecção. Estes dados apontam para a importância de espécies não-identificadas anteriormente na etiologia das periodontites e podem orientar futuras investigações sobre o papel real desses possíveis novos patógenos no aparecimento e progressão das doenças periodontais destrutivas.

Palavras-chave: *Archaea*, *Bactéria*, placa dental, microbiologia, doença periodontal, DNA

ABSTRACT

There is substantial evidence in the literature supporting the role of certain oral bacteria species in the onset and progression of periodontitis. Nevertheless, results of independent-culture diagnostic methods introduced around a decade ago have pointed to the existence of new periodontal pathogens. However, the data of these studies have not been evaluated together, which may generate some misunderstanding on the actual role of these microorganisms in the etiology of periodontitis. The aim of this systematic review was to determine the current weight of evidence for newly identified periodontal pathogens based on the results of "association" studies. This review was conducted and reported in accordance with the PRISMA statement. The MEDLINE, EMBASE and Cochrane databases were searched up to September 2013 for studies comparing microbial data of subgingival plaque samples collected from subjects with periodontitis and periodontal health, and evaluating at least one microorganism other than the already known periodontal pathogens. From 1,450 papers identified, 41 studies were eligible. The data were extracted and registered in predefined piloted forms. The results suggested that there is moderate evidence in the literature to support the association of 17 species or phylotypes from the phyla *Bacteroidetes, Spirochaetes, Firmicutes, Synergistetes* and *Proteobacteria* with periodontitis. The phylum *Candidatus Saccharibacteria* and the Archaea domain also seem to have an association with this infection. These data point out the importance of previously unidentified species in the etiology of periodontitis and might guide future investigations on the actual role of these suspected new pathogens in the onset and progression of periodontitis.

Key-words: *Archaea, Bacteria*, dental plaque, microbiology, periodontal disease, DNA

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1. Introdução e Justificativa

A periodontite é uma doença infecciosa que envolve uma complexa interação entre os microrganismos orais, organizados em uma estrutura de biofilme e a resposta imune do hospedeiro. A manifestação clínica dessa doença é a destruição dos tecidos que suportam e protegem o dente (Page et al.,1997). Como em qualquer outra doença infecciosa, a identificação dos agentes patogênicos microbianos associados com a etiologia da infecção periodontal, é o primeiro passo no desenvolvimento de abordagens terapêuticas eficazes. No entanto, esse não é um objetivo fácil de ser alcançado quando se trata de infecções mistas, como no caso das infecções periodontais (Feres & Figueiredo, 2009). O estabelecimento de um microrganismo como um verdadeiro patógeno periodontal deve basear-se em dois níveis principais de evidência: (1) o organismo deve estar presente em maior prevalência e/ou níveis na doença do que na saúde (estudos de "associação"), e (2) a sua supressão ou eliminação deve reduzir ou parar a progressão da doença (Socransky, 1979). Adicionalmente, investigações mais aprofundadas dos mecanismos de patogenicidade desses microrganismos e da capacidade de estimular ou evadir a resposta imune do hospedeiro também são necessárias para se confirmar a associação desses patógenos com a etiologia das periodontites (Socransky 1979, Hajjishenegalli et al., 2012)

A busca pelo fator etiológico relacionado às doenças periodontais tem sido objeto de diversas pesquisas ao longo dos últimos 120 anos. Infelizmente, por muitas décadas, a pesquisa neste campo foi consideravelmente retardada devido a dificuldades técnicas para se identificar a complexa microbiota subgengival (Socransky & Haffajee, 1994, Feres et al., 2004). O início do século XX foi considerado a ”era de ouro da

microbiologia". Neste período, a etiologia microbiana específica da doença periodontal foi sugerida pela primeira vez e, microrganismos como espiroquetas, fusobactérias e *estreptococos*; foram relacionados ao inicio da destruição periodontal (Barret,1914 a,b; Le Clear, 1915; Kolle, 1917; Kritchevsky & Seguin, 1918; Hartzell, 1925; Fisher, 1927). Entretanto, devido às limitações técnicas em se identificar os verdadeiros patógenos periodontais, observou-se que muitas dessas espécies não tinham relação direta com a etiologia da doença, como no caso dos *estreptococos*. Por outro lado, havia uma dificuldade em se controlar espécies que hoje são consideradas agentes etiológicos das periodontites, como as espiroquetas, pois agentes antimicrobianos efetivos ainda não estavam disponíveis. Uma vez que as terapias sugeridas não foram efetivas para controlar a periodontite, seguiu-se uma fase de grande frustração clínica. Sendo assim, o entusiasmo inicial em se estabelecer a etiologia microbiana específica das periodontites foi abandonado, e por quatro décadas, fatores sistêmicos como deficiências nutricionais e defeitos constitucionais foram apontados como responsáveis pelo início e progressão dessas doenças (Bunting, 1922; Printz, 1926; Haupl & Lang, 1927; Gottlieb, 1921 a,b,1928, Feres et al., 2004). Somente na década de 60, com os estudos de transmissão de microorganismos subgengivais e consequente desenvolvimento de doença periodontal em animais enjaulados (Keyes & Jordan, 1964; Gibbons & Socransky, 1966) e os estudos mostrando a presença maciça de espiroquetas em lesões de GUNA (Listgarten et al.,1965), a etiologia infecciosa específica da doença periodontal voltou a ser sugerida. Porém, o maior impacto veio dos estudos de gengivite experimental em humanos (Loe et al.,1965). Esses autores mostraram de forma simples e definitiva que o acúmulo de bioflime supragengival levava

ao desenvolvimento de gengivite e que a remoção desse biofilme levaria a remissão do processo inflamatório. A repercussão dos resultados desses estudos foi grande e disseminou-se a idéia de que periodontite poderia ser tratada por meio do controle de placa supragengival. Acreditava-se que a composição da placa era similar de indivíduo para indivíduo, que todos apresentariam susceptibilidade semelhante à perda de inserção e que o desenvolvimento da doença era totalmente dependente da quantidade de acúmulo de biofilme supragengival (Löe et al., 1965, Theilade et al., 1966). Esta teoria, denominada “Hipótese da Placa não Específica”, foi questionada após os estudos epidemiológicos longitudinais realizados em populações do Sri Lanka e do Kenya observarem que alguns indivíduos não desenvolviam doença periodontal mesmo na presença de grande acúmulo de biofilme, enquanto outros indivíduos apresentavam grande destruição periodontal e pouco acúmulo de biofilme (Löe et al., 1986, Baelum et al.,1988). Paralelamente, estudos de microscopia eletrônica e de cultura ressaltaram diferenças na morfologia da placa dentária de indivíduos com saúde periodontal e com periodontite (Listgarten et al.,1976, 1978). Essas observações levaram a comunidade científica a retomar as pesquisas em torno da especificidade microbiana das doenças periodontais. Diversos estudos começaram a mostrar diferenças importantes na composição da microbiota subgengival entre indivíduos com saúde periodontal, periodontite crônica e periodontite agressiva, ou mesmo entre sítios saudáveis e doentes dos mesmos indivíduos (Slots, 1976, Haffajee et al.,1984, Mandel et al.,1987), dando origem ao conceito aceito até os dias atuais, chamado de “Hipótese da Placa Específica”(Loesche, 1976).

O conceito da especificidade na etiologia das doenças periodontais deu início a uma era extremamente produtiva na pesquisa da microbiologia periodontal, e expandiu de forma substancial o conhecimento sobre a composição da microbiota (Teles et al., 2013). Durante mais de três décadas esses estudos foram realizados utilizando técnicas de cultura (Moore & Moore,1994) e diversas espécies de microrganismos foram associadas com o início e progressão das periodontites, culminando com o reconhecimento do *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis e Tannerella forsythia* como verdadeiros patógenos periodontais, em 1996, pela Academia Americana de Periodontia (Proceedings of the 1996 World Workshop). Embora este tenha sido um período fértil nas pesquisas em microbiologia oral, a necessidade de se ter as bactérias viáveis para sua identificação limitou enormemente o trabalho dos pesquisadores em termos de diagnóstico e de avaliação do efeito das diversas terapias (Socransky & Haffajee, 1994, Hajjishenegalli et al., 2012). O desenvolvimento de novas técnicas imunológicas e as de biologia molecular a partir da década de 1990 permitiu um maior conhecimento da composição microbiológica do ambiente subgengival. Microrganismos como *T. forsythia e Treponema denticola*, que se acreditava ser relativamente incomuns na placa subgengival pelos métodos de cultura, mostraram estar presentes em níveis elevados em pacientes com periodontite quando avaliadas por métodos moleculares (Haffajee et al., 1997, Taubman et al.,1992, Riviere et al., 1992.). Os estudos utilizando essas técnicas possibilitaram a avaliação de grandes números de amostras de placa bacteriana e foram responsáveis pelos conhecimentos acerca da diversidade da composição dos biofilmes supra e subgengivais, e do estabelecimento de perfis microbiológicos relacionados com as

diferentes formas de doença periodontal e peri-implantar (Socransky et al., 1998, Haffajee et al.,1997, Shibli et al., 2008, Faveri et al., 2008)

Neste período, Socransky e colaboradores (1998) utilizando a técnica do Checkerboard DNA-DNA hybridization, avaliaram 13.261 amostras de biofilme subgengival, de 165 indivíduos com periodontite crônica e 25 indivíduos periodontalmente saudáveis, e descreveram 5 complexos bacterianos nos indivíduos com doença. Três deles, os complexos roxo, amarelo e verde, considerados colonizadores primários da superfície dentária, compostos por espécies de *Streptococcus*, *Capnocytophaga* e *Veillonella*, microrganismos considerados compatíveis com a saúde periodontal. Os outros dois complexos, o vermelho e o laranja, foram considerados colonizadores tardios do biofilme, estavam intimamente relacionados entre si e foram encontrados em maiores proporções em sítios doentes, principalmente os três patógenos do complexo vermelho (Socransky et al.,1998). O complexo vermelho é composto pelas espécies *T. forsythia, P. gingivalis e T. denticola,* e o laranja abriga as espécies *Fusobacterium nucleatum, Fusobacterium periodonticum, Prevotella intermedia, Prevotella nigrescens* e *Parvimonas micra, Eubacterium nodatum, Campylobacter rectus, Campylobacter showae, Campylobacter gracilis* e *Streptococcus constellatus*. Posteriormente, algumas espécies de actinomicetos (*Actinomyces gerencseriae*, *Actinomyces israelii*, *Actinomyces naeslundii* e *Actinomyces oris*) foram agrupadas e consideradas compatíveis com o hospedeiro, ou seja, relacionadas diretamente com o estado de saúde periodontal (Socransky & Haffajee, 2002). Esses resultados foram muito importantes para se estabelecer a ideia de que as diferentes terapias periodontais devem ser avaliadas não somente quanto à capacidade de reduzir

certos patógenos, mas também de aumentar a proporção de espécies compatíveis com o hospedeiro. Em outras palavras, os dados desse estudo ajudaram na definição de metas microbiológicas (e não somente clínicos) para as terapias periodontais. Pesquisadores e clínicos reconheceram que o sucesso clínico das diferentes terapias periodontais dependeria da alteração de um perfil microbiano subgengival associado à doença para um perfil compatível com saúde periodontal, ou seja, contendo baixos níveis e proporções de patógenos e altas proporções de espécies benéficas (Feres et al., 2004, Haffajee et al., 2006; Teles et al., 2013).

Seguiu-se assim um longo e profuso período – que dura até os dias atuais - aonde técnicas de biologia molecular alvo-específico, que utilizam sondas de DNA ou iniciadores, foram largamente utilizadas para se estabelecer os efeitos de diversas terapias periodontais na composição da microbiota subgengival (Feres et al., 2001, Carvalho et al., 2005, Teles et al., 2006, Haffajee et al., 2006, Haffajee et al,. 2008, Mestnik et al., 2010, Sampaio et al., 2011, Feres et al., 2014, Socransky et al., 2013, Soares et al., 2014, Figueiredo et al., 2014). Porém, a busca por novos patógenos periodontais ficou suspensa por muitos anos.

A partir da década de 2000 técnicas de sequenciamento (*open ended*) começaram a ser utilizadas para se avaliar a composição da microbiota oral, retomando assim a busca por novos patógenos periodontais. Essas abordagens permitiram a detecção e classificação de inúmeros filotipos até então não descritos e não cultivados (Matarazzo et al., 2011, Faveri et al., 2008, Dymock et al., 1996, Harper-Owen et al., 1999, Paster et al., 2001, Hutter et AL., 2003, Kumar et al., 2003, de Lillo et al., 2006). Utilizando a técnica de clonagem e sequanciemanto de Sanger, Paster et al. (2001)

estimaram que a diversidade da microbiota oral poderia estar perto de 500 espécies, sendo 415 da microbiota subgengival. Em estudo subsequente, esse mesmo grupo de pesquisadores (Paster et al., 2006) estimaram que a diversidade da microbiota bucal poderia chegar a 700 espécies e que um único indivíduo poderia apresentar entre 100 e 200 espécies bacterianas diferentes na cavidade oral. Estas técnicas possibilitam o sequenciamento de DNA metagenômico empregando duas abordagens: 1) o sequenciamento de uma parte específica do DNA bacteriano (DNA-alvo) com iniciadores universais; como por exemplo, a amplificação por PCR do marcador filogenético 16S rDNA (Xie et al., 2010, Belda-Ferre et al., 2011, Liu et al., 2012, Griffen et al., 2012, Abusleme et al., 2013, Dewhirst et al., 2010, Teles et al., 2011, Duran-Pinedo et al., 2011, Dias et al., 2012), ou 2) o sequenciamento direto do microbioma, sem passar pelas etapas de amplificação ou clonagem do DNA (Liu et al., 2012, Wang et al., 2013).

Os dados gerados nos últimos 14 anos pelos estudos que vêm utilizando técnicas de sequenciamento ou técnicas de biologia molecular alvo-específico para a investigação de espécies bacterianas incomumente associadas à etiologia das periodontites, sugerem a existência de novos patógenos periodontais. Entretanto, esses estudos diferem substancialmente em termos do teste de diagnóstico utilizado, da forma de avaliação e interpretação dos dados, do número de amostras avaliadas, dentre outros aspectos. Essas diferenças dificultam o estabelecimento de conclusões objetivas sobre o real papel desses microrganismos na etiologia das doenças periodontais. Sendo assim, uma revisão sistemática da literatura que compile os resultados destes estudos pode ser útil para a interpretação precisa da literatura atual sobre esse tema.

2- Proposição

O objetivo desse estudo foi determinar o peso atual de evidência para a existência de novos patógenos periodontais, com base no resultado dos estudos de “associação”.

3- Materiais e Métodos

Esta revisão sistemática foi conduzida de acordo com os critérios preconizados pelos protocolos de preparo de revisões sistemática PRISMA (Preferred Reporting Itens for Systematic Reviews and Meta-Analyses) (Moher et al.,2009).

3.1- Questão foco

A questão foco pesquisada foi: *"Qual o peso atual da evidência cientifica para a existência de novos patógenos periodontais com base em estudos de associação?*

3.2- Estratégia de busca

Foi realizada uma busca nas bases de dados do MEDLINE (via PubMed), EMBASE e biblioteca Cochrane, utilizando a estratégia de pesquisa descrita na Tabela S1. A pesquisa foi realizada por dois revisores independentes e incluiu estudos realizados até 10 setembro de 2013. Além disso, foi realizada uma busca manual com base na lista de referência dos manuscritos selecionados e artigos de revisão.

3.3- Critérios de inclusão

- Estudos que compararam os dados microbiológicos de amostras de placa bacteriana subgengival coletadas de pacientes sistemicamente saudáveis com periodontite e saúde periodontal;

- Estudos que avaliaram pelo menos um novo microrganismo diferente das espécies já sugeridas como possíveis patógenos periodontais ou patógenos periodontais (*P. gingivalis, T. denticola, T. forsythia, F. nucleatum, F. periodonticum, P. intermedia, P. nigrescens, P.micra, C.gracilis, C.rectus, C.showae, E. nodatum, Streptococcus constellatus* e *A. actinomycetemcomitans*) (Proceedings of the 1996 World Workshop in Periodontics, Socransky et al., 1998; Teles et al., 2013).

3.4- Critérios de exclusão

- Estudos publicados em outros idiomas além do Inglês, Espanhol, Francês ou Português;

- A falta de uma comparação transversal direta dos dados microbiológicos entre indivíduos com doença e saúde periodontal;

- Falta de dados de amostras de placa bacteriana subgengival em indivíduos com doença e/ou saúde periodontal;

- Estudos que avaliaram apenas pacientes com periodontite agressiva localizada ou periodontite refratária;

- Estudos de Revisão;

- Estudos que avaliaram apenas vírus.

3.5- Extração de dados

Os estudos foram selecionados de forma independente por dois pesquisadores e qualquer desacordo foi resolvido por meio de discussão. Quando o desacordo persistiu outro pesquisador foi consultado, a fim de alcançar um consenso. Os estudos que preencheram os critérios de inclusão e exclusão foram processados para extração de dados, que foi realizada por outros dois pesquisadores independentes. As seguintes informações foram coletadas de cada artigo e registradas em formulários específicos (Anexo A):(1) local de estudo, (2) tipo de estudo, (3) descrição da população, (4) tipo de avaliação microbiológica (ex.:amostras avaliadas individualmente ou em “pool”, número de amostras avaliadas, método de diagnóstico empregado, (5) desfechos microbiológicos (ex.: microrganismos avaliados: bactérias e/ou *Archaea*), níveis e/ou proporção e/ou abundância e/ou prevalência dos microrganismos mais elevados em

periodontite do que nos voluntários com saúde periodontal (desfecho primário) ou os relatados pelos autores como sendo associados com a periodontite, (6) conflito de interesse (7), fonte de financiamento.

