UNIVERSIDADE DE BRASÍLIA
Faculdade de Ciências de Saúde
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Biogeografia do Arqueoma Oral

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília, como requisito parcial à obtenção de título de Doutor em Odontologia.

Orientadora: Prof. Dra. Nailê Damé-Teixeira

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Tese aprovada, como requisito parcial para obtenção do grau de Doutor(a) em Odontologia, Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília.

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Maria e Raimundo Cena, por me oferecerem Amor e apoio incondicional.
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“O coração do que tem discernimento adquire o conhecimento, e o ouvido dos sábios busca o conhecimento.”

Provérbios 18:15

RESUMO

Evidências crescentes indicam que as arqueias são parte da microbiota residente em vários sítios orais e desempenham um papel ainda pouco compreendido. Estudos sugerem que a diversidade dessas arqueias na cavidade oral humana pode estar subestimada e não limitada apenas a organismos metanogênicos, como se acreditava anteriormente. Na intenção de colaborar com a ampliação do conhecimento do arqueoma no contexto do microbioma oral e suas possíveis funções, o projeto "Biogeografia do arqueoma oral" foi desenvolvido nesta tese de doutorado. Seu objetivo geral foi mapear a ocorrência de membros do domínio *Archaea* em diferentes sítios orais. Identificamos que os desafios metodológicos relacionados à detecção e isolamento, incluindo dificuldades na cultura e no desenho de iniciadores específicos, contribuem para essa lacuna no conhecimento. Há necessidade de iniciadores para detectar arqueias além das metanogênicas, como os grupos *Nanoarchaeota* e *Thaumarchaeota*, o que poderia ampliar a compreensão da diversidade das arqueias na biogeografia oral. Nossa revisão sistemática com meta-análise mostrou que indivíduos com periodontite têm maior probabilidade de apresentar biofilmes subgengivais positivos para arqueias em comparação com indivíduos periodontalmente saudáveis (OR 6,68, IC 95% 4,74-9,41 para análise do gene 16S rRNA e OR 9,42, IC 95% 2,54-34,91 para análise do gene *mcrA*), sugerindo que as arqueias podem atuar como colonizadoras secundárias em processos inflamatórios periodontais. Além disso, a prevalência de arqueias em canais radiculares foi estimada em cerca de 20% (IC 95% 8-32), com a predominância de metanogênicas, mas também com a detecção de *Thaumarchaeota* e *Crenarchaeota*. Demonstramos também que, embora de baixa abundância, arqueias estão presentes tanto em amostras de biofilme e saliva associados a cárie em análise *in silico*. Genes relacionados à metanogênese estão superexpressos em amostras de pessoas livres de cárie, o que sugere um possível papel na manutenção da homeostase do microbioma oral. Por fim, também observamos a biogeografia de arqueias metanogênicas na cavidade oral, analisando diferentes tipos de amostras como saliva, biofilmes supragengivais e subgengivais, dentina cariada e biofilme lingual. Um total de 142 amostras foram analisadas e a amplificação do gene *mcrA* foi realizada por PCR e qPCR. Os resultados indicaram a presença de arqueias metanogênicas em múltiplos tipos de amostras. Conclui-se que, apesar da relevância das arqueias no

microbioma oral ainda requerer estudo, elas surgem como componentes importantes de baixa abundância, da microbiota bucal associada a doença no biofilme subgengival e endodônticos, mas também em saúde em biofilme supragengival e saliva.