Para atribuir com precisão os nomes mais atualizados para os microrganismos, e evitar a repetição dos mesmos, o banco de dados *Human Oral Microbiome* (HOMD, http: //www.homd. org / index.php, 28 de outubro de 2013) foi interrogado para cada microrganismo citado nos 41 artigos incluídos. Para esta etapa foi utilizada a nomenclatura dada por cada autor no estudo. Quando essa consulta não encontrou resultado, o HOMD foi usado para consultar a seqüência 16S rDNA disponível, com comprimento >1.300 nt. Nos casos em que ambas as consultas não tiveram sucesso, foi mantida a nomenclatura do autor. Filos, classes, espécies e filotipos foram indexados de acordo com o Centro Nacional de Informações sobre Biotecnologia (NCBI) (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi, 29 de Outubro de 2013), quando disponível, caso contrário, a classificação HOMD foi mantida.

4. Artigo Científico

ARTIGO CIENTÍFICO PUBLICADO NO JOURNAL OF DENTAL RESEARCH ***(Newly identified pathogens associated with periodontitis: a systematic review. J Dent Res. 2014 Sep; 93(9): 846-58. doi: 10.1177/0022034514542468. Epub 2014)***

Keywords: Archaea/ bacteria/ dental plaque/ microbiology/ periodontal disease/review
Running Title: A review of newly identified pathogens in periodontitis

Abstract

There is substantial evidence supporting the role of certain oral bacteria species in the onset and progression of periodontitis. Nevertheless, results of independent-culture diagnostic methods introduced around a decade ago have pointed to the existence of new periodontal pathogens. However, the data of these studies have not been evaluated together, which may generate some misunderstanding on the actual role of these microorganisms in the etiology of periodontitis. The aim of this systematic review was to determine the current weight of evidence for newly identified periodontal pathogens based on the results of “association” studies. This review was conducted and reported in accordance with the PRISMA statement. The MEDLINE, EMBASE & Cochrane databases were searched up to September 2013 for studies (1) comparing microbial data of subgingival plaque samples collected from subjects with periodontitis and periodontal health, and (2) evaluating at least one microorganism other than the already known periodontal pathogens. From 1,450 papers identified, 41 studies were eligible. The data were extracted and registered in predefined piloted forms. The results suggested that there is moderate evidence in the literature to support the association of 17 species or phylotypes from the phyla *Bacteroidetes*, *Candidatus Saccharibacteria*, *Spirochaetes*, *Firmicutes*, *Synergistetes* and *Proteobacteria* with periodontitis. The phylum *Candidatus Saccharibacteria* and the *Archaea* domain also seem to have an association with this disease. These data point out the importance of previously unidentified species in the etiology of periodontitis and might guide future investigations on the actual role of these suspected new pathogens in the onset and progression of this infection.

Introduction

Periodontitis is an infectious disease involving a complex interaction between the oral microorganisms organized in a biofilm structure and the host immune response. Its clinical consequence is the destruction of the tissues that support and protect the tooth. As with any other infection, identification of the microbial pathogens associated with the etiology of periodontitis is the first step towards the development of effective therapeutic approaches. The establishment of a microorganism as a true pathogen should be based on two main levels of evidence: 1) the organism should be present in higher prevalence and/or levels in disease than in health ("association" studies), and 2) its suppression or elimination should reduce or stop disease progression ("elimination" studies) (Socransky, 1979).

The composition of the oral microbiota, more specifically the subgingival microbiota has been studied for over a century. Unfortunately, for many decades, research in this field was considerably delayed due to technical difficulties, such as the need to identify microorganisms to the species level using only culture techniques. The use of immunological and molecular diagnostic tests for the identification of microorganisms independent on cultivation, such as DNA probes, PCR and immunoassays, began in the 1990´s and allowed a great progress in the understanding about the composition of the subgingival microbiota. Using one of these molecular tests, namely checkerboard DNA-DNA hybridization, Socransky *et al.,* (1998) described the role of 5 main microbial complexes in the subgingival biofilm. Some species/complexes were associated with periodontal health, such as the yellow (*Streptococcus species*) and purple (*Veillonela parvula* and *Actinomyces odontolyticus*) complexes, while others were

closely associated with disease, such as the red (*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia)* and orange complexes *(Fusobacterium, Prevotella and Campylobacter species).* Afterwards, other association and elimination studies have confirmed the involvement of the three members of the red complex, some members of the orange complex, such as *Prevotella intermedia*, *Parvimonas micra*, *Fusobacterium nucleatum, Eubacterium nodatum* and *Aggregatibacter actinomycetemcomitans* with the etiology of different periodontal conditions (Teles *et al.* 2013).

In 2001, using cloning and Sanger sequencing, Paster *et al.,* suggested a possible role of cultivable and not-yet cultivable/unrecognized microbial species in the etiology of periodontitis, confirming the idea that the diversity of the oral microbiota was more complex than previously known. Subsequently, a number of other studies using several molecular approaches, including next generation sequencing techniques were published in the periodontal literature (Kumar *et al.,* 2005; Matarazzo *et al.,* 2011; Teles *et al.,* 2011; Griffen *et al.,* 2012; Abusleme *et al.*, 2012). The overall data provided by these studies for over 12 years suggested the existence of new periodontal pathogens. However, studies are diverse in terms of the diagnostic test used, taxa assessed and number of samples evaluated, which may generate some misunderstanding while trying to draw objective conclusions on the actual role of these microorganisms in the etiology of periodontitis. Thus, a thorough review compiling the results of these studies could be helpful for the accurate interpretation of the present literature on this topic. Therefore, the aim of this systematic review was to determine the current weight of evidence for newly identified periodontal pathogens based on the results of “association” studies.

Materials and Methods

This systematic review was conducted in accordance with the recommendations of PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-analysis). (Moher *et al*., 2009).

Focused question

"What is the weight of evidence for the existence of newly identified periodontal pathogens based on "association" studies?"

Inclusion Criteria

The manuscripts meeting the following criteria were included:

- Studies of any design that compared microbial data of subgingival plaque samples collected from systemically healthy patients with periodontitis and periodontal health;

- Studies evaluating at least one new microorganism other than the species already suggested as periodontal pathogens or putative periodontal pathogens (*P. gingivalis, T. denticola, T. forsythia, F. nucleatum*, *Fusobacterium periodonticum, P. intermedia*, *Prevotella nigrescens*, *P. micra, Campylobacter gracilis, Campylobacter rectus, Campylobacter showae, E. nodatum, Streptococcus constellatus and A. actinomycetemcomitan*s) (Proceedings of the World Workshop in periodontics, 1996, Socransky *et al.,* 1998; Teles *et al*., 2013).

Exclusion Criteria

- Studies published in languages other than English, Spanish, French or Portuguese;

- Lack of baseline data;
- Lack of a direct comparison of baseline microbial data between periodontitis and periodontally healthy groups;
- Lack of data from subgingival plaque samples in periodontitis and/or periodontally healthy groups;
- Lack of data from subgingival plaque samples of systemically healthy subjects;
- Studies that evaluated only subjects with Localized Aggressive Periodontitis or Refractory Periodontitis;
- Review studies;
- Studies that evaluated only viruses.

Search Strategy and Data Extraction

The MEDLINE (via PubMed), EMBASE, and Cochran library databases were searched up to September 10th 2013, by two independent reviewers (PJPC and PD) using the search strategy described in Appendix Table S1. In addition, a manual search was conducted based on the reference list of the selected manuscripts and review articles. The studies were screened independently by two researchers (EL, MFa) and any disagreement was solved through discussion. When disagreement persisted, another researcher was consulted to achieve consensus (MFe). Those studies that fulfilled the inclusion and exclusion criteria were processed for data extraction, conducted by another two independent researchers (PJPC and CG). The following information was collected from each manuscript and registered in predefined piloted forms: (1) study location, (2) type of trial, (3) characteristics of participants (e.g. systemically health status, number of patients per group, age, periodontal condition), (4) type of

microbiological evaluation (e.g. individually or pooled strategy, number of samples evaluated, employed diagnostic method), (5) microbiological outcomes [(e.g. microorganisms appraised (e.g. *Bacteria* and/or *Archaea*), taxa in higher levels and/or proportion and/or abundance and/or prevalence in periodontitis than in periodontal health or those reported by the authors as being associated with periodontitis (Primary Outcome of Interest)], (6) conflict of interest, (7) source of funding.

To accurately assign the most updated names to the microorganisms, so that we could avoid taxa repetition and to assign a Human Oral Taxon number (HOT) whenever available, the Human Oral Microbiome database (HOMD, http://www.homd.org/index.php, 28th October, 2013) was interrogated for each microorganism cited on the 41 included studies by three researchers (PJPC, LCF, NT). For this step, we used the nomenclature given by each author (i.e. the microorganism/strain/isolate name or the Genbank accession number). When this query did not return any result, the local HOMD blast tool was used to query the available 16S rDNA sequence with length >1,300 nt. In cases in which both queries were unsuccessful, the author's nomenclature was retained. Phyla, class, species and phylotypes were indexed according to the National Center for Biotechnology Information (NCBI) taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi, 29Th October 2013) when available, otherwise HOMD classification was retained.

Results

Studies Included

A total of 1,450 titles were found during the electronic search. After title screening, 1,303 studies were excluded, and 147 were selected. After abstract reading, 116 studies were excluded and 31 full-text publications were comprehensively evaluated. In addition, 15 studies were selected during the manual search. After reading these 46 studies, five were excluded for not meeting the inclusion criteria (Appendix Table S2). Therefore, 41 studies were included in this study (Figure).

Study Designs: Periodontal Conditions/Samples Evaluated and Diagnostic Techniques Used

Table 1 presents the studies included and their main methodological features. The majority of the studies had more patients and samples in the periodontitis than in the periodontally healthy group. A total of 912 individuals with periodontal health and 1,918 with periodontitis were evaluated. Subgingival biofilm samples were processed individually in 24 studies and pooled in 13 studies. One study used both sampling methods (Liu *et al.,* 2012), two studies did not provide information about the number of samples collected (Dewhirst *et al.,* 2000; Paster *et al.,* 2001) and one study (Bringuier *et al.,* 2013) did not clarify whether the samples were analyzed individually or pooled. A total of 3,508 and 10,800 subgingival plaque samples were evaluated from subjects with periodontal health or periodontitis, respectively.

Three studies used culture methods (Macuch and Tanner, 2000; Murdoch *et al.,* 2004; Canabarro *et al.,* 2012), but Macuch and Tanner (2000) also used a protein electrophoresis technique (SDS-PAGE). The other 38 studies used technologies based

on nucleic acid detection as follows: 22 used targeted techniques, 10 used open-ended techniques, and 6 used both approaches. Most of the studies used techniques based on DNA detection, only two studies (Teles *et al.,* 2011; Gonçalves *et al.,* 2012) used a RNA based detection method, more specifically the RNA-oligonucleotide quantification technique (ROQT).

Microbial data

The microorganisms found in statistically significantly higher levels and/or proportion and/or abundance and/or prevalence in periodontitis than in periodontal health or those reported by the authors as being associated with periodontitis were catalogued and data are summarized in Appendix Table S3.

Table 2 presents the taxa found in at least one study in statistically significantly higher levels and/or proportion and/or abundance and/or prevalence in periodontitis than in periodontal health. Three domain systems were identified: *Bacteria*, *Archaea* and *Eukaryote* (represented by Fungi). *Bacteria* was the main domain detected, and included 10 phyla (*Bacteroidetes*, *Spirochaetes*, *Firmicutes*, *Synergistetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Chloroflexi*, *Tenericutes* and the *Candidatus Saccharibacteria* (syn. Candidate division TM7), the Candidate division Sulphur River 1 (SR1, no rank http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=221235&lvl=3&lin=f&keep=1&srchmode=1&unlock, 29th October, 2013), 63 bacterial genera, and 108 species/phylotypes. *Firmicutes*, which harbors mostly Gram-positive bacteria, was the phylum with the highest number of species associated with periodontitis (n=39), in contrast with *Chloroflexi* (n=1). One species from the *Archaea* domain

(*Methanobrevibacter oralis* HOT 815) and the total levels and proportions of this domain were also associated with periodontitis.

In order to estimate the current weight of evidence of newly identified pathogens associated with periodontitis, the data of Table 2 were subsetted into the following categories: taxa found in statistically significantly higher levels and/or proportion and/or prevalence and/or abundance in periodontitis than in periodontal health from 3 to 5 studies (moderate evidence) or in 2 studies (some evidence) (Table 3). Seventeen species/phylotypes, the phylum *Candidatus Saccharibacteria,* and the *Archaea* domain were included in the Moderate evidence category and other 15 taxa in the some evidence category.

Appendix Table S4 presents the same type of data of Table 2 but for the known pathogens. Recognized periodontal pathogens such as the members of the red complex *A. actinomycetemcomitans* and certain members of the orange complex were found in statistically significantly higher levels and/or proportions and/or prevalence in a number of studies using targeted and open-ended techniques. For example, *P. gingivalis*, *T. forsythia* and *T. denticola* were statistically significantly elevated in periodontitis than in health in 9 studies.

Discussion

This is the first systematic review that assessed the current weight of evidence concerning new candidate periodontal pathogens after 12 years of what could be considered the “modern era” of oral microbiology. We estimated that at this point no

microorganism could be set as a true new periodontal pathogen with strong evidence, since the number of studies that associated each of the taxa with periodontitis is still low - from 1 to 5. Therefore, the highest evidence category specified was moderate.

Four microorganisms out of the 17 taxa included in the moderate evidence category are not-yet cultivable, and 13 have been cultivated before. Five of the cultivable species are Gram-positive (*Eubacterium saphenum, Mogibacterium timidum*, *Peptostreptococcus stomatis, Filifactor alocis and Enterococcus faecalis*) while all the other eight (*Bacteroidales [G-2] sp. oral taxon 274*, *Porphyromonas endodontalis*, *Treponema lecithinolyticum*, *Treponema medium*, *Treponema vincentii*, *Anaeroglobus geminatus* - also known by *Megasphaera* oral clone BB166, *Selenomonas sputigena*, *Fretibacterium fastidiuosum*) are Gram-negative and anaerobic, characteristics of most of the microorganisms involved in polymicrobial infections. Five of these new candidate periodontal pathogens belong to the phyla *Bacteroidetes* and *Spirochaetes*, which include several known periodontal pathogens, such as *P. gingivalis*, *T. forsythia*, *T. denticola* and *T. socranskii* and species from the genera *Prevotella* (Socransky *et al.,* 1998). Seven species were from the *Firmicutes* phylum, and the other five species or phylotypes were distributed among the *Proteobacteria*, *Synergistetes* and *Candidatus Saccharibacteria* phyla. The phylum *Firmicute*s harbors genera previously associated with periodontal health (e.g. *Streptococcus*) or disease (e.g. *Eubacterium* and *Selenemonas*) (Socransky *et al.,* 1998; Kumar *et al.,* 2003), and several other cultivable or not-yet cultivable microorganisms from this phylum fell into the moderate (e.g. *F. alocis, E. faecalis*) or some evidence (*Dialister pneumosintes, Lachnospiraceae [G-8] sp.* HOT 500) categories.

Almost all bacterial species listed as a suspected periodontal pathogen in the present study are mostly found in the oral cavity and rarely involved in extra-oral infections. One exception was *E. faecalis,* wich is part of the commensal microbiota of the human gastrointestinal tract but may also act as an opportunistic pathogens when spreading to other mucosa or skin tissues (Vu and Carvalho, 2011). With respect to oral diseases, *E. faecalis* has been associated with root canal treatment failure (Wang *et al.,* 2012). It was interesting to note that all the evidence supporting *E. faecalis* as a candidate periodontal pathogen came out of studies that evaluated Brazilian patients (Colombo *et al.,* 2002; Souto *et al.,* 2006; Souto and Colombo 2008; da Silva-Boghossian *et al.,* 2011). This could be an example of a geographic specificity, since it has been previously suggested that the periodontal microbiota may show specific differences between different countries (Haffajee *et al.,* 2004). However, this information would need to be confirmed by future studies evaluating the prevalence and levels of this microorganism in other populations. The other exceptions of microorganisms associated with periodontitis in the present review, which may inhabit extra-oral environments are *S. sputigena*, *T. medium,* species from the *Synergistetes* and *Candidatus Saccharibacteria* phyla. *S. sputigena* is a normal resident of the upper respiratory tract and has been associated with a case of septicemia (McCarthy and Carlson, 1981) while *T. medium* has been detected in the human brain cortex of subjects with Alzheimer, but not in healthy controls (Riviere *et al.,* 2002). Species from the *Synergistetes* phylum, such as *Synergistetes jonesii* and *Peritoneal fluid isolate RMA 16088* have been isolated from the peritoneal fluid (Horz *et al.,* 2006). Species from the *Candidatus Saccharibacteria* phylum have been detected in vaginosis and bowel disease (Fredricks *et al.,* 2005; Kuehbacher

et al., 2008). The presence of microorganisms in the subgingival biofilm that are also associated with extra-oral diseases may be an important link between oral and systemic infections and should be considered in further studies.

Another finding that deserves attention in the present review concerns to the *Archaea* domain, which also fell into the moderate evidence category. Among the 41 studies included in this review only five searched for *Archaea*, and four of them showed an association between this domain and periodontitis (Lepp *et al.,* 2004; Li *et al.,* 2009; Bringuier *et al.,* 2013; Matarazzo *et al.*, 2001). Although the fifth study (Vianna *et al.,* 2008) did not find statistically significant higher prevalence or counts of metanogenic *Archaea* in subjects with periodontitis in comparison with periodontally healthy subjects, this taxa was not detected in any of the healthy subjects evaluated. Hence, while the number of studies that examined *Archaea* is still modest, all of them have suggested some type of association between this domain and periodontitis, and it would be important to conduct future investigations, in order to elucidate this evidence more clearly. To date, *Archaea* has not been associated with other infections in the body.

Some of the microorganisms showing moderate evidence of being periodontal pathogens have not yet been cultivated. It was possible to detect these species due to molecular diagnostic approaches, such as polymerase chain reaction and DNA probes introduced in the late 1990s, and more recently, the open-ended polymerase chain reaction/sequencing techniques. The results of studies using these techniques have broadened our knowledge about oral cavity ecology, including the possible role of some not-yet cultivable taxa in the etiology of periodontitis. The *Candidatus Saccharibacteria* and *Synergistetes* phyla, for example, comprise mainly uncultivated species and many of

them fell into the moderate or some evidence categories. Some of the studies using independent-culture techniques have also contributed to showing that the diversity of certain genera already associated with periodontitis, such as *Treponema*, might be greater than previously reported. It is interesting to observe that 21 species from the *Treponema* genus, other than those already recognized as periodontal pathogens, have been found in statistically significant higher levels and/or proportions and/or abundance in subjects with periodontitis in nine different studies (Table 2).

The number of plaque samples evaluated by the various studies is also an important point to consider. It has been advocated that the evaluation of large number of plaque samples per patient is a crucial requirement for obtaining reliable information about the etiology of periodontitis (Haffajee and Socransky, 2006). In this regard, there is an important difference between the targeted and open-ended molecular techniques. For instance, while the open-ended *16S rDNA* pyrosequencing approaches allow an in-depth characterization of microbial diversity, these techniques are still relatively costly, therefore, the studies using pyrosequencing have evaluated a limited number of plaque samples. However, some of the target techniques, such as checkerboard DNA-DNA hybridization and RNA-oligonucleotide quantification technique, allow the evaluation of thousands of plaque samples at a relatively low cost. Specifically, one-third of the studies included in this review used open-ended diagnostic tests and evaluated approximately 230 and 630 subgingival plaque samples from periodontally healthy or periodontitis subjects, respectively; in contrast to 3,220 and 10,160 analyzed by the two-thirds of the studies using targeted approaches. Thus, the combination of open-ended and targeted methods seems to be our best option towards full understanding of the etiology and,

consequently, of the treatment of periodontitis. Probes or primers for the suspected new pathogens detected by the *16S rDNA* pyrosequencing studies might be developed and used on a large scale by target techniques. In an even more optimistic future perspective, the cost associated with this next generation sequencing technology will be reduced and the processing of data would be simplified, allowing for the sequencing of large numbers of samples.

Overall, the data of this systematic review support the notion that the subgingival pocket is a complex environment that harbors a highly diverse microbiota. It seems evident that other microorganisms other than the already known periodontal pathogens might be involved in the onset and/or progression of periodontitis. Nonetheless, it is essential to emphasize that this review provides only the first evidence necessary to associate a microorganism with the etiopathogenesis of periodontitis; that is, higher levels and/or proportions of the species in cases than in controls (association studies). Indeed, the etiologic role of these microorganisms would need to be confirmed by risk assessment and interventional (i.e. elimination) studies to evaluate whether their reduction or elimination would be accompanied by clinical improvements and whether their persistence would lead to disease progression (Socransky, 1979). In addition, further investigation into their mechanisms of pathogenicity and their ability to promote or evade host immune response would be required.

Another important idea to keep in mind while interpreting the results of association studies is the “causal versus the casual” concept. The fact that a microorganism is found in higher levels and proportions in disease than in health might not be sufficient to determine whether it actually initiated the disease process or was merely favored by the

inflammatory environment associated with periodontitis. In recent years, this discussion around causality/casualty has gained new momentum with the introduction of novel theories about the ecological events associated with periodontal destruction (Marsh *et al.,* 2003; Socransky and Haffajee, 2005; Darveau, 2010; Hajishengallis *et al.,* 2011; Hajishengallis and Lamont, 2012). Although they differ in several aspects, a common principle of these theories is that there is a reciprocal interaction between the environment and the microbiota; specifically, environmental factors may lead to the selection or overgrowth of certain pathogens. An interesting hypothesis has suggested that certain known periodontal pathogens - termed “keystone pathogens” - that have the capacity of evade host response would be able to mediate the microbial community’s conversion into dysbiosis, and a wide perturbation of this community would cause and/or sustain the process of periodontal breakdown (Hajishengallis *et al.,* 2011). Apparently, these keystone pathogens might elevate the virulence of the entire biofilm through specific interactions with accessory pathogens (Hajishengallis and Lamont, 2012). The results of the present review might serve as the initial step for the identification of new keystone or accessory pathogens, contributing to future preventive and therapeutic strategies for periodontitis.

In summary, the results of this systematic review support moderate evidence for the association of 17 species/phyolypes from the *Bacterial* domain; the *Candidatus Saccharibacteria* phylum and the *Archaea* domain with the etiology of periodontitis. These findings would be useful to guide future investigations on the actual role of these suspected new pathogens in the onset and progression of this disease.

Acknowledgments

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Figura - Flowchart of the search strategy

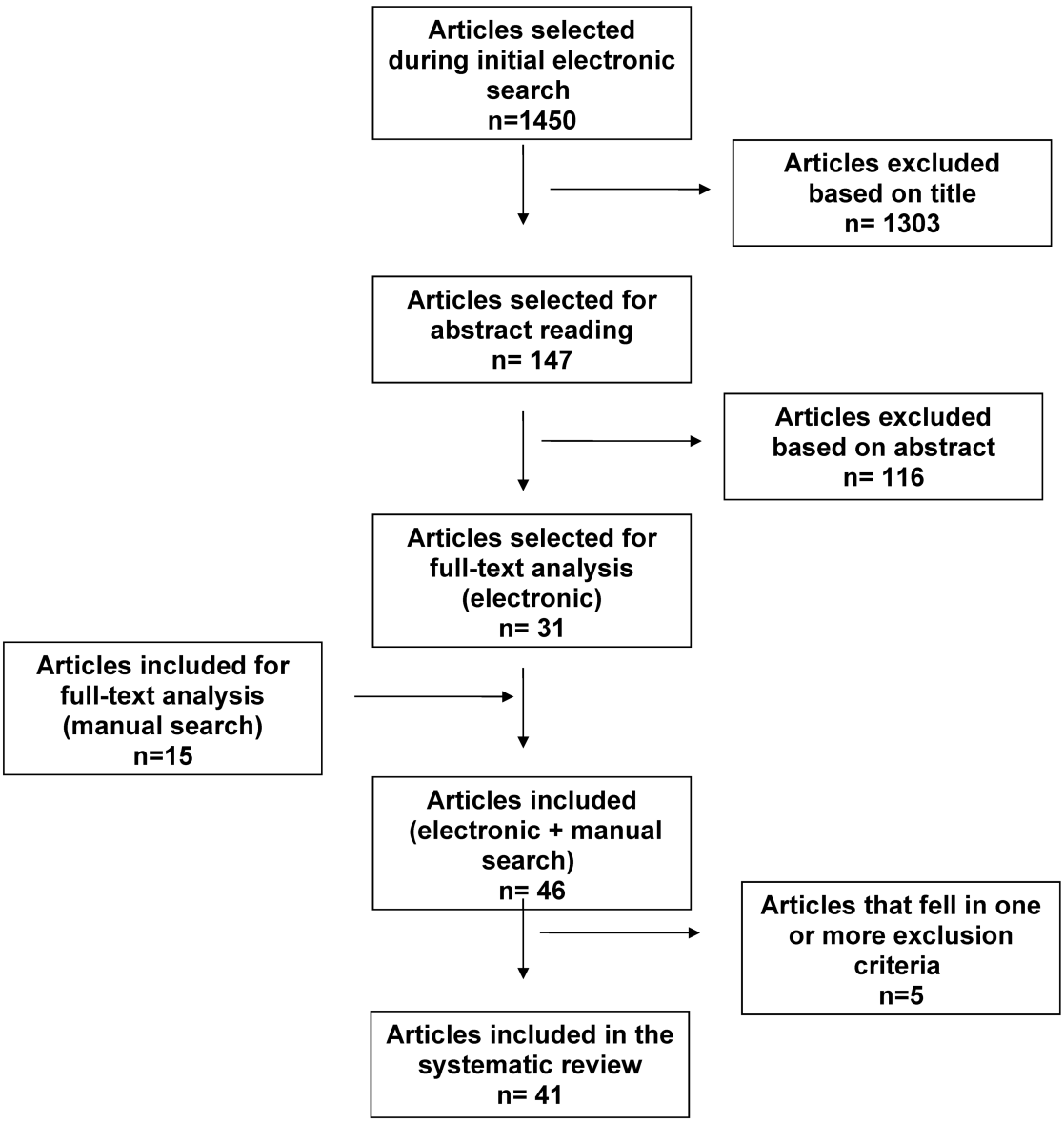

Table 1. Summary of the methodological features of the included studies

	Periodontal status (n)				**Sample (n)**		**Method/taxaevaluated**
	H	GAgP	ChP	RP	H	P	
Willis *et al.*, (1999)	10		21		10 (I)	21 (I)	Nested PCR 7 *Treponema* species
Harper-Owen *et al.*, (1999)	20		28		40 (I)	56 (I)	PCR/Sanger sequencing Phylotype PUS3.422, PUS9.170, PUS9.180
Dewhirst *et al.*, (2000)	2		1	8	NA	NA	PCR/cloning/Sanger sequencing *Spirochaetes* phylum
Sawada *et al.*, (2000)	20		40		20 (I)	40 (I)	PCR *Selenomona sputigena*, *Centipeda periodontii*
Macuch and Tanner, (2000)	18		52		44 (I)	52 (I)	Culture and SDS- Page *Campylobacter* species
Paster *et al.*, (2001)	5		9	11	NA	NA	PCR/cloning/Sanger sequencing *Bacteria* domain and *Spirochaetes*, *Bacteroidetes* phyla
Colombo *et al.*, (2002)	14		25		1492 (I)	2540 (I)	Checkerboard DNA-DNA hybridization 42 bacterial species
Leys *et al.*, (2002)	172		121		172 (P)	121 (P)	PCR/Sanger sequencing *Bacteroides forsythus* and oral clone BU063
Asai *et al.*, (2002)	13		37		13 (P)	37 (P)	PCR and qPCR Total Treponemes, *T.denticola*, *T.medium* and *T. vincentii*
Hutter *et al.*, (2003)	6	26			6 (I)	26 (I)	PCR/cloning/Sanger sequencing *Bacteria* domain
Brinig *et al.*, (2003)	4		42		18 (I)	53 (I)	PCR/cloning/Sanger sequencing, qPCR and FISH TM7 (*Candidatus Saccharibacteria*) division and TM7 I025 subgroup
Ouverney *et al.*, (2003)	4		12		9 (I)	12 (I)	FISH TM7 subgroup
Kumar *et al.*, (2003)	66		66		66 (P)	66 (P)	Nested PCR/Sanger sequencing 39 bacterial species or

							phylotypes
Zijnge *et al.*, (2003)	6		9		6 (P)	9 (P)	PCR/DGGE and PCR/Sanger sequencing *Bacteria* domain
Booth *et al.*, (2004)	40		40		40 (P)	80 (P)	Slot-blot hybridization *Bulleidia extructa, Eubacterium nodatum, Mogibacterium timidum and Slackia exigua*
Murdoch *et al.*, (2004)	28		28		84 (I)	168 (I)	Culture Oral *staphylococci*
Lepp *et al.*, (2004)	8		50		29 (I)	205 (I)	PCR/cloning/Sanger sequencing, FISH and qPCR *Archaea* and *Bacteria* domains
Mayanagi *et al.*, (2004)	12		18		12 (I)	18 (I)	Nested PCR 25 putative or probable periodontal pathogens
Kumar *et al.*, (2005)	15		15		15 (P)	30 (P)	PCR/Cloning/Sanger sequencing *Bacteria* domain
Li *et al.*, (2006)	20		35		20 (P)	35 (P)	PCR/Sanger sequencing Phylotype AU 126 and X 112
Souto *et al.*, (2006)	3		14		200 (I)	400 (I)	Checkerboard DNA-DNA hybridization 11 putative periopathogen *bacteria*
Ledder *et al.*, (2007)	18		29		18 (I)	29 (I)	PCR/DGGE, PCR/Sanger sequencing for *Bacteria* and Multiplex PCR for *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythensis*
Souto and Colombo, (2008)	56		169		56 (P)	169 (P)	PCR *Enterococcus faecalis*
Vianna *et al.*, (2008)	65		102		65 (P)	102 (P)	qPCR and Sanger sequencing *Hydrogenotrophic Archaea* and *Bacteria*
Li *et al.*, (2009)	15		41		15 (P)	41 (P)	PCR and PCR/cloning/Sanger sequencing (n=8) *Archaea* domain
Riep *et al.*, (2009)	21	44	46		105 (I)	450 (I)	Dot blot hybridization 10 Putative periodontal

							pathogen bacteria
Vartoukian *et al.*, (2009)	5		5		5 (P)	10 (P)	PCR/cloning/Sanger sequencing and FISH *Synergistetes* phylum
Schlafer *et al.*, (2010)*	19	72	30		82 (I)	408 (I)	Dot blot hybridization *Filifactoralocis*, red complex, *A.actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*
Abiko *et al.*, (2010)	12		28		12 (I)	28 (I)	qPCR Total Bacteria and 13 bacterial species
Drescher *et al.*, (2010)*	19	62	82		82 (I)	660 (I)	Dot blot hybridization *Selenomonas genus, Centipeda genus*
Da Silva-Boghossian *et al.*, (2011)	51	90	219		357 (I)	4326 (I)	Checkerboard DNA-DNA hybridization Red Complex, *A. actinomycetemcomitans*, *Acinetobacter baumannii, Escherichia coli, E. faecalis, Pseudomonas aeruginosa, Staphylococcus aureus*
Matarazzo *et al.*, (2011)	30	30			60 (I)	103 (I)	qPCR and PCR/cloning/Sanger sequencing(10 samples from each group) *Bacteria* and *Archaea* domains
Teles *et al.*, (2011)	8		11		112 (I)	154 (I)	RNA-Oligonucleotide Quantification Technique – (ROQT) 43 bacterial species
Canabarro *et al.*, (2012)	20		40		20 (I)	60 (I)	Culture *Candida albicans* and other yeast
Griffen *et al.*, (2012)	29		29		29 (I)	58 (I)	*16S rDNA*PCR 454 Pyrosequencing *Bacteria* domain
Gonçalves *et al.*, (2012)	15	15			135 (I)	135 (I)	ROQT 10 bacterial species
Liu *et al.*, (2012)	3		2		12 (I)	12 (I)	*16S rDNA* PCR 454 pyrosequencing and Illumina Metagenome high-throughput sequencing *Bacteria* domain
Bringuier *et*	10		22		10	22	qPCR

***al.*, (2013)**					(NA)	(NA)	*Methanobrevibacter oralis*
Abusleme *et al.*, (2013)	10		22		17 (I)	44 (I)	*16S rDNA* PCR 454 pyrosequencingand qPCR *Bacteria* domain
You *et al.*, (2013a)	10	1	9		10 (P)	10 (P)	PCR/Cloning/Sanger sequencing *Bacteria* domain
You *et al.*, (2013b)	10		10		10 (P)	10 (P)	PCR/Cloning/Sanger sequencing *Bacteria* domain

- ***** FISH from this study was not taken into account since no group control was performed for this experiment
- **NA:** not available, **H:** periodontal health, **GAgP:** general aggressive periodontitis, **ChP:** Chronic periodontitis, **RP:** Refractory periodontitis, **P**: Periodontits
- **(P):** samples processed in pool, **(I):** samples processed individually
- **PCR,** polymerase chain reaction; **qPCR**,quantitative polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrilamide gel electrophoresis; **FISH,** fluorescence *in situ* hybridization; **DGCE**, denaturating gradient gel electrophoresis, **ROQT,** RNA-oligonucleotide quantification techinique

Table 2. Summary of the data of the included studies. Newly taxa associated with periodontitis

TAXA	Chronic periodontitis	Generalized Agressive periodontitis
Bacteria		
Phylum *Actinobacteria*		
Actinobacteria class		
Actinomyces naeslundii HOT 176 (1)	Kumar *et al.*, 2003	
Bifidobacterium dentium HOT 588 (1)	Griffen *et al.*, 2012	
Cryptobacterium curtum HOT 579 (1)	Kumar *et al.*, 2003	
Corynebacterium diphtheria HOT 591 (1)	Souto *et al.*, 2006	
Rothia dentocariosa HOT 587 (1)	Kumar *et al.*, 2003	
Slackia exigua HOT 602 (1)	Abiko *et al.*, 2010	
Phylum*Bacteroidetes*		
Bacteroidia class		
Bacteroidetes[G-1] genus (1)	Abusleme *et al.*, 2013	
Bacteroidaceae[G-1]sp. oral taxon 272 HOT 272 [*Bacteroidetes[G-1]*sp.OT 272] (1)	Abusleme *et al.*, 2013	
Bacteroidales[G-2]sp. oral taxon 274 HOT 274[*Bacteroidete*s clone AU126/Phylotype AU126/ *Bacteroidales*OT 274] (3)	Kumar *et al.*, 2003; Li *et al.*, 2006; Griffen *et al.*, 2012	
Bacteroidetes[G-3] genus (1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-3]sp. oral taxon 280 HOT 280(1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-3] sp. oral taxon 365 HOT 365 (1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-6] genus(1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-6]sp. oral taxon 516 HOT 516(1)	Abusleme *et al.*, 2013	
Porphyromonas endodontalis HOT 273 (4)	Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Prevotella denticola HOT 291 (2)	Kumar *et al.*, 2003; Griffen *et al.*, 2012	
Prevotella sp. oral taxon 526 HOT 526 [*Prevotella* genomo *sp*. P4](1)	Griffen *et al.*, 2012	
Prevotella sp. oral taxon 304 HOT 304(1)	Abusleme *et al.*, 2013	
Alloprevotella tannerae HOT 466 [*Prevotella tannerae*](2)	Mayanagi *et al.*, 2004; Griffen *et al.*, 2012	
Phylum *Chloroflexi*		
Chloroflexi class		
*Chloroflexi [G-1] genus (*1)	Abusleme *et al.*, 2013	