PALAVRAS-CHAVE: Microbiota bucal; Domínio *Archaea*, Arqueoma Oral; biofilmes, biogeografia, cárie dentária, periodontite, infecções endodônticas, saliva, in silico, Revisão, Bioinformática

ABSTRACT

Growing evidence indicates that archaea are part of the resident microbiota in various oral sites and play a still poorly understood role. Recent studies suggest that the diversity of these archaea in the human oral cavity may be underestimated and not limited to methanogenic organisms, as previously believed. With the aim of contributing to the expansion of knowledge about the archaeome within the context of the oral microbiome and its potential functions, the project titled *"Biogeography of the Oral Archaeome"* was developed as part of this PhD thesis. Its overarching objective was to map the occurrence of members of the domain *Archaea* in different oral sites. We identified that methodological challenges related to detection and isolation, including difficulties in culturing and designing specific primers, contribute to this gap in knowledge. There is a need for designing primers to detect archaea beyond methanogens, such as to detect *Nanoarchaeota* and *Thaumarchaeota* groups, which could broaden the understanding of archaea diversity in oral biogeography. Our systematic review with meta-analysis showed that individuals with periodontitis are more likely to have subgingival biofilms positive for archaea compared to periodontally healthy individuals (OR 6.68, 95% CI 4.74-9.41 for 16S rRNA gene analysis and OR 9.42, 95% CI 2.54-34.91 for *mcrA* gene analysis), suggesting that archaea may act as secondary colonizers in periodontal inflammatory processes. Additionally, the prevalence of archaea in root canals was estimated at about 20% (95% CI 8-32), predominantly methanogenic but also detecting *Thaumarchaeota* and *Crenarchaeota*. We also demonstrated that archaea are present in biofilm and saliva samples associated with caries, although in low abundance. Genes related to methanogenesis were overexpressed in samples from caries-free individuals, suggesting a potential role in maintaining oral microbiome homeostasis. Finally, we also observed the biogeography of methanogenic archaea in the oral cavity, analyzing different types of samples such as saliva, supragingival and subgingival biofilms, carious dentin, and lingual biofilm. A total of 142 samples were analyzed, and *mcrA* gene amplification was performed by PCR and qPCR. The results indicated the presence of methanogenic archaea in multiple types of samples. We conclude that the relevance of archaea in the oral microbiome still requires further exploration, but they emerge as important, low-abundance components of the oral microbiota associated with disease in

subgingival and endodontic biofilm, but also in health in supragingival biofilm and saliva.

Key words: Oral microbiota; *Archaea* domain; Oral Archaeome; biofilms, biogeography, dental caries, periodontitis, endodontic infections, saliva, in silico, Review, Bioinformatics

APRESENTAÇÃO

A cavidade oral humana abriga a segunda comunidade microbiana mais abundante e diversa do corpo humano (1). Diversos estudos buscam entender sua real composição e funções na saúde e em doenças orais ou sistêmicas (8). Como nos demais sítios do corpo, a presença e funções de organismos pertencentes ao domínio *Archaea* no microbioma oral ainda estão em fase inicial de compreensão, principalmente devido à detecção de arqueias ser fortemente sensível a questões metodológicas (2-4).

Na intenção de colaborar com a ampliação do conhecimento do arqueoma no contexto do microbioma oral e suas possíveis funções, o projeto "Biogeografia do arqueoma oral" foi desenvolvido nesta tese de doutorado. Seu objetivo geral foi mapear a ocorrência de membros do domínio *Archaea* em diferentes sítios orais e assim traçar seu perfil biogeográfico nos diferentes sítios da cavidade oral. Considerou-se como hipóteses bases as premissas que (I) microrganismos membros do domínio *Archaea* fazem parte da microbiota oral residente; (II) a diversidade de arqueias na cavidade oral vai além das espécies metanogênicas e; (III) as arqueias podem atuar como patobiontes secundários em áreas de disbiose, sendo favorecidas no ambiente inflamofílico, pelo menos no caso das metanogênicas em sítios periodontais e endodônticos (IV) arqueias metenogênicas podem ter uma ação protetiva no processo de desenvolvimento da cárie.