Chloroflexi [G-1] sp. otal taxon 439 HOT 439(1)	Abusleme *et al.*, 2013	
Phylum *Firmicutes*		
Clostridia class (1)	Kumar *et al.*, 2005	
Clostridiales[F-1][G-1] sp. HOT 093 [Oral clone MCE_107](1)	Griffen *et al.*, 2012	
Catonella genus(1)	Liu *et al.*, 2012	
*Catonella sp.oral taxon*164 HOT 164 [*Catonella sp*. oral clone BR063] (1)	Kumar *et al.*, 2005	
Shuttle worthia C1 (1)	Griffen *et al.*, 2012	
Johnsonella sp. oral taxon 166 HOT 166 [*Johnsonella* CK051](2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Eubacterium [XI] [G-1] genus(1)	Abusleme *et al.*, 2013	
Eubacterium [XI] [G-3] brachy HOT 557 [*Eubacteriumbrachy*] (2)	Griffen *et al.*, 2012;Abusleme*et al.*, 2013	
Eubacterium[XI][G-5] saphenum HOT 759 [*Eubacterium saphenum*](5)	Kumar*et al.*, 2003; Mayanagi*et al.*, 2004; Abiko*et al.*, 2010; Griffen*et al.*, 2012; Abusleme *et al.*, 2013	
Eubacterium [XI] [G-6] genus(1)	Abusleme *et al.*, 2013	
Eubacterium[XI][G-6] minutum HOT 673 (1)	Abusleme *et al.*, 2013	
Mogibacterium genus(1)	Abusleme *et al.*, 2013	
Mogibacterium timidum HOT 042 (3)	Mayanagi *et al.*, 2004; Abiko*et al.*, 2010; Abusleme *et al.*, 2013	
Peptostreptococcaceae [XI] [G-2] genus(1)	Abusleme *et al.*, 2013	
*Peptostreptococcaceae [XI] [G-2] sp. oral taxon*091HOT 091 (1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[XI][G-4]genus(1)	Abusleme *et al.*, 2013	
*Peptostreptococcaceae[XI][*G-4] *sp. oral taxon103* HOT103 [*phylotype PUS9.170*](1)	Harper-Owen *et al.*, 1999	
Peptostreptoccaceae[XI] [G-4] sp. oral taxon 369 HOT 369(1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[XIII][G-1]genus(1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[*XIII][G-1*] *sp. oral taxon*113HOT 113[*Peptoniphilus* oral taxon 113](2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Peptostreptococcus genus(1)	Abusleme *et al.*, 2013	
Peptostreptococcus stomatis HOT 112 [*Peptostreptococcus sp.*oral clone CK035] (3)	Kumar*et al.*, 2005; Griffen*et al.*, 2012; Abusleme *et al.*, 2013	
Peptococcus sp. oral taxon 167 HOT 167 (1)	Abusleme *et al.*, 2013	

Pseudoramibacter genus (1)	Abusleme *et al.*, 2013	
Pseudoramibactera lactolyticus HOT 538(1)	Abusleme *et al.*, 2013	
Filifactor genus(1)	Abusleme *et al.*, 2013	
Filifactor alocis HOT 539 (5)	Kumar *et al.*, 2003; Kumar *et al.*, 2005; Schlafer *et al.*, 2010; Griffen*et al.*, 2012, Abusleme *et al.*, 2013	Schlafer *et al.*, 2010
Lachnospiraceae [G-8] genus (1)	Abusleme 2013	
Lachnospiraceae [G-8]sp. oral taxon 500 HOT 500 [*Lachnospiraceae*JM048](1)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Lachnospiraceae [G-4] genus (1)	Abusleme *et al.*, 2013	
Stomatobaculum sp. oral taxon 373 HOT 373[*Lachnospiraceae [G-4]* sp.OT 373](1)	Abusleme *et al.*, 2013	
Unclassified clostridialesord(1)	Abusleme *et al.*, 2013	
Negativicutes class		
Anaeroglobus geminates HOT 121 [*Megasphaerao* oral clone BB166](3)	Kumar *et al.*, 2003; Kumar *et al.*, 2005; Griffen *et al.*, 2012	
Centipeda genus (1)	Drescher *et al.*, 2010	Drescher *et al.*, 2010
Dialister invisus HOT 118 [*Dialister sp*. oral strain GBA27] (1)	Kumar *et al.*, 2003	
Dialister sp. oral taxon 119 HOT 119 [*Dialister sp*. oral clone MCE7_134] (1)	Kumar *et al.*, 2005	
Dialister pneumosintes HOT 736 (2)	Mayanagi *et al.*, 2004; Kumar *et al.*, 2005	
Megasphaera sp. oral clone MCE3_141 (1)	Kumar *et al.*, 2005	
Megasphaera sp. oral taxon 123 HOT 123 [*Megasphaera sp*. oral clone BS073](1)	Kumar *et al.*, 2005	
Mitsuokella sp. HOT 131 [*Selenomonas* CS002](1)		Gonçalves *et al.*, 2012
Selenomonas genus (2)	Liu *et al.*, 2012; Drescher *et al.*, 2010	Drescher *et al.*, 2010
Selenomonas sputigena HOT 151 (5)	Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Griffen *et al.*, 2012; Abusleme *et al.*, 2012	Gonçalves *et al.*, 2012
Selenomonas sp. oral clone D0042 (1)	Kumar *et al.*, 2005	
Selenomonas sp. oral clone 126 HOT 126 [*Selenomonas sp.* oralclone EY047/*Selenomonas* EY0471) (1)	Griffen *et al.*, 2012	
Selenomonas dianae HOT 139 (1)	Griffen *et al.*, 2012	
Veillonellaceae [G-1] genus(1)	Abusleme *et al.*, 2013	
Veillonellaceae [G-1] sp. oral taxon 129 HOT 129 (1)	Griffen *et al.*, 2012	

Veillonellaceae [G-1] sp. oral taxon 132 HOT132 (1)	Abusleme *et al.*, 2013	
Veillonellaceae [G-1] sp. oral taxon 155 HOT 155(1)	Abusleme *et al.*, 2013	
Bacilliclass		
Enterococcus faecalis HOT 604 (4)	Colombo *et al.*, 2002; Souto *et al.*, 2006; Souto and Colombo, 2008; Silva- Boghossian *et al.*, 2011	
Streptococcus sp. oral strain 9F (1)	Kumar *et al.*, 2005	
Streptococcus sp. oral taxon 061 HOT 061 [*Streptococcus sp*. oral clone DP009](1)	Kumar *et al.*, 2005	
Streptococcus constellatus HOT 576(1)	Abusleme *et al.*, 2013	
Streptococcus anginosus HOT 543 (1)	Abusleme *et al.*, 2013	
Streptococcus sp. oral taxon 071 HOT 071 (1)	Abusleme *et al.*, 2013	
Staphylococcus aureus HOT 550 (1)	Souto *et al.*, 2006	
Phylum*Fusobacteria*		
Fusobacteriia class		
Fusobacterium oral taxon A71 (1)	Griffen *et al.*, 2012	
Fusobacterium nucleatum subsp.animalis HOT 420 [*Fusobacterium animalis*](1)	Abusleme *et al.*, 2013	
Leptotrichiaceae [G-1] sp. oral taxon 210 HOT 210 (1)	Griffen *et al.*, 2012	
Leptotrichia sp. oral taxon 498 HOT 498 [*Leptotrichia* IK040](1)	Griffen *et al.*, 2012	
Leptotrichia EX103 (1)	Griffen *et al.*, 2012	
Sneathia sanguinegens HOT 837(1)	Abusleme *et al.*, 2013	
Phylum*Proteobacteria*		
Alphaproteobacteria class		
Bartonellasp. (1)	Colombo *et al.*, 2002	
Gammaproteobacteriaclass		
*Acinetobacter baumannii*HOT 554 (2)	Silva- Boghossian *et al.*, 2011; Souto *et al.*, 2006	Silva- Boghossian *et al.*, 2011
Aggregatibacter sp. oral taxon 458 HOT 458 *[Aggregatibacter*AY349380](1)	Griffen *et al.*, 2012	
Escherichiacoli HOT 574 (2)	Colombo *et al.*, 2002; Souto *et al.*, 2006	
Klebsiella pneumoniae HOT 731 (1)	Souto *et al.*, 2006	
Pseudomonas sp. (1)	Ledder *et al.*, 2007	
Pseudomonas aeruginosa HOT 536 (1)	Souto *et al.*, 2006	
Deltaproteobacteria class		
Desulfobulbus genus (1)	Abusleme *et al.*, 2013	

Desulfobulbos sp. oral taxón 041 [Clone *Desulfobulbus sp.* R004/ *Desulfobulbus sp*. oral clone R004/*Desulfobulbos sp.* OT 041/*Desulfobulbus*R004] (3)	Kumar *et al.*, 2005; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Desulfobulbus oral clone CH031 (1)	Kumar *et al.*, 2005	
Epsilonproteobacteria class		
Campylobacter sputorum HOT 776 (1)	Kumar *et al.*, 2005	
Campylobacter sp. oral taxon 044 HOT 044 [*Campylobac*ter *sp*. oral clone BB120] (1)	Kumar *et al.*, 2005	
Phylum *Spirochaetes*		
Spirochaetia class		
Treponema phylogroup II (2)	You *et al.*, 2013a	Riep *et al.*, 2009; You *et al.*, 2013a
Treponema phylogroup III (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema phylogroup V (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema phylogroup I:OTU 8P68 (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema sp. IIC:T1 HOT 246(1)	Griffen *et al.*, 2012	
Treponema phylogroup II:OTU 1P26 (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema amylovorum HOT 541 (1)	Griffen *et al.*, 2012	
Treponema lecithinolyticum HOT 653 (4)	Kumar *et al.*, 2003;, Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Riep *et al.*, 2009
Treponema maltophilum HOT 664 (2)	Dewhirst *et al.* 2000; Abusleme *et al.*, 2013,	
Treponema medium HOT 667 (5)	Asai *et al.*, 2002; Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Treponema vincentii HOT 029 (3)	Willis *et al.*, 1999; Asai *et al.*, 2002; Griffen *et al.*, 2012	
Treponema sp. oral taxon 230 HOT 230 (1)	Griffen *et al.*, 2012	
Treponema sp. oral taxon 490 HOT 490[*Treponema E25-8*](1)	Griffen *et al.*, 2012	
*Treponema*E_D_05_72 (1)	Griffen *et al.*, 2012	
*Treponema sp. oral taxon*237HOT 237 (1)	Abusleme *et al.*, 2013	
Treponema maltophilum HOT 664 (1)	Abusleme *et al.*, 2013	
Treponema sp. oraltaxon 257 HOT 257 [*Treponema* D36ER-1](2)	Dewhirst *et al.*, 2000; Abusleme *et al.*, 2013	
Treponema sp. oraltaxon 249 HOT 249 (1)	Abusleme *et al.*, 2013	
Treponema sp. parvum HOT 274 (1)	Abusleme *et al.*, 2013	
Treponema sp. oral taxon 253 HOT 253 (1)	Abusleme *et al.*, 2013	

Treponema sp. oral taxon 258 HOT 258 (1)	Abusleme *et al.*, 2013	
Treponema sp. (1)	Abusleme *et al.*, 2013	
Phylum *Synergistetes*(1)	Vartoukian *et al.*, 2009	
Unclassified class		
Synergistetes oral clone A2F 22 ["*Synergistetes*" OTU 4.2 A2F_22-OTU 4.2 FJ490414](1)	Vartoukian *et al.*, 2009	
Synergistes oral taxon G36 (1)	Griffen *et al.*, 2012	
Fretibacterium sp. oral taxon 359 HOT 359 [*Deferribacteres sp.*oral clone BH007/ *Synergistetes*OTU 7P1] (2)	Kumar *et al.*, 2005; You *et al.*, 2013b	
Fretibacterium sp. oral taxon 360 HOT 360 [*Deferribacteres* clone BH017/ Synergistes oral taxon 360/*Synergistetes* OTU 7P22*/Synergistes [G-3]* sp. OT 360] (4)	Kumar *et al.*, 2003; Griffen *et al.*, 2012; You *et al.*, 2013b; Abusleme *et al.*, 2013	
Fretibacterium sp. oral taxon 361 HOT 361[*Synergistes [G-3]sp. OT361*](1)	Abusleme *et al.*, 2013	
Fretibacterium sp. oral taxon 362 HOT 362[*Deferribacteres* clone D084/ *Synergistetes [G-3]* sp.OT362/ *Synergistetes* OTU 2P9/*Synergistetes* OTU 6P18](3)	Kumar*et al.*, 2003; You *et al.*, 2013b; Abusleme *et al.*, 2013	
Fretibacterium fastidiosum HOT 363 [*Deferribacteres*sp. oral clone W090/ *Synergistetes[G-3]* sp. OT 363/ *Synergistetes* OT 4P12] (3)	Kumar *et al.*, 2005; You *et al.*, 2013b; Abusleme *et al.*, 2013	
Fretibacterium sp.oral taxon 453 HOT 453[*Synergistes* OT 453] (1)	Griffen *et al.*, 2012	
Phylum *Tenericutes*		
Mollicutes class		
Mycoplasma genus (1)	Abusleme *et al.*, 2013	
Mycoplasma facium HOT 606 (1)	Abusleme *et al.*, 2013	
Phylum *CandidatusSaccharibacteria* (Syn. Candidatedivision TM7) (3)	Brinig *et al.*, 2003; Ouverney *et al.*, 2003; Liu *et al.*, 2013	
TM7 [G-1]sp.oral taxon 346 HOT 346 [*TM7* 401H12](2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
TM7[G-1]sp. oral taxon 347 HOT 347 (1)	Griffen*et al.*, 2012	
TM7 [G-1] *sp. oral taxon 349* HOT 349 (2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
TM7 [G-5]genus (1)	Abusleme*et al.*, 2013	
TM7 [G-5]sp. oral taxon 356 HOT 356 [TM7 Clone I025] (3)	Kumar *et al.*, 2003; Brinig *et al.* 2003; Abusleme *et*	

	al., 2013	
Candidate division Sulphur River 1 (Candidatedivision SR1)		
SR1[G-1] sp. oral taxon 345 HOT 345[OP11 clone X112/Phylotype X112](2)	Kumar *et al.*, 2003; Li *et al.*, 2006	
Archaea(3)	**Lepp *et al.*, 2004; Li *et al.*, 2009**	**Matarazzo *et al.*, 2011**
Phylum *Euryarchaeota*		
Methanobacteria class		
Methanobrevibacter oralis HOT 815 [Uncultured *Methanobrevibacter* isolate mcrA-II] (1)	Bringuier *et al.*, 2013	
Eukaryote		
Fungi Kingdom (1)	Canabarro *et al.*, 2012	

GAgP: generalized aggressive periodontitis, **ChP**: Chronic periodontitis, **HOT**: Human Oral Taxon (designations are provided in accordance with the Human Oral Microbiome Database), **()**:number of studies, **[ ]:** Other nomenclatures for the specie/phylotype used on the different studies

Table 3. Weight of evidence for newly identified periodontal pathogens in the etiology of Periodontitis.

Evidence	Studies (n)	Taxa
Moderate[@]		**Phyum *Bacteroidetes***
	3	1. *Bacteroidales [G-2] sp. oral taxon 274* HOT 274 (-)* [Bacteroidetes clone AU126/Phylotype AU126/ BacteroidalesOT 274]
	4	2. *Porphyromonas endodontalis* HOT 273 (-)*
		Phylum *Firmicutes*
	5	*3. Eubacterium [XI] [G-5] saphenum*HOT 759 (+)* *[Eubacteriumsaphenum*]
	3	4. *Mogibacterium timidum*HOT 042 (+)*
	3	*5. Peptostreptococcus stomatis*HOT 112 (+)* [*Peptostreptococcus sp*. oral clone CK035]
	5	*6. Filifactor alocis*HOT 539(+)*
	3	7. *Anaeroglobus geminatus* HOT 121 (-)* [*Megasphaera* oral clone BB166]
	5	*8. Selenomonas sputigena*HOT 151(-)*
	4	9. *Enterococcus faecalis*HOT 604(+) ´
		Phylum *Proteobacteria*
	3	10. *Desulfobulbus sp. oral taxon 041* HOT 041 [*Desulfobulbus sp. oral clone* R004/ *Desulfobulbos sp.* OT 041/*Desulfobulbus* R004]
		Phylum *Spirochaetes*
	4	11. *Treponema lecithinolyticum* HOT 653 (-)*
	5	12. *Treponema médium* HOT 667 (-)*
	3	13. *Treponema vincentii* HOT 029 (-)*
		Phylum *Synergistetes*
	4	14. *Fretibacterium sp. oral taxon 360* HOT 360 [*Deferribacteres* clone BH017/ *Synergistes* oral taxon 360/*Synergistetes* OTU 7P22/*Synergistes [G-3] sp. OT 360*]
	3	15. *Fretibacterium sp. oral taxon 362 HOT* 362 [*Deferribacteres* clone D084/ *Synergistetes [G-3] sp.* OT362/ *Synergistetes* OTU 2P9/*Synergistetes*OTU 6P18]
	3	16. *Fretibacterium fastidiuosum* HOT 363 (-)* [*Deferribacteres sp.* oral clone W090/ *Synergistetes[G-3] sp*. OT 363/ *Synergistetes* OT 4P12]
	3	17. **Phylum *Candidatus saccharibacteria*** (Syn. Candidatedivision TM7)
	3	
	3	18. *TM7 [G-5] sp. oral taxon 356* HOT 356 [TM7 clone I025]
		19. ***Archaea* domain**
Some[&]		**Phylum *Bacteroidetes***
	2	1. *Prevotella denticola*HOT 291 (-)*
	2	2. *Alloprevotella tannerae*HOT 466 (-)* [*Prevotella tannerae*]
		Phylum *Firmicutes*
	2	3. *Selenomonas genus* (-)*
	2	4. *Johnsonella sp. oral taxon 166* HOT 166 [*Johnsonella*CK051]
	2	5. *Eubacterium [X1] [G-3] brachy*HOT 557(+)* [*Eubacterium brachy*]
	2	*6. Peptostreptococcaceae* [*XIII][G-1*] *sp.* HOT 113

		[*Peptoniphilus* oral taxon 113]
	2	7. *Lachnospiraceae [G-8]sp.* HOT 500 [*Lachnospiraceae*JM048]
	2	8. *Dialister pneumosintes*HOT 736 (-)*
		Phylum *Proteobacteria*
	2	9. *Acinetobacter baumannii*HOT 554 (-)*
	2	10. *Escherichia coli* HOT 574 (-)´
		Phylum *Spirochaetes*
	2	11. *Treponema phylogroup*II(-)*
	2	12. *Treponema altophilum* HOT 254 (-)*
	2	13. *Treponema sp. oraltaxon 257*HOT 257(-)* [Treponema D36ER-1]
		Phylum *Synergistetes*
	2	14. *Fretibacterium sp. oral taxon 359* HOT 359 [*Deferribacteres sp.* Oral Clone BH007/ *Synergistetes* OTU 7P1]
		Phylum *Candidatus saccharibacteria*(Syn. Candidatedivision TM7)
	2	15. *TM7* [*G-1] sp.oral taxon 346* HOT 346 [TM7401H12]
	2	16. *TM7 [G-1] sp. oral taxon 349* HOT 349
		Candidate division Sulphur River 1(Candidatedivision SR1)
	2	17. *SR1[G-1] sp. oral taxon 345* HOT 345 [OP11 clone X112/Phylotype X112]

(+): Gram positive, **(-):** Gram negative, ***:** anaerobic, **´:**facultative anaerobic, **[]:** Other nomenclatures for the specie/phylotype used on the different studies**; Species in light grey**: not-yet cultivable, **P:** Periodontitis, **H:** periodontal health

Specie/phylothype/phylum/domain found in statistically significantly higher level and/or prevalence and/or proportion and/or abundance in periodontitis than in periodontal health in 3, 4 or 5 (Moderate evidence - @) OR in 2 (Some evidence - &) studies

Table S1- Search Strategy

	Term sused	**Number of studies**
	periodontitis OR periodontal infection OR chronic periodontitis OR aggressive periodontitis OR refractory periodontitis OR periodontal disease	76.286
And	bacteria OR microorganism OR archaea OR yeast OR prokaryotic OR pathogen OR uncultivated OR yet cultivated OR novel species OR novel pathogens OR microorganisms OR biofilm OR bacterial community OR subgingival plaque OR oral microbiota OR microbiome OR biofilm OR operational taxonomic unit OR Phylogroup OR *Bacteroidetes* OR *Firmicutes* OR *Treponema* OR *Proteobacteria* OR Synergistetes OR Actinobacteria OR *Fusobacteria* OR *Deferribacteres* OR *Dialister* OR *Selenomonas* OR *Streptococcus* OR *Acinetobacter* OR clone AU 126 OR OP11 OR TM7 OR *Cryptobacterium curvum*OR *Enterococcus faecalis* OR *Escherichia coli* OR *Eubacterium saphenous* OR *Exiguobacterium aurantiacum* OR *Megasphaera* OR *Mogibacterium timidus*OR *Peptostreptococcus magnus*OR *Porphyromonas endodontalis* OR *Prevotella corporis* OR *Prevotella denticola* OR *Prevotella disiens* OR *Slackia exigua*	14.274
And	Metagenome OR HOMIM OR DNA-DNA hybridization OR DGGE OR PCR OR high throughput sequencing OR sanger sequencing OR cloning OR independent-culture methods OR FISH OR pyrosequencing OR ROQT OR dot blot hybridization	1.450

Table S2. Excluded studies and reasons.

Excluded due to the lack of microbiological data from subgingival plaque sample for the periodontally healthy group
Sakamoto M, Huang Y, Umeda M, Ishikawa I, Benno Y (2002). Detection of novel oral phylotypes associated with periodontitis. *FEMS Microbiol Lett* 217:65–69.
Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, *et al.* (2013). Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease. *Sci Rep* 3:1843.
Kistler JO, Booth V, Bradshaw DJ, Wade WG (2013). Bacterial Community Development in Experimental Gingivitis. *PLoS One* 8:e71227.
Excluded due to the lack of direct microbial comparison between periodontally healthy and diseased groups at baseline
Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL (2006). Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J Clin Microbiol* 44:3665–3673.
Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, *et al.* (2009). Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol* 80:1421–1432.

Table S3. Microorganisms associated with periodontitis in the included studies (i.e. found in higher leveland/orprevalenceand/orproportionand/orabundance in periodontitis than in periodontal health, with or without statistical significance).

TAXA	Chronic Periodontitis	Generalized Aggressive periodontitis
Bacteria		
Phylum*Actinobacteria*		
Actinobacteria class		
Actinomyces israelii HOT 645(1)	Zijnge *et al.*, 2003	
Actinomyces gerencseriae HOT 618(1)	Teles *et al.*, 2011	
Actinomyces naeslundii HOT 176 (1)	Kumar*et al.*, 2003	
Atopobium parvulum HOT 119 [*Atopobium*sp. *oral clone* C019]*(*1)	Kumar *et al.*, 2005	
Atopobium parvulum HOT 723 (1)	Paster *et al.*, 2001	
Atopobium rimae HOT 750(1)	Paster *et al.*, 2001	
Bifidobacterium dentium HOT 588 (1)	Griffen *et al.*, 2012	
Cryptobacterium curtum HOT 579(1)	Kumar *et al.*, 2003	
Corynebacterium diphtheria HOT 591 (1)	Souto *et al.*, 2006	
Rothia dentocariosa HOT 587(1)	Kumar*et al.*, 2003	
Slackia exigua HOT 602 (2)	Booth *et al.*, 2004; Abiko *et al.*, 2010	
Phylum *Bacteroidetes*(1)	Griffen *et al.*, 2012	
Bacteroidia class		
Bacteroidales[G-2]sp. oral taxon 274 HOT 274[*Bacteroidetes* clone AU126/Phylotype AU126/ *Bacteroidales* OT 274] (3)	Kumar *et al.*, 2003; Li *et al.*, 2006; Griffen *et al.*, 2012	
Bacteroidetes[G-1] genus (1)	Abusleme *et al.*, 2013	
Bacteroidaceae[G-1]sp. oral taxon 272 HOT 272 [*Bacteroidetes[G-1]sp.*OT 272] (1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-3] genus (1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-3]sp. oral taxon 280 HOT 280(1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-3] sp. oral taxon 365 HOT 365 (1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-6] genus(1)	Abusleme *et al.*, 2013	
Bacteroidetes[G-6]sp. oral taxon 516 HOT 516(1)	Abusleme *et al.*, 2013	
Porphyromonas genus(1)	Abusleme *et al.*, 2013	

Porphyromonas gingivalis HOT 619(13)	Paster *et al.*, 2001; Colombo *et al.*, 2002; Leys *et al.*, 2002; Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Abiko *et al.*, 2010; Silva-Boghossian *et al.*, 2011; Griffen *et al.*, 2012; Drescher *et al.*, 2010; Teles *et al.*, 2011; Abusleme *et al.*, 2013	Hutter *et al.*, 2003; Silva-Boghossian *et al.*, 2011; Gonçalves *et al.*, 2012; Drescher *et al.*, 2010
Porphyromonas endodontalis HOT 273 (6)	Paster *et al.*, 2001; Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Hutter *et al.*, 2003
Tannerella genus(2)	Liu *et al.*, 2012; Abusleme *et al.*, 2013	
Tannerella forsythia HOT 613 [*Bacteroides forsythus*](11)	Paster *et al.*, 2001; Colombo *et al.*, 2002; Leys *et al.*, 2002; Kumar *et al.*, 2003; Kumar *et al.*, 2005; Griffen *et al.*, 2012; Abiko *et al.*, 2010; Silva-Boghossian *et al.*, 2011; Abusleme *et al.*, 2013	Schlafer *et al.*, 2010; Drescher *et al.*, 2010; Silva-Boghossian *et al.*, 2011
Prevotella genus(1)	Liu *et al.*, 2012	
Prevotella denticola HOT 291(3)	Paster *et al.*, 2001; Kumar *et al.*, 2003; Griffen *et al.*, 2012	
Prevotella sp. oraltaxon 526 HOT 526 [*Prevotella* genomo *sp* P4](1)	Griffen*et al.*, 2012	
Prevotella intermedia HOT 643(5)	Colombo *et al.*, 2002; Mayanagi *et al.*, 2004; Abiko *et al.*, 2010; Griffen *et al.*, 2012; Drescher *et al.*, 2010	Drescher *et al.*, 2010

Prevotella nigrenscens HOT 693(1)	Colombo *et al.*, 2002	
Prevotella melaninogenica HOT 469(1)	Colombo *et al.*, 2002	
Prevotella oris HOT 311 (1)	Paster *et al.* 2001	
Prevotella sp. oral taxon 306 HOT 306(1)	Teles *et al.*, 2011	
Prevotella sp. oral taxon 304 HOT 304(1)	Abusleme *et al.*, 2013	
Alloprevotella tannerae HOT 466 [*Prevotella tannerae*](2)	Mayanagi *et al.*, 2004; Griffen *et al.*, 2012	
Flavobacteria class		
Capnocytophaga gingivalis HOT 337(1)	Paster *et al.*, 2001	
Phylum *Chloroflexi*		
Chloroflexi class		
*Chloroflexi [G-1] genus (*1)	Abusleme *et al.*, 2013	
Chloroflexi [G-1] sp. HOT 439(1)	Abusleme *et al.*, 2013	
Phylum *Firmicutes*		
Erysipelotrichia class(1)	Griffen *et al.*, 2012	
Clostridia class (2)	Kumar *et al.*, 2005; Griffen *et al.*, 2012	
Clostridiales[F-1][G-1] sp. oral taxon 093 HOT093 [Oral clone MCE_107](1)	Griffen *et al.*, 2012	
Catonellagenus(1)	Liu *et al.*, 2012	
Catonella sp. oral taxon 164 HOT 164 [*Catonella sp.* oral clone BR063] (1)	Kumar *et al.*, 2005	
ShuttleworthiaC1 (1)	Griffen *et al.*, 2012	
Johnsonella sp. oral taxon 166 HOT 166 [*Johnsonella* CK051] (2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Parvimonas genus (1)	Abusleme *et al.*,2013	
Parvimonas micra HOT 111 [*Peptostreptococcus micros*] (4)	Kumar *et al.*, 2003; Kumar *et al.*, 2005; Teles *et al.*, 2011; Abusleme *et al.*, 2013	
Eubacterium [XI] [G-1] genus (1)	Abusleme *et al.*, 2013	
Eubacterium[XI][G-1] infirmum HOT 105 [*Eubacterium s*p. oral strain A35MT] (1)	Kumar *et al.*, 2005	
Eubacterium [XI] [G-3] brachy HOT 557 [*Eubacterium brachy*](2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	

Eubacterium nodatum [XI] [G-6] HOT 694 [*Eubacterium nodatum*](2)	Booth *et al.*, 2004; Abusleme *et al.*, 2013	
Eubacterium[XI][G-5] saphenum HOT 759 [*Eubacterium saphenum*](6)	Paster *et al.*, 2001; Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Abiko *et al.*, 2010; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Eubacterium [XI] [G-6] genus (1)	Abusleme *et al.*, 2013	
Eubacterium[XI][G-6] minutum HOT 673 (1)	Abusleme *et al.*, 2013	
Mogibacterium genus(1)	Abusleme *et al.*, 2013	
Mogibacterium timidum HOT 042 (3)	Mayanagi *et al.*, 2004; Abiko *et al.*, 2010; Abusleme *et al.*, 2013	
Peptostreptococcaceae [XI][G-2]genus(1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae [XI] [G-2] sp. oral taxon 091 HOT 091 (1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[XI][G-4] genus (1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[XI][G-4] sp. HOT 103 [phylotypePUS9.170/Uncultured*Eubacterium*PUS9.170](3)	Harper-Owen *et al.*, 1999; Paster *et al.*, 2001; Zijnge *et al.*, 2003	
Peptostreptoccaceae[XI] [G-4] HOT 369(1)	Abusleme *et al.*, 2013	
Peptostreptococcaceae[XIII][G-1] genus(1)	Abusleme *et al.*, 2013	
*Peptostreptococcaceaesp. [XIII] [G-1]*sp. *oral taxon 113*HOT 113 [*Peptoniphilus* oral taxon 113](2)	Abusleme *et al.*, 2013; Griffen *et al.*, 2012	
Peptostreptococcus genus (1)	Abusleme *et al.*, 2013	
Peptostreptococcus anaerobius HOT 542 (1)	Kumar *et al.*, 2005	
Peptostreptococcus stomatis HOT 112 [*Peptostreptococcus sp.*oral clone CK035](3)	Kumar *et al.*, 2005; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Peptococcus sp oral taxon 167 HOT 167 (1)	Abusleme *et al.*, 2013	

Pseudoramibacter genus(1)	Abusleme *et al.*, 2013	
Pseudoramibacter alactolyticus HOT 538(1)	Abusleme *et al.*, 2013	
Filifactor genus(1)	Abusleme *et al.*, 2013	
Filifactor alocis HOT 539(8)	Paster *et al.*, 2001; Kumar *et al.*, 2003; Kuma *et al.*, 2005; Griffen *et al.*, 2012; Schlafer *et al.*, 2010; Zijnge *et al.*, 2003; Abusleme *et al.*, 2013	Hutter *et al.*, 2003; Schlafer*et al.*, 2010
Lachnospiraceae [G-8] genus(1)	Abusleme *et al.*, 2013	
Lachnospiraceae [G-8] sp. HOT *500* [*Lachnospiraceae* JM048] (2)	Griffen*et al.*, 2012;Abusleme*et al.*, 2013	
Lachnospiraceae [G-4]genus (1)	Abusleme *et al.*, 2013	
Stomatobaculum sp. oral taxon 373 HOT 373[*Lachnospiraceae [G-4]* sp. OT 373](1)	Abusleme *et al.*, 2013	
Unclassified clostridialesord (1)	Abusleme *et al.*, 2013	
Negativicutes class(1)	Griffen*et al.*, 2012	
Anaeroglobus geminatus HOT 121 [*Megasphaera* oral clone BB166] (4)	Paster *et al.*, 2001; Kumar*et al.*, 2003; Kumar*et al.*, 2005; Griffen*et al.*, 2012	
Centipedagenus(1)	Drescher *et al.*, 2010	Drescher *et al.*, 2010
Dialister invisus HOT 118 [*Dialister sp*. oral *strain* GBA27](1)	Kumar *et al.*, 2003	
Dialister pneumosintes HOT 736 (4)	Paster *et al.*, 2001; Mayanagi *et al.*, 2004; Kumar *et al.*, 2005	Hutter *et al.*, 2003
Dialister sp. oral taxon *119* HOT 119 [*Dialister sp.* oral clone MCE7_134] (1)	Kumar *et al.*, 2005	
Megaesphaera sp. oral clone MCE3_141(1)	Kumar *et al.*, 2005	
Megasphaera sp. oral taxon 123 HOT 123 [*Megasphaera sp.* oral clone BS073](1)	Kumar *et al.*, 2005	
Mitsuokellasp. oral taxon 131 HOT 131 [*Selenomonas* oral clone CS002](1)		Gonçalves *et al.*, 2012
Selenomas genus (2)	Drescher *et al.*,	Drescher *et al.*, 2010

	2010; Liu *et al.*, 2012	
Selenomonas sputigena HOT 151 (5)	Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Griffen*et al.*, 2012; Abusleme *et al.*, 2013	Gonçalves *et al.*, 2012
Selenomonas noxia HOT 130(1)	Teles *et al.*, 2011	
*Selenomonas sp.*oral cloneD0042 (1)	Kumar *et al.*, 2005	
Selenomonas flueggei 125 [*Selenomonas flueggei*-likesp.clone AH132] (1)	Kumar *et al.*, 2005	
Selenomonas dianae HOT 139 (1)	Griffen *et al.*, 2012	
Selenomonas sp. oral clone 126 HOT 126 [*Selenomonassp.* Oral clone EY047/*Selenomonas*EY047](2)	Kumar*et al.*, 2005; Griffen*et al.*, 2012	
Veillonellaceae [G-1] genus(1)	Abusleme *et al.*, 2013	
Veillonellaceae [G-1]sp. oral taxon 129 HOT 129 [*Veillonellaceae* oral taxon 129](1)	Griffen *et al.*, 2012	
Veillonellaceae [G-1]sp. oral taxon 132 HOT132 (1)	Abusleme *et al.*, 2013	
Veillonellaceae [G-1] sp. oraltaxon 155 HOT 155 (1)	Abusleme *et al.*, 2013	
Uncultured *Veillonella* sp. clone BL013[*Veillonella sp*. DQ188766](1)	Ledder *et al.*, 2007	
Bacilli class		
Exiguobacterium aurantiacum (1)	Zijnge *et al.*, 2003	
Gemella haemolysans HOT 626(1)	Paster *et al.*, 2001	
Staphylococcus aureus HOT 550(1)	Souto *et al.*, 2006	
*Staphylococcus epidermidisisolate*CV64 [*Staphylococcus epidermidis*AJ717377](1)	Ledder *et al.*, 2007	
Granulicatella adiancens HOT 534 [*Abiotrophiaadiacens*](1)	Paster *et al.*, 2001	
Enterococcus faecalis HOT 604(4)	Colombo *et al.*, 2002; Souto *et al.*, 2006; Souto and Colombo, 2008; Silva- Boghossian *et al.*, 2011	
Streptococcus constellatus HOT 576(1)	Paster *et al.*, 2001; Abusleme *et al.*, 2013	
Streptococcus anginosus/gordonii(1)	Teles *et al.*, 2011	
Streptococcus anginosus HOT 543 (1)	Abusleme *et al.*,	