Os objetivos específicos dessa tese são:

1. Descrever a presença do domínio *Archaea* em amostras de diferentes sítios bucais, incluindo biofilme supragengival, biofilme subgengival, biofilme lingual e saliva;
2. Revisar os primers utilizados para identificação de arqueias e apresentar suas limitações
3. Estudar a prevalência geral do arqueoma periodontal, avaliando diferenças entre saúde e doença;
4. Estudar a prevalência geral do arqueoma endodôntico;
5. Estudar o arqueoma relacionado à cárie;
6. Detectar a presença do gene *mcrA* de arqueias em amostras de diferentes sítios bucais.

Esta tese será apresentada em forma de capítulos, onde os capítulos já publicados serão apresentados no formato final em língua inglesa.

O **capítulo 1** apresenta uma revisão de literatura a fim de resumir o conhecimento atual sobre o arqueoma humano.

Já o **capítulo 2** descreve uma análise *in silico* dos iniciadores usados na amplificação de arqueias por qPCR, publicado no periódico *Archives of Oral Biology.*

O **capítulo 3** consiste em uma revisão sistemática que investiga o "arqueoma periodontal" e a prevalência de arqueias nesse sitio e foi publicada em formato de capítulo no livro "Periodontitis: Advances in Experimental Research", da editora Springer.

O **capítulo 4** é outra revisão sistemática publicada no periódico *Journal of Endodontics*, que tinha como objetivo analisar a prevalência de arqueias em canais endodônticos.

Por último, o **capítulo 5** apresenta um estudo transversal com análise em *wet lab,* que teve como objetivo mapear a presença de arqueias metanogênicas em amostras orais, utilizando PCR e qPCR com iniciadores diretos para o gene *mcrA*. Os participantes do estudo, doadores de amostras, foram recrutados em atendimentos realizados na Unidade de Saúde Bucal do Hospital Universitário de Brasília (USBUC/HUB/Ebserh).

Ao final deste documento, será feita uma discussão geral, demonstrando as dificuldades da realização dessa pesquisa.

Foi acrescentado como anexo**,** um estudo realizado um estudo *in sílico* de dados disponíveis em bases de dados através de uma análise personalizada de bioinformática com foco em arqueias. Esse trabalho foi desenvolvido em colaboração com a Universidade de Leeds, para avaliar presença e funções de arqueias em bancos de dados disponíveis publicamente, compreendendo biofilmes e saliva de indivíduos livre de cárie e cárie-ativos.

SUMÁRIO

1 CAPÍTULO I: Revisão de Literatura

1.1 INTRODUÇÃO

Diversos processos fisiológicos no corpo humano se dão em decorrência das interações entre suas células e as células de seus microrganismos residentes. Esse fato torna o ser humano um holobionte, ou seja, somos seres eucariotos multicelulares, formado em associação com suas comunidades microbianas (1). As interações que acontecem dentro dos mais diversos microambientes que podem ser encontrados ao longo do corpo ainda continuam envoltas em mistérios. Contudo, a inovação das ferramentas moleculares, sobretudo de sequenciamento genético, possibilitou iniciar o entendimento das interações do microbioma humano, incluindo espécies que estão presentes em baixa abundância, e/ou não são comumente cultiváveis (2, 3).

As arqueias são um exemplo de espécies de baixa abundância presente em nosso organismo. O arqueoma, comunidades de arqueias associadas ao corpo humano, vem se mostrando cada vez mais como parte relevante do microbioma humano. As principais relações humano-arqueias atualmente conhecidas são associadas a arqueias metanogênicas presentes no trato gastrointestinal em processos relevantes para saúde e doença (4). Todavia, além do intestino, membros do domínio *Archaea* já foram identificados em amostras vaginais, de pulmão, nariz, pele e boca (5-7).

Ainda é incerto se existe uma interação parasita-hospedeiro entre arqueias e humanos ou se arqueias possuem seus próprios fatores de virulência, uma vez que estudos associando membros deste domínio da vida a patologias específicas são escassos, sendo os casos mais bem explorados ligados ao intestino ou a doenças periodontais (8). Todavia, a falta de espécies de arqueias estabelecidas como patógeno humano, tem sido usado como justificativa para o moroso avanço em seu estudo (9).