	2013	
Streptococcus sp. oral strain 9F(1)	Kumar *et al.*, 2005	
Streptococcus sp. oral taxon 061 HOT 061[*Streptococcus* sp. oral clone DP009](1)	Kumar *et al.*, 2005	
Streptococcus sp. oral taxon 071 HOT 071 (1)	Abusleme *et al.*, 2013	
Streptococcus sp. Clone BL045B32 [*Streptococcus sp*. DQ188664] (1)	Ledder *et al.*, 2007	
Phylum*Fusobacteria*		
Fusobacteriiaclass		
Fusobacteriumnucleatum subsp.animalis HOT 420 [*Fusobacteriumanimalis*](2)	Paster *et al.*, 2001; Abusleme *et al.*, 2013	
Fusobacterium nucleatum subsp. Polymorphum HOT 202(1)	Teles *et al.* 2011	
Fusobacterium nucleatum sub sp. nucleatum HOT 698[*Fusobacteriumnucleatum*](4)	Schlafer *et al.*, 2010; Drescher *et al.*, 2010; Teles *et al.*, 2011	Riep*et al.*, 2009; Schlafer *et al.*, 2010
Fusobacterium oral taxon A71(1)	Griffen *et al.*, 2012	
Leptotrichiaceae [G-1] HOT 210(1)	Griffen *et al.*, 2012	
Leptotrichia sp. oral taxon 498 HOT 498 [*Leptotrichia* IK040](1)	Griffen *et al.*, 2012	
Leptotrichia EX103(1)	Griffen*et al.*, 2012	
Sneathia sanguinegens HOT 837(1)	Abusleme *et al.*, 2013	
Phylum *Proteobacteria*		
Alphaproteobacteria class		
Bartonella sp. (1)	Colombo *et al.*, 2002	
Betaproteobacteria class		
Azohydromonas lata [*Alcali geneslatus*D88007](1)	Ledder *et al.*, 2007	
Eikenella corrodens HOT 577(1)	Kumar *et al.*, 2003	
Gammaproteobacteria class		
Acinetobacter baumannii HOT 554 (2)	Souto *et al.*, 2006; Silva-Boghossian *et al.*, 2011	Silva-Boghossian *et al.*, 2011
Aggregatibacter actinomycetemcomitans HOT 531 [*Actinobacillus actinomycetemcomitans*] (4)	Colombo *et al.*,2002; Zijnge *et al.*, 2003; Ledder *et al.*, 2007; Silva-Boghossian *et al.*, 2011	Silva-Boghossian *et al.*, 2011
Aggregatibacter sp. oral taxon 458 HOT 458 *[Aggregatibacter* AY349380](1)	Griffen *et al.*, 2012	

Escherichia coli HOT 574(2)	Colombo *et al.*,2002; Souto *et al.*, 2006	
Haemophilus parainfluenzae HOT 718(1)	Paster *et al.*, 2001	
Klebsiella pneumoniae HOT 731(1)	Souto *et al.*, 2006	
Pseudomonas sp. Ps8 [*Pseudomonas* sp. AY365082](1)	Ledder *et al.*, 2007	
*Pseudomonas aeruginosa*HOT 536(1)	Souto *et al.*, 2006	
Deltaproteobacteria class		
Desulfobulbus genus (1)	Abusleme *et al.*, 2013	
Desulfobulbos sp. Oral taxón 041 [Clone *Desulfobulbus sp*. R004*/ Desulfobulbus sp*. oral clone R004*/Desulfobulbos sp*.OT 041/*Desulfobulbus* R004](6)	Paster *et al.* 2001; Kumar *et al.*, 2005; Teles *et al.*, 2011; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Desulfobulbus sp.oral clone CH031 (1)	Kumar *et al.*, 2005	
*Desulfovibrio fairfieldensis*HOT 605 (1)	Zijnge *et al.*, 2003	
Uncultured *Desulfovibrio sp. Isolated* dsrABII (1)	Vianna *et al.*, 2008	
Epsilonproteobacteria class		
Campylobacter sp. (1)	Zijnge *et al.*, 2003	
*Campylobacter rectus*HOT 748(6)	Macuchand Tanner 2000; Colombo *et al.*, 2002; Abiko *et al.*, 2010; Griffen *et al.*,2012; Abusleme *et al.*, 2013	Riep*et al.*, 2009
Campylobacter X [*Campilobacter showae* HOT 763] (1)	Macuch and Tanner 2000	
Campylobacter sputorum HOT 776 [*Campylobacter sputorumsputorum*](1)	Kumar *et al.*, 2005	
*Campylobac*ter *sp. oral taxon 044* HOT 044 [*Campylobac*ter *sp*. oral clone BB120] (1)	Kumar *et al.*, 2005	
Phylum *Spirochaetes*(2)	Griffen *et al.*, 2012; You *et al.*, 2013a	
Spirochaetiaclass		
Treponema genus (2)	Liu *et al.*, 2012; Abusleme *et al.*, 2013	
*Treponema phylogroup*II (2)	You *et al.*, 2013a	Riep*et al.*, 2009; You *et al.*, 2013a
Treponema phylogroup III (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema phylogroup V (1)	You *et al.*, 2013a	You *et al.*, 2013a

Treponema sp. I:G:T21 HOT 231 [Clone *Treponema sp*.I:G:T21](1)	Paster *et al.*, 2001	
Treponema sp. IIC:T1 HOT 246(1)	Griffen *et al.*, 2012	
Treponema sp. oral taxon 237HOT 237 (1)	Abusleme *et al.*, 2013	
Treponema amylovorum HOT 541(2)	Dewhirst *et al.*, 2000; Griffen *et al.*, 2012	
Treponema denticola HOT 584 [OTU8P47](10)	Colombo *et al.*, 2002; Kumar *et al.*, 2003; Mayanagi *et al.* 2004; Asai *et al.*, 2012; Griffen *et al.*, 2012; Silva-Boghossian *et al.*, 2011; Drescher *et al.*, 2010; Dewhirst *et al.*, 2000; Abusleme *et al.*, 2013; You *et al.*, 2013a	Silva- Boghossian *et al.*, 2011; Drescher *et al.*, 2010; You *et al.*, 2013a
Treponema phylogroup II:OTU 1P26 (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema phylogroup I:OTU 8P68 (1)	You *et al.*, 2013a	You *et al.*, 2013a
Treponema lecithinolyticum HOT 653 (4)	Kumar *et al.*, 2003; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Riep *et al.*, 2009
Treponema maltophilum HOT 664 (2)	Abusleme *et al.*, 2013; Dewhirst *et al.* 2000	
Treponema medium HOT 667(5)	Kumar *et al.*, 2003; Mayanagi *et al.*, 2004; Asai *et al.*, 2012; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
Treponema socranskii HOT 769 [*Treponema socranskii subsp. Buccale/ Treponema socranskii subsp.*](8)	Paster *et al.*, 2001; Dewhirst *et al.* 2000; Kumar*et al.*, 2003; Ledder *et al.*, 2007; Mayanagi *et al.*, 2004; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Hutter *et al.* 2003
Treponemasp. oral taxon 269 HOT 269 [*Treponema*sp. strain 6:H:D15A-4] (1)	Kumar *et al.*, 2005	

Treponema vincentii HOT 029(4)	Willis *et al.*, 1999; Dewhirst *et al.*, 2000; Asai *et al.*, 2012; Griffen *et al.*, 2012	
Treponema sp. oral taxon 230 HOT 230 (1)	Griffen *et al.*, 2012	
Treponema sp. oral taxon 490 HOT 490 [*Treponema E25-8*](1)	Griffen *et al.*, 2012	
Treponema E_D_05_72 (1)	Griffen*et al.*, 2012	
Treponema sp. oral taxon 257 HOT 257 [*Treponema* D36ER-1](2)	Dewhirst *et al.* 2000; Abusleme *et al.*, 2013	
Treponema sp. oral taxon 249 HOT 249 (1)	Abusleme *et al.*, 2013	
Treponema sp. parvum HOT 724 (1)	Abusleme *et al.*, 2013	
Treponema sp. oral taxon 253 HOT 253(1)	Abusleme *et al.*, 2013	
Treponema sp. oral taxon 258 HOT 258 (1)	Abusleme *et al.*, 2013	
Treponema sp.(1)	Abusleme *et al.*, 2013	
Phylum *Synergistetes* (Prev. *Deferribacteres*) (4)	Kumar *et al.*, 2005; Vartoukian *et al.*, 2009; Griffen *et al.*, 2012; You *et al.*, 2013b	
Unclassified class		
Synergistetes Oral Clone A2F_22 ["*Synergistetes*" OTU 4.2 A2F_22-OTU 4.2 FJ490414] (1)	Vartoukian *et al.*, 2009	
Synergistes oral taxon G36 (1)	Griffen *et al.*, 2012	
Fretibacterium sp. oral taxon 359 HOT 359 [*Deferribacteres sp.* oral clone BH007/ *Synergistetes*OTU 7P1] (2)	Kumar *et al.*, 2005; You *et al.*, 2013b	
Fretibacterium sp. oral taxon 360 HOT 360[*Deferribacteres* clone BH017/ Synergistes oral taxon 360/*Synergistetes* OTU 7P22/*Synergistes* [*G-3*] *sp.* OT 360](4)	Kumar *et al.*, 2003; Griffen *et al.*, 2012; Abusleme *et al.*, 2013; You *et al.*, 2013b	
Fretibacterium sp. oral □P□on 361 [*Synergistes [G-3]*□P. OT361](1)	Abusleme *et al.*, 2013	
Fretibacterium sp. oral taxon 362 HOT 362 [*Deferribacteres* clone D084/ *Synergistetes* [*G-3]sp.*OT362/ *Synergistetes* OTU 2P9/	Paster *et al.*, 2001; Kumar *et al.*, 2003; Abusleme *et al.*,	

Synergistetes OTU 6P18] *(*4)	2013; You *et al.*, 2013b	
Fretibacterium fastidiuosum HOT 363 [*Deferribacteres*sp. oral clone W090/*Synergistetes[G-3] sp.* OT 363/ *Synergistetes* OT 4P12] (3)	Kumar *et al.*, 2005; Abusleme *et al.*, 2013; You *et al.*, 2013b	
Fretibacterium sp. oral taxon 453 HOT 453[*Synergistes*OT 453] (1)	Griffen *et al.*, 2012	
Phylum *Tenericutes*		
Mollicutes class		
Mycoplasma genus (1)	Abusleme *et al.*, 2013	
Mycoplasma facium oral taxon 606 HOT 606 (1)	Abusleme *et al.*, 2013	
Phylum *CandidatusSaccharibacteria* (Syn. Candidate division TM7)	Brinig *et al.*, 2003; Ouverney*et al.*, 2003; Liu *et al.*, 2012	
TM7 [G-1] sp.oral taxon 346 HOT 346 [*TM7* 401H12](3)	Teles *et al.*, 2011; Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
TM7 [G-1] sp. oral taxon 349 HOT 349(2)	Griffen *et al.*, 2012; Abusleme *et al.*, 2013	
TM7 [G-5] genus (1)	Abusleme *et al.*, 2013	
TM7 [G-5] sp. oral taxon 356 HOT 356 [TM7clone I025](3)	Kumar *et al.*, 2003; Brinig *et al.*, 2003; Abusleme *et al.*, 2013	
*TM7[G-5]sp. oral taxon 437*HOT 437 (1)	Griffen *et al.*, 2012	
Candidate divisionSulphur River 1 (Candidate division SR1)		
SR1[G-1] sp. oral taxon 345 HOT 345 [OP11 clone X112/Phylotype X112] (2)	Kumar *et al.*, 2003; Li *et al.*, 2006	
Archaea(3)	**Lepp *et al.*, 2004; Li *et al.*, 2009**	**Matarazzo *et al.*, 2011**
Euryarchaeota		
Methanobacteria class(1)	Vianna *et al.*, 2008	
Uncultured *Methanobrevibacter sp.* like SBGA-1 (1)	Lepp *et al.*, 2004	
Methanobrevibacteroralis HOT 815 [Uncultured *Methanobrevibacter* isolatemcrA-II](3)	Li *et al.*, 2009; Bringuier *et al.*, 2013; Vianna *et al.*,	

	2008	
Eukarya		
Fungi(1)	Canabarro *et al.*, 2012	
Basidiomycota		
Rhodotorulasp.(1)	Canabarro *et al.*, 2012	
Ascomycota		
Candida albicans(1)	Canabarro *et al.*, 2012	
Candida parapisilosis (1)	Canabarro *et al.*, 2012	
Candida dubliniensis(1)	Canabarro *et al.*, 2012	
Candida tropicalis(1)	Canabarro *et al.*, 2012	

(): number of studies, **[ ]** other nomenclatures for the species/phylotype used on the different studies

Table S4. Summary of the data of the included studies: known periodontal pathogens

TAXA	Chronic Periodontitis	Generalized Aggressive periodontitis
Bacteria		
Phylum *Bacteroidetes*		
Bacteroidetia class		
Porphyromonas genus (1)	Abusleme *et al.*, 2013	
Porphyromonas gingivalis HOT 619 (9)	Colombo *et al.*, 2002; Kumar*et al.*, 2003; Mayanagi *et al.,* 2004; Abiko *et al.*, 2010; Drescher *et al.*, 2010; Silva-Boghossian *et al.*, 2011, Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Drescher *et al.*, 2010; Silva-Boghossian *et al.*, 2011, Gonçalves *et al.*, 2012
Tannerella genus(2)	Liu *et al.*, 2012; Abusleme *et al.*, 2013	
Tannerella forsythia HOT 613 [*Bacteroidesforsythus*](8)	Colombo *et al.*, 2002; Kumar*et al.*, 2003; Kumar*et al.*, 2005; Abiko*et al.*, 2010; Silva-Boghossian *et al.*, 2011, Griffen *et al.*, 2012; Abusleme *et al.*, 2013	Drescher *et al.*, 2010; Schlafer*et al.*, 2010; Silva- Boghossian *et al.*, 2011,
Prevotella genus(1)	Liu *et al.*, 2012	
Prevotella intermedia HOT 643 (5)	Colombo *et al.*, 2002; Mayanagi *et al.*, 2004; Abiko *et al.*, 2010; Drescher *et al.*, 2010; Griffen *et al.*, 2012	Drescher *et al.*, 2010
Prevotella nigrenscens HOT 693 (1)	Colombo *et al.*, 2002	
Prevotella melaninogenica HOT 469 (1)	Colombo *et al.*, 2002	
Phylum *Firmicutes*		
Clostridia class (1)	Griffen *et al.*, 2013	
Eubacteriumnodatum [11] [G-6] HOT 694 [*Eubacteriumnodatum*] (2)	Booth *et al.*, 2004; Abusleme *et al.*, 2013	
Parvimonas genus(1)	Abusleme *et al.*, 2013	
Parvimonas micra HOT 111 [*Peptostreptococcus micros*](2)	Kumar *et al.*, 2003; Abusleme *et al.*,2013	
Phylum *Fusobacteria*		
Fusobacteriia Class		

Fusobacterium nucleatum subp. Nucleatum HOT 698 [*Fusobacteriumnucleatum*](3)	Schlafer *et al.*, 2010; Drescher *et al.*, 2010	Riep *et al.*, 2009; Schlafer *et al.*, 2010
Phylum*Proteobacteria*		
Gammaproteobacteria Class		
Aggregatibacter actinomycetemcomitans HOT 531 [*Actinobacillus actinomycetemcomitans*] (3)	Colombo *et al.*, 2002; Ledder *et al.* 2007; Silva- Boghossian *et al.*, 2011	Silva- Boghossian *et al.*, 2011
Epsilonbacteria class		
Campylobacter rectus HOT 748 (6)	Macuchand Tanner. 2000; Colombo *et al.*, 2002; Abiko *et al.*, 2010; Griffen *et al.,* 2012; Abusleme *et al.*, 2013	Riep *et al.*, 2009
Campylobacter showae [*Campylobacter X*]HOT 763 (1)	Macuchand Tanner, 2000	
Eikenella corrodens HOT 577 (1)	Kumar *et al.*, 2003	
Phylum*Spirochaetes*	You *et al.*, 2013a	
Spirochaetia class		
Treponema genus(2)	Liu *et al.*, 2012; Abusleme *et al.*, 2013	
Treponema denticola HOT 584 [OTU8P47](10)	Colombo *et al.*, 2002; Kumar*et al.*, 2003; Mayanagi *et al.*, 2004; Asai *et al.*, 2002; Drescher *et al.*, 2010; Silva- Boghossian *et al.*, 2011; Griffen *et al.*, 2012; You *et al.*, 2013a; Abusleme *et al.*, 2013	Drescher *et al.*, 2010; Silva- Boghossian *et al.*, 2011; You *et al.*, 2013a
Treponema socranskii HOT 769 [*Treponema socranskii subsp. Buccale/ Treponema socranskii subsp.*] (5)	Kumar*et al.*, 2003; Mayanagi *et al.*, 2004; Ledder *et al.* 2007; Griffen *et al.*, 2012;Abusleme *et al.*, 2013	

(): number of studies ,**[ ]** other nomenclatures for the species/phylotype used on the different studies

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5- Conclusão

Existe uma evidência moderada sustentando a associação de 17 novas espécies/filotipos do domínio bacteriano, do filo *Candidatus Saccharibacteria* e do domínio *Archaea* com a etiologia das doenças periodontais destrutivas. Estes dados apontam para a importância de espécies não-identificadas anteriormente na etiologia das periodontites e podem orientar futuras investigações sobre o papel real desses possíveis novos patógenos no aparecimento e progressão das doenças periodontais destrutivas.

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Anexos

Anexo A: Formulário de coleta de dados

Formulário de Avaliação

Publicação:

Localização do estudo:
__ Pratica privada ___Hospital /Universidade País_____

I.Tipo de estudo
1. ___Randomizedcontrolledclinicaltrials (RCTs)
2.___ Outros

II. Tipo de participantes
1. Sistemicamente saudáveis ___Sim ____Não
2. Numero de pacientes por grupo _____
3. Amostra total: ____ M___F ___idade/média____/___
4. Fumo:_____
5. Periodontite crônica _____ Periodontite agessiva____ Saudáveis____
6. Critérios utilizados para definir doenças/ saúde_____

III. Diagnóstico microbiológico
1. Método utilizado:_____forma de coleta_____
2. Patogénos avaliados_____
IV. Tipo de intervenção realizada:
V. Resultados microbiológicos:
VI Risco de vieses: (A) Low risk of bias (B) Unclear risk of bias (C) High risk of bias
VII.Fonte de financiamento_____
VIII. Conflito de Interesse _____

Observações:

Anexo B: Outras Publicações

Effect of systemic metronidazole and amoxicillin administered during the active phase of periodontal treatment or after the healing phase: a systematic review

Running title: Moment of antibiotic administration

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Effect of systemic metronidazole and amoxicillin administered during the active phase of periodontal treatment or after the healing phase: a systematic review

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Please use keywords available at http://decs.bvs.br/.:	Periodontal disease, Chronic periodontitis, Aggressive periodontitis, Periodontal therapy, Systemic antibiotics

ABSTRACT

Objective: The aim of this systematic review was to compare the clinical effectiveness of systemic antibiotics administered in the active stage of periodontal treatment or after the healing phase. **Material and Methods:** An electronic search was performed in the EMBASE, MEDLINE and Cochrane Central Register of Controlled Trials CENTRAL databases, in accordance with the PRISMA statement. A manual search of the reference list of selected studies and of Review articles was also performed up to November 2013. Randomized Clinical Trials (RCT) that evaluated the systemic administration of antibiotics as adjuvants to scaling and root planning (SRP), at different phases of periodontal treatment were included. Systematic reviews and studies that evaluated subjects with systemic diseases and those that used sub-antimicrobial doses of antibiotics were excluded. **Results:** The initial search identified 1039 articles, of which seven were selected, and only one met the inclusion criteria. The study showed that subjects taking MTZ+AMX at the initial phase of treatment exhibited significantly greater reduction in pocket depth and gain in clinical attachment level in initially deep sites (PD ≥ 7 mm) than subjects taking antibiotics after healing ($p<0.05$). This comparison was conducted at 2 months post-antibiotic intake at the healing phase. **Conclusion:** To date, only one short-term RCT has directly compared different moments of systemic antibiotics administration, as adjuncts to SRP, in the treatment of periodontitis. Although the results of this study suggested some benefits for antibiotics intake during the active phase of therapy, these findings need to be confirmed by larger placebo-controlled randomized clinical trials with longer follow-up periods.

Key Words: Periodontal disease. Chronic periodontitis. Aggressive periodontitis. Periodontal therapy. Systemic antibiotics.

INTRODUCTION

Periodontal diseases are infectious-inflammatory conditions that may lead to the loss of teeth. Scaling and root planning (SRP) is the most commonly therapy used for the treatment of periodontitis. Although SRP promotes an improvement in the clinical periodontal parameters in the majority of cases, it is frequently insufficient to change the bacterial profile associated with periodontitis to a profile compatible with periodontal health. Therefore, the clinical beneficial results achieved with this procedure in the short-term are frequently not maintained in the long term, particularly in more advanced cases [3,18] or in those associated with risk factors, such as smoking[13]. For this reason, other treatments such as the administration of systemic antibiotics have been proposed in association with SRP, with the goal of potentiating the effects of this therapy.

In spite of the clinical relevance and effective use of systemic antibiotics in the treatment of diverse infectious diseases, clinical studies on the effects of these agents in the treatment of periodontal diseases only began in the 1970s[22]. However, over the course of the last few decades, antibiotics have been widely studied as adjuvants to mechanical periodontal therapy[4,9-10]. The combination of metronidazole (MTZ) and amoxicillin (AMX) has been shown to be especially effective for the treatment of aggressive periodontitis[14-15,20] and chronic periodontitis[2,3,6,19,25]. However, although a notable therapeutic advantage has been shown when MTZ+AMX are used in periodontal therapy, some questions relative to the protocol for the use of these medications remain obscure, such as for example, which would be the best phase of mechanical treatment for the administration of antibiotics?

In clinical practice the decision to use antibiotics associated with SRP treatment is commonly taken after the healing phase of the mechanical treatment, which may vary from 2 to 5 months[7]. However, some biologic concepts suggest that the effects of antibiotics could be potentiated if they were used during the active phase of treatment[4]. Apparently, a rapid and more profound reduction of the subgingival microbial load may lead to a more beneficial recolonization of the recently scaled pockets in the long term. Lighter and sequential

disturbances of the biofilm may not be sufficient to change the highly stable climax community of biofilm[21,23]. Therefore, since there is no consensus in the literature about the ideal time for the administration of these agents, a systematic review of the literature could help with taking the correct clinical decision and could guide future studies about this topic.

MATERIAL AND METHODS

This systematic review was conducted in accordance with the recommendations of PRISMA (*Preferred Reporting Items for Systematic Reviews and Meta-analysis*)[16].

FOCUSED QUESTION

"At which phase of mechanical therapy would systemic antibiotics promote the best clinical results, including reduction in probing depth (PD) and gain in clinical attachment level (CAL): during the active phase of treatment or after the healing phase of mechanical therapy?

Inclusion Criteria:

- Randomized clinical trials (RCTs) with at least two groups: one test (systemic antibiotic used in the active phase of mechanical treatment) and one control (systemic antibiotic used after the healing phase of the mechanical therapy).

- Studies that included individuals with chronic or aggressive periodontitis.

- Studies that evaluated PD and CAL.

Exclusion Criteria

- Studies published in languages other than Portuguese, English and Spanish and Reviews of the Literature.

- Studies that used antibiotics in sub-antimicrobial doses.

Evaluation Variables

Changes in full-mouth mean PD and CAL as well as changes in mean PD and CAL in different categories of pockets.

Search Strategy

The EMBASE, MEDLINE and (*Cochrane Central Register of Controlled Trials*) were searched up to November 2013, by two researchers (ARFS e CG), using the search strategy described in Figure 1. A manual search of the reference list of selected studies and of Review articles about the topic was also performed. Studies were selected by the two researchers in and independent manner, and any disagreement was resolved by a third researcher (MFe).

Data Extraction

Studies that fulfilled the inclusion and exclusion criteria were submitted to data extraction by one of the researchers (ARFS). The following items of information were collected and recorded on a piloted form: 1) place where the research was conducted - country and environment in which the patients were treated (private clinic or hospital/university clinics), 2) randomization method, 3) sample characteristics - sample size, age, gender, local and systemic health conditions, characteristics of the interventions performed, statistical significance, 4) clinical results, 5) conflict of interest, 6) founding.

RESULTS

The flow diagram of this study is represented in Figure 2. In the electronic search 1039 studies were identified. After reading the titles and abstracts, 1032 studies were excluded, and 7 selected. No article was identified in the manual search. After reading the full text of the 7 studies selected 6 were excluded [1-2, 8, 12, 17, 24] because they had not compared the time of administration of MTZ+AMX. Therefore, only 1 study [7] was included in this review. In this study, the authors compared the clinical efficacy of MTZ+AMX administered in the active phase of periodontal treatment, i.e. together with the SRP procedure (Antibiotic initially- Group A n=20) or after the healing phase, i.e. 6 months after the completion of the SRP (Antibiotic after healing – Group B n=21). Clinical parameters were registered at 2 months after the treatment of Group B with antibiotics (i.e. 8 months after treatment of group A/Baseline). The data showed that subjects taking antibiotics at the initial phase of treatment exhibited statistically significant greater reduction in pocket depth and gain in clinical attachment level in initially deep sites (PD ≥ 7 mm) than subjects taking antibiotics at re-assessment (difference between groups of 0.9 mm and 0.7 mm, respectively). In addition, the percentage of sites converting from PD ≥ 5 mm to ≤ 4 mm was 83% in Group A and 67% in Group B (p=0.041) (Table 1)

DISCUSSION

The results of this systematic review demonstrated that up until today, only one study in the literature compared the effects of systemic antibiotics administered in different phases of mechanical therapy. This study, published by Griffiths, et al.[7], suggested there were greater clinical benefits when MTZ+AMX were prescribed during the active phase of periodontal therapy than after the healing phase. Individuals treated with antibiotics together with SRP in the initial phase of treatment, showed greater reduction in PD and greater gain in CAL in deep pockets than individuals treated with antibiotics 6 months after SRP. It is worth pointing out that this study presents some limitations, such as for example, the fact of not having used placebo and having had a short longitudinal evaluation time (the comparison between the two groups was conducted at 2 months post-antibiotic intake at the healing phase). A more longitudinal evaluation would be important to observe whether these results were maintained over the course of time.

Apart from the study included in this review, Kaner et. al.[11] conducted a retrospective evaluation of individuals with generalized aggressive periodontitis treated with SRP+MTZ+AMX administered immediately or 3 months after mechanical treatment, and observed that the time of administration of the antibiotics influenced the clinical response. At 3 months after the second group had taken the antibiotics, the group treated in the active phase of therapy obtained statistically significantly higher reductions in the mean PD (4.09 mm) and relative gain in CAL (2.50 mm) in deep sites (with PD > 6 mm) in comparison with the group that took MTZ+AMX at the time of re-evaluation (2.80 mm and 1.41 mm, respectively)($p<0.05$).

The results of the studies of Griffiths, et al.[7] and Kaner, et al. [11] suggest a benefit for the administration of MTZ+AMX in the active phase of mechanical treatment. These results suggest that the chemical treatment administered together with the mechanical treatment in the initial phase of therapy has a greater potential to change the pathogenic bacterial

community to a community compatible with health. This statement is in agreement with the concept that a rapid and remarkable reduction in the subgingival microbiota would be necessary in order to obtain more beneficial and stable recolonization over time in the recently scaled pockets [21,23]. In addition, a hypothesis related to the high degree of inflammation observed in non treated periodontal patients may also help to explain the greater clinical benefit observed in the individuals who took antibiotics in the initial phase of treatment[5,11]. In the presence of an intense inflammatory process, there are higher levels of gingival fluid; therefore, higher levels of antibiotic are also released into the subgingival environment[5,11]. In addition, inflammation is also associated with great capillary permeability, which may contribute to better absorption of these agents[5,11].

Implications for research and clinical practice

In daily clinical practice, periodontists tend to postpone the administration of antibiotics to the re-assessment phase, rather than as part of the initial treatment. An evidence-based appraisal of current literature presented in this manuscript showed that to date, only one short-term RCT has directly compared different moments of systemic antibiotics administration, as adjuncts to SRP in the treatment of periodontitis. Although the results of this study suggested some benefits for antibiotics intake during the active phase of therapy, these findings need to be confirmed by larger placebo-controlled randomized clinical trials with longer follow-up periods. This information could help clinicians with their decision-making in the daily practice.

ACKNOWLEDGMENTS

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Figure 1. Descriptors, free terms and key words used in the search strategy.

	Terms used	Number of studies
	periodontitis OR periodontal infection OR chronic periodontitis OR aggressive periodontitis OR periodontal disease	79.029
And	“periodontal disease” OR “periodontitis” OR “alveolar bone loss” OR “ora conditions” OR “tooth diseases” OR "anti-bacterial agents" OR "anti-bacterial" OR "anti-bacterial agents" OR "antibiotics" OR "anti-bacteria agents" AND "OR "anti-infective agents" OR"anti-infective" AND "agents" OR "anti-infective agents" OR "antimicrobials" OR "anti-infective agents" AND "OR "systemic AND "anti-infective agents" OR "anti-infective" AND "agents" OR "anti-infective agents" OR "antimicrobials" OR "anti-infective agents" AND "OR "AND systemic AND "anti-infective agents" OR "anti-infective" AND "agents" OR "anti-infective agents" OR "antimicrobial”	1.039

Figure 2. Flow chart of the search strategy.

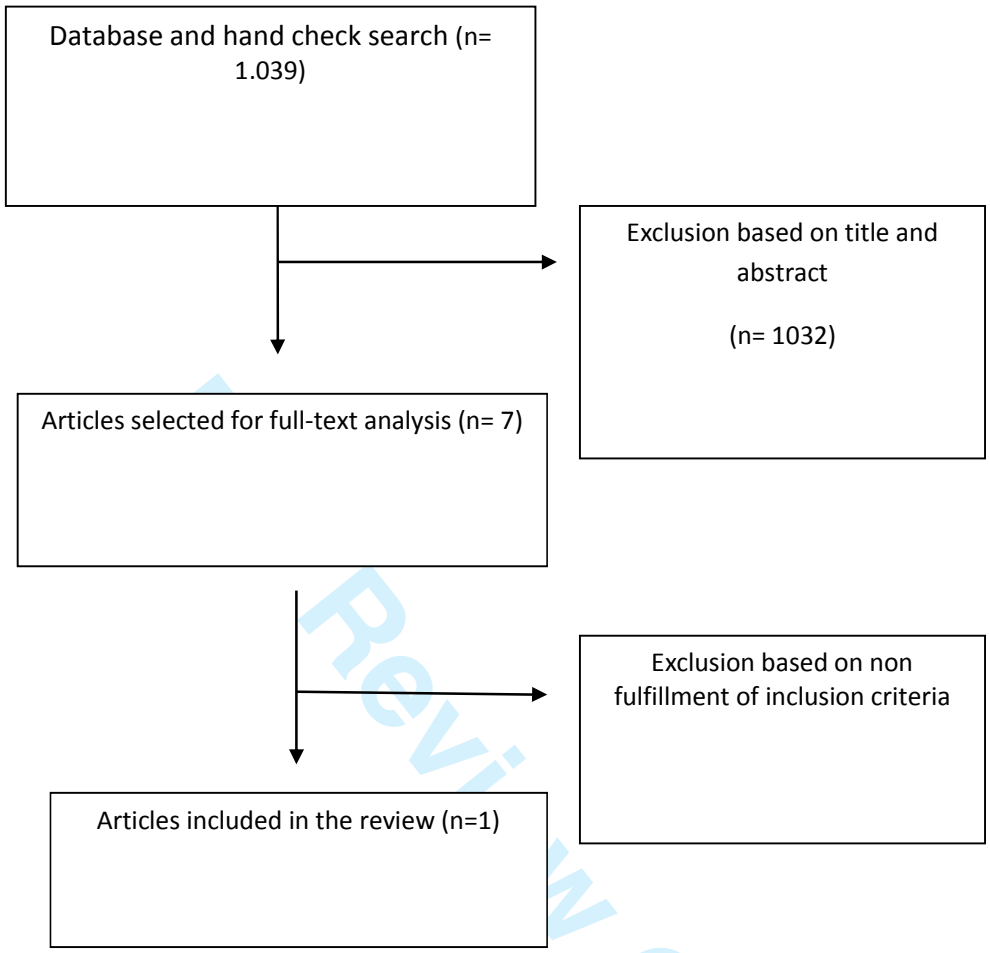

Table 1. Description of the study analyzed.

Study	Study Design	Sample	Intervention	Follow-up	Results	Conclusion
Griffiths et al. 2011	RCT	**Group A** n= 20 5 smokers 16 females GAgP	**Group A** FMRSD + AMX 500mg + MTZ 500mg (TID for 7 days) STARTING RIGHT AFTER THE COMPLETION OF SRP + CHX 0,2% for two weeks	8 months after SRP (2 months post antibiotic intake in Group B)	Group A: showed greater reduction in mean PD and gain in CAL in initially deep sites (with PD ≥ 7 mm) than Group B: difference between groups of 0.9 mm (PD reduction) and 0.7 mm (CAL gain) ($p<0.05$).	Subjects who received MTZ+AMX at the initial therapy showed statistically significant additional benefits compared with those who received the same antibiotic regimen after the healing phase.
		Group B n= 21 4 smokers 12 females GAgP	**Group B** FMRSD + AMX 500mg + MTZ 500mg (TID for 7 days) STARTING 6 MONTHS AFTER THE COMPLETION OF SRP + CHX 0,2% for two weeks		The percentage of sites converting from PD ≥ 5 mm to ≤ 4 mm was 83% in Group A and 67% in Group B ($p=0.041$)	

RCT: randomized clinical trial, GAgP: generalized aggressive periodontitis, FMRSD: full mouth root surface debridement, AMX: amoxicillin, MTZ: metronidazole, SRP: scaling and root planning, PD: probing depth, CAL: clinical attachment level, CHX: chlorhexidine.

Identification of three putative periodontal pathogens in subjects with chronic periodontitis

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Word count: 1828

Number of figures: 3

Number of tables: 1

Short running title: Novel pathogens in periodontitis

A one sentence summary describing the key finding(s) from the study: *F. alocis* and *D. pneumosintes* may be associated with the etiology of ChP,

ABSTRACT

Background: The aim of this study was to evaluate the association of *Porphyromonas endodontalis, Filifactor alocis* and *Dialister pneumosintes* with periodontitis, by comparing their levels and prevalence between subjects with chronic periodontitis (ChP) and periodontal health (PH). **Methods**: Thirty subjects with ChP and 10 with PH entered the study. Nine biofilm samples were collected per subject and analyzed by checkerboard DNA-DNA hybridization for the presence and levels of *F. alocis D. pneumosintes* and *P. endodontalis*. **Results**: The three species evaluated were found in a statistically significantly higher percentage of sites and at higher levels in the group with ChP than in that with PH ($p<0.05$, Mann-Whitney test). These differences were also observed when the samples from shallow (PD ≤4 mm) or deep pockets (PD ≥5 mm) of subjects with ChP were compared with the samples from subjects with PH ($p<0.05$, Mann-Whitney test). In addition, the prevalence and levels of *D. pneumosintes*, and especially of *F. alocis*, were very low in healthy subjects. The mean levels of *F. alocis D. pneumosintes* and *P. endodontalis* in the PH group were $0.01x10^5$, $0.12x10^5$ and $1.89x10^5$, respectively; and in subjects with ChP were $1.77x10^5$, $7.61x10^5$ and $4.01x10^5$, respectively. **Conclusion**: *F. alocis* and *D. pneumosintes* may be associated with the etiology of ChP, and their role in the onset and progression of this infection should be further investigated. The role of *P. endodontalis* was less evident, since this species was also found in relatively high levels and prevalence in PH.

Key words: maximum of 6 with Mesh documentation *(Ju, por favor acrescente essas palavras de acordo com as que já acrescentamos lá no site)*

Introduction

The oral cavity naturally hosts several microorganisms that together constitute the oral microbiome. Most of these microorganisms are compatible with the oral health status, while some of them are considered pathogens and might trigger infectious processes, such as periodontitis. The knowledge about the periodontal pathogens associated with the onset and progression of periodontal diseases has been largely concentrated on the microorganisms that comprise the "subgingival microbial complexes" previously described by Socransky et al.[1,2] However, results from studies that have used molecular diagnostic approaches to study the composition of oral microbiota have advocated the existence of other candidate periodontal pathogens[3]. *Porphyromonas endodontalis, Filifactor alocis* and *Dialister pneumosintes* are among the newly identified candidate periodontal pathogens. Some recent investigations have suggested that these species are present in higher prevalence and levels in patients with periodontitis than in periodontally health subjects[4-9]. However, the number of studies assessing these species and the number of plaque samples per subjects evaluated by these studies is still low. Therefore, further studies determining the actual role of these suspected novel pathogens in the etiology of periodontitis would be of importance to the field of periodontology. This knowledge might help to establish more effective preventive and treatment strategies for these infections.