Atualmente está começando a ser traçado um padrão de espécies de arqueias de acordo com a sua biogeografia (7, 10). Esses estudos têm mostrado que a

ocorrência e diversidade de arqueias são maiores em dois locais do corpo, o intestino e a cavidade oral (10). Apesar de termos disponíveis na literatura revisões que abordaram a abundância geral de arqueias em humanos (11-15), esta revisão visa atualizar o conhecimento atual sobre a biogeografia do arqueoma humano, com ênfase no arqueoma oral.

1.2 BIOGEOGRAFIA

1.2.1 Definindo Biogeografia

As comunidades microbianas associadas ao homem diferem em composição, tanto entre os locais do corpo como entre os sítios dentro de um mesmo local. Os padrões de variação na distribuição dessas comunidades ao longo do tempo e espaço são chamados de 'biogeografia' (16). A biogeografia é relevante para compreender a ecologia das comunidades microbianas em situação de simbiose, bem como seus efeitos em doenças humanas. Afinal, a cooperação mútua de espécies um determinado sítio pode alterar suas caraterísticas fisiológicas, bem como causar “distúrbios ecológicos” no microambiente. (17).

A importância do conceito de biogeografia para o estudo do microbioma humano foi demonstrada no estudo de Costello e colaboradores (18) que, ao analisar espécimes de 27 locais de nove adultos saudáveis, demonstrou que a composição da comunidade foi determinada principalmente pelo habitat do corpo e que, dentro dos habitats, a variabilidade interpessoal foi alta, enquanto os indivíduos exibiram variabilidade temporal mínima.

Ressalta-se que a cavidade oral difere de todos os outros habitats microbianos humanos pela presença simultânea de distintas superfícies e micronichos para colonização microbiana (19). Aas e colaboradores (20) investigaram a colonização bacteriana em nove sítios da cavidade oral de cinco indivíduos saudáveis, utilizando sequenciamento Sanger. O estudo revelou que diferentes locais apresentam comunidades bacterianas específicas, influenciadas por características anatômicas, e destacou a importância desses perfis na definição da saúde oral. No epitélio bucal, predominam espécies do filo *Firmicutes*, como *Streptococcus mitis* e *Gemella hemolysans*. No vestíbulo anterior maxilar, observa-se a presença marcante de *Firmicutes* (*Streptococcus mitis*) e *Actinobacteria* (*Granulicatella* spp.). No dorso da

língua, há diversidade de *Firmicutes* (*S. mitis, Streptococcus salivarius*) e *Proteobacteria* (*Veillonella* spp.). A superfície lateral da língua apresenta perfil semelhante ao dorso, com predominância de *Firmicutes* (*S. mitis* biovar 2) e *Proteobacteria.* No palato duro, predominam *Firmicutes* (*S. mitis*, *Granulicatella elegans*) e *Proteobacteria* (N*eisseria subflava*). Já no palato mole, as comunidades são compostas principalmente por *Firmicutes* (*Streptococcus* spp., *G. hemolysans*). Nas superfícies dentárias, observa-se abundância de *Firmicutes* (*S. sanguinis, Streptococcus gordonii)* e *Actinobacteria* (*Rothia dentocariosa*). Por fim, na placa subgengival, diversas espécies de *Firmicutes* (*Streptococcus e Gemella*) são frequentemente encontradas, reforçando a especificidade dos perfis bacterianos na cavidade oral e sua relação com diferentes condições anatômicas e funcionais (20).

Segata e cols. (21) descreveram a composição da comunidade bacteriana de mais de 200 adultos saudáveis inscritos no Projeto Microbioma Humano, através de sequenciamento de alto rendimento. Os dados mostraram que os sítios bucais podem ser agrupados de acordo com a composição de sua microbiota