Therefore, the aim of this study was to evaluate the association of three putative periodontal pathogens, *P. endodontalis, F. alocis* and *D. pneumosintes* with

periodontitis, by comparing their levels and prevalence between subjects with chronic periodontitis (ChP) and periodontal health (PH).

Material and Methods

Study population

Ten periodontally healthy subjects and 30 subjects with ChP were selected from the population referred to the Periodontal Clinic of Guarulhos University (Guarulhos, SP, Brazil) for treatment. Detailed medical and dental records were obtained, and one trained and calibrated examiner performed a full-mouth periodontal examination. Subjects who fulfilled the inclusion criteria were invited to participate in the study. All eligible subjects were informed of the nature, potential risks and benefits of their participation in the study and signed a term of informed consent. The Guarulhos University's Ethics Committee in Clinical Research approved the study protocol.

Inclusion and exclusion criteria

Inclusion criteria for periodontally healthy subjects were: > 30 years, ≥ 24 teeth, no sites with probing depth (PD) and/or clinical attachment level (CAL) ≥ 3 mm and fewer than 20% of sites with gingival bleeding and/or bleeding on probing (BOP). Inclusion criteria for subjects with periodontitis were: > 30 years, ≥ 20 teeth, ≥ 8 sites in different teeth with PD ≥5 mm, CAL ≥3 mm and BOP.

Exclusion criteria were pregnancy, lactation, current smoking, antimicrobial therapies during the previous 6 months, medical conditions requiring

prophylactic antibiotic coverage, continuous use of mouthrinses containing antimicrobials in the preceding 3 months, systemic conditions that could affect the progression of periodontitis and long-term administration of anti-inflammatory and immunosuppressive medications.

Clinical evaluation

Clinical monitoring was performed by one examiner who carried out all clinical measurements in a given subject. Visible plaque (0/1), gingival bleeding (0/1), BOP (0/1), suppuration (0/1), PD and CAL were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth, excluding third molars. PD and CAL measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

Microbiological evaluation

Sample collection

After having recorded the clinical parameters, supragingival plaque was removed and then nine individual subgingival plaque samples were collected per subject using sterile 11/12 Mini-Gracey curettes as follows: Periodontally healthy subjects: samples were collected from the mesial/buccal aspect of each tooth in two randomly chosen contralateral quadrants. Subjects with ChP:

samples were collected from three sites in each of the following PD categories: shallow (PD≤3 mm), moderate (PD 4-6 mm) and deep (PD≥7 mm).

The samples were placed in separate plastic tubes containing 0.15 ml of TE (10mM Tris- HCl, 1mM EDTA, pH 7.6). One hundred microliters of 0.5M NaOH were immediately added to each tube and the samples were stored at -80 °C.

Checkerboard DNA-DNA hybridization

The samples were evaluated by checkerboard DNA–DNA hybridization [10,11] at the Guarulhos University Laboratory of Microbiology. The samples were boiled for 10 min and neutralized using 0.8 ml of 5M ammonium acetate. The DNA released was then placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated on a 15/15 cm positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) and fixed to the membrane by baking it at 120°C for 20min. The membrane was then placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° C to the lanes of the device. Digoxigenin-labelled whole genomic DNA probes for *P. endodontalis*, *F. alocis and D. pneumosintes* were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using the antibody to digoxigenin conjugated with alkaline phosphatase, and chemiluminescence detection. The last two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. Signals were evaluated by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane. The sensitivity of this assay was adjusted to allow detection of each DNA probe.

This procedure was carried out in order to provide the same sensitivity of detection for each of the species.

Statistical analysis

Each individual clinical parameter as well as mean counts ($x10^5$) of each bacterial species evaluated and the percentage of sites colonized by these species were computed per subject and then across subjects in both groups. The significance of differences between the two groups for age, clinical and microbiological parameters was sought by Mann–Whitney test. Chi-square test was used to compare the differences in the frequency of gender. Levels of the three bacterial species were averaged separately within two PD categories (PD ≥ 5 mm and PD≤ 4 mm) per subject, and then across subjects with ChP. The significance of differences between these two categories was sought by Wilcoxon Test. The level of significance was set at 5%.

Results

Demographic and clinical characteristics of the subjects with chronic periodontitis and periodontal health are presented in Table 1. As expected, statistically significant differences were observed between the two groups for all clinical parameters evaluated ($p<0.05$). Mean age and percentage of females did not differ between groups ($p>0.05$).

Figures 1 and 2 present the mean percentage of sites colonized by *F. alocis*, *D. pneumosintes* and *P. endodontalis* and the mean counts of these species, respectively, in the two clinical groups. All three species were present in

statistically significantly higher percentage of sites and mean counts in subjects with ChP than in those with PH ($p<0.05$). The mean levels of *F. alocis*, *D. pneumosintes* and *P. endodontalis* in the healthy subjects were 0.01 x 10^5, 0.12 x 10^5 and 1.89 x 10^5, respectively; and 1.77 x 10^5, 7.61 x 10^5 and 4.01 x 10^5, respectively, in periodontitis subjects. The mean counts ($X10^5$) of the three microbial species in subjects with PH and in sites with PD ≥ 5 mm and ≤ 4 mm in subjects with ChP are presented in Figure 3. The three bacterial species were present in statistically significantly higher mean levels in shallow and deep sites of periodontitis subjects in comparison with the levels observed in healthy subjects ($p<0.05$).

Discussion

The three putative novel periodontal pathogens evaluated in this study were found in statistically significantly higher percentage of sites and in higher levels in subjects with ChP than in periodontally healthy individuals. These data are in agreement with previous investigations that also showed an association of *P. endodontalis* [4,8,9], *F. alocis* [4,6,7,8,9] and *D. pneumosintes* [5,6] with periodontal diseases. In addition, a recent Systematic Review, which evaluated the weight of evidence for the existence of novel periodontal pathogens suggested that 32 newly identified taxa might be associated with the etiology of periodontitis. The authors proposed that there is “moderate evidence” in the literature to support the role of *P. endodontalis* and *F. alocis* as periodontal pathogens, and “some evidence” for *D. pneumosintes*[3]. Although the three bacterial species evaluated in this study were elevated in subjects with ChP in comparison with

periodontally healthy individuals (Figures 1 and 2), their levels and prevalence in healthy subjects also provided essential information as regards their possible role in the disease etiopathogenesis. *F. alocis* was present in only 8% of the sites of healthy individuals and at insignificant levels. An average of 1000 cells of this species were detected in the 10 periodontally healthy subjects evaluated. *D. pneumosintes* was also at very low levels in healthy individuals, but was detected in 42% of the sites evaluated. *P. endodontalis* have been found in almost 50% of the sites of healthy subjects and at relatively high levels. These data might indicate that *P. endodontalis* is an opportunistic bacterial species or an accessory pathogen that may not trigger the disease process, but whose levels may increase when the inflammation process begins, thus contributing to the disease process[12]. *F. alocis* appears to retain the characteristics of a keystone pathogen, microorganisms that have the potential to initiate periodontal destruction by causing a dysbiosis in the subgingival ecosystem[13]. In addition to being in very low prevalence and levels in healthy subjects, Aruni et al.[14,15] showed that *F. alocis* has some unique virulence factors that favor its persistence in inflammatory environments and may mediate *P. gingivalis* proliferation, suggesting that this specie can play a pivotal role in microbial community dynamics.

A strength of this study was the number of subgingival plaque samples individually analyzed for the levels and prevalence of the three bacterial species. To date, all the association studies that have investigated *F. alocis*, *D. pneumosintes* or *P. endodontalis* have evaluated from 30 to 160 plaque samples, [4-9] while 360 plaque samples were individually analyzed in the present investigation. In addition, most of these studies have only determined the

prevalence or abundance (proportion) of these microbial species in the subgingival plaque. Indeed, it has been recognized that the evaluation of a large number of plaque samples and the quantification of the microorganisms are critical requisites when trying to establish an association between certain microorganism and the onset and progression of periodontitis[16].

It is important to mention that association studies only provide the initial information necessary to suggest a possible link between a microorganism and an infection. Further evaluations are necessary to confirm this type of association, such as clinical (i.e. risk assessment and interventional studies), and host-response studies, and investigations into the mechanisms of pathogenicity of the suspected pathogens.

In conclusion, the results of the present investigation suggested that *F. alocis* and *D. pneumosintes* have a high potential to be periodontal pathogens. The role of *P. endodontalis* was less evident, since this species was found in relatively high levels and prevalence in periodontally healthy subjects. These data might guide future studies on the actual role of these three bacterial species in the etiology of periodontitis and help to establish more effective treatments for these infections.

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FIGURE LEGENDS

Figure 1. Mean percentage of sites colonized by the three bacterial species evaluated. The significance of differences between groups was assessed using the Mann Whitney test; **p<0.01 and ***p<0.001.

Figure 2. Mean counts ($X10^5$) of the three bacterial species evaluated. The significance of differences between groups was assessed using the Mann Whitney test; **p<0.01 and ***p<0.001.

Figure 3. Mean counts ($X10^5$) of the three bacterial species evaluated in Health and in sites with probing depth ≥ 5 mm and ≤ 4 mm in subjects with periodontitis. The significance of differences between the healthy group and each of the PD category subgroup was assessed using the Mann Whitney test; *p<0.05, **p<0.01 and ***p<0.001.

Table 1 – Clinical parameters and demographic characteristics of the two groups

	Health	**Periodontitis**	
	(n=10)	(n=30)	Mann-Whitney
	Mean ± SD	Mean ± SD	(p)
Number of females	9	22	0.73889
Age	38.3 ± 6.8	42.2 ± 6.4	0.26000
PD	1.8 ± 0.2	3.7 ± 0.7	0.00000
CAL	0.9 ± 0.2	4.5 ± 1.3	0.00000
% sites with:			
Plaque	25.5 ± 10.1	80.0 ± 15.6	0.00000
Gingival bleeding	2.0 ± 0.8	27.7 ± 15.3	0.00000
Bleeding on probing	3.1 ± 1.2	78.9 ± 14.1	0.00000
Suppuration	0.0 ± 0.0	1.1 ± 0.8	0.00001

PD, probing depth; CAL, clinical attachment level; SD, standard deviation

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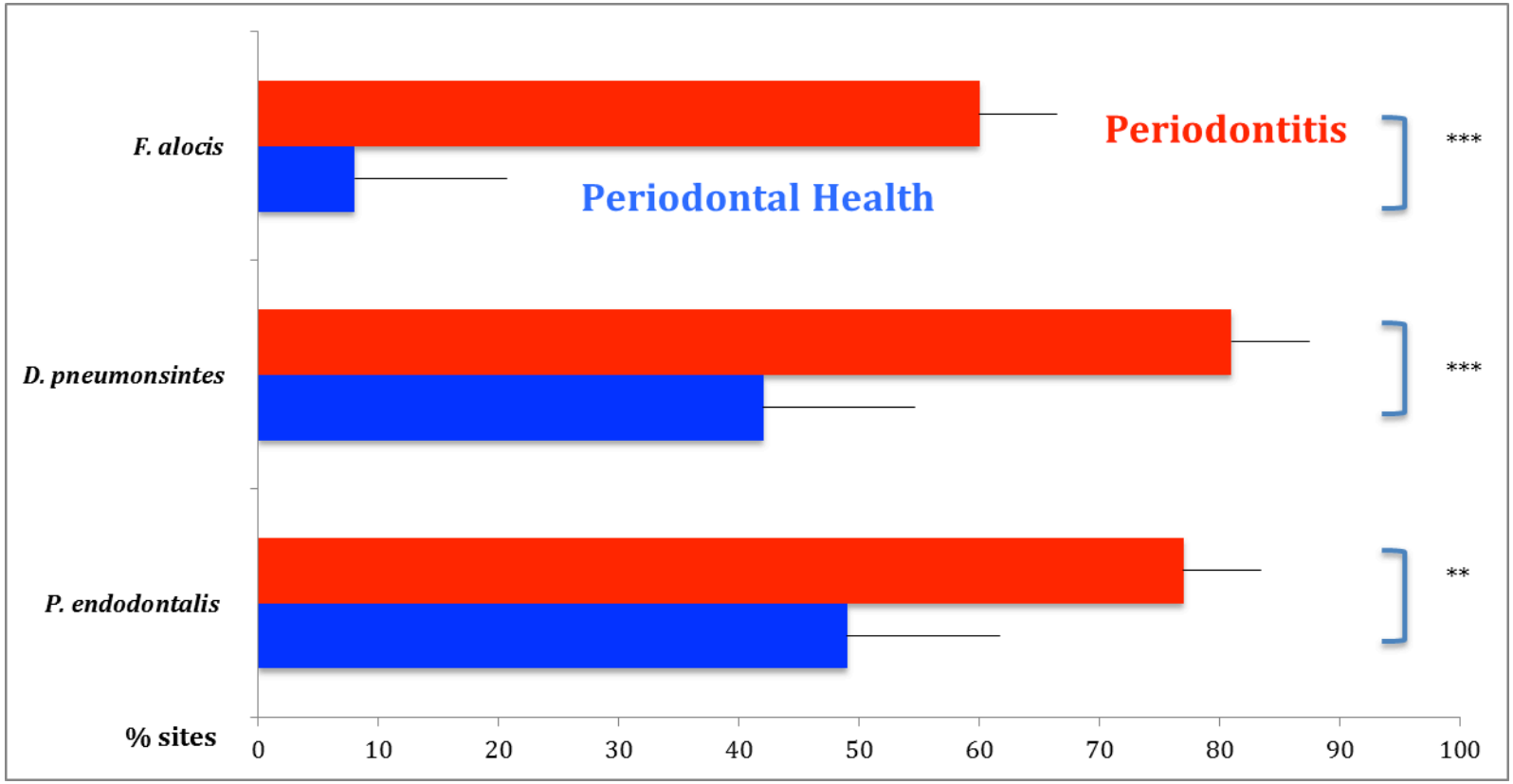

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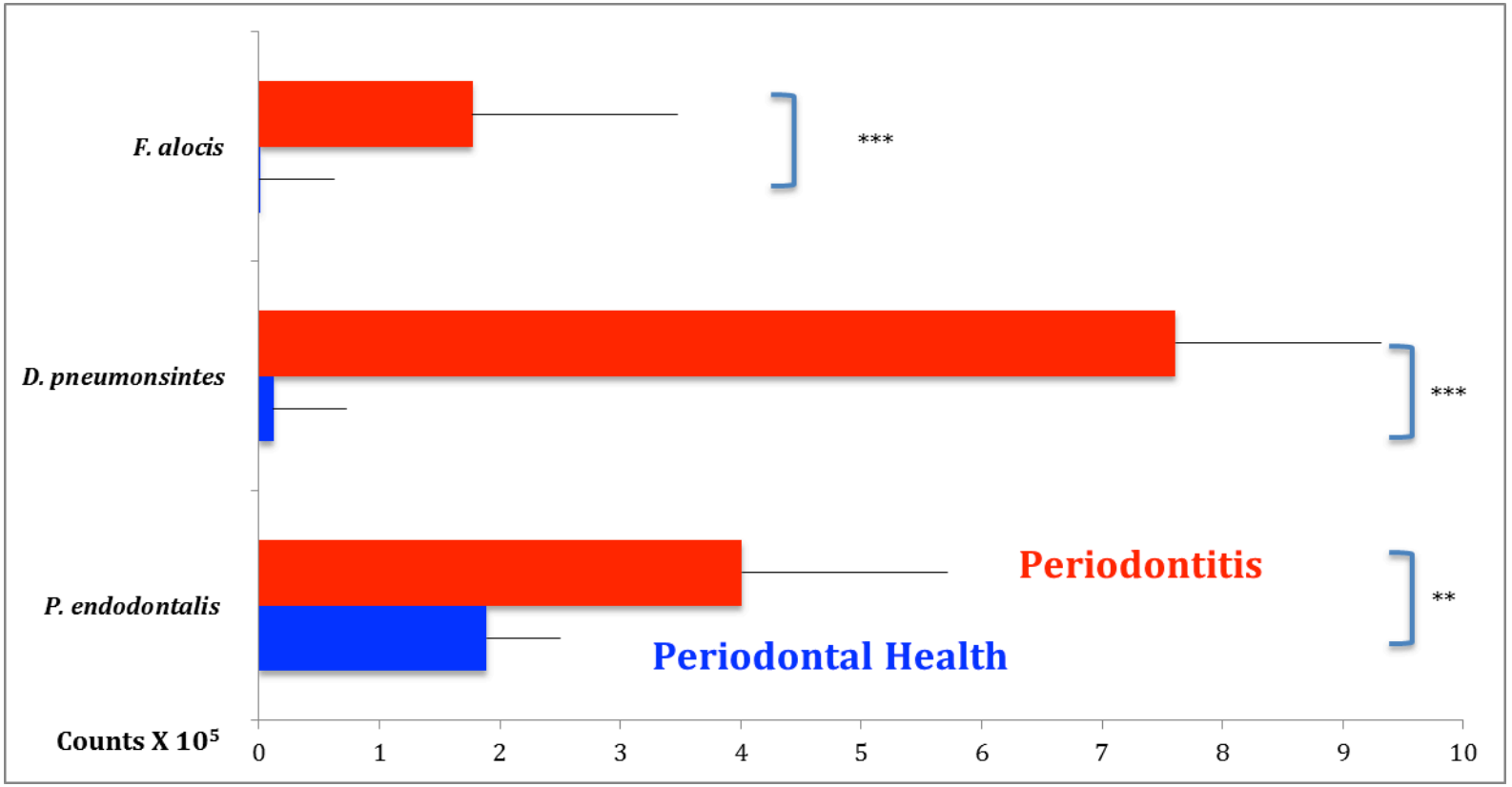

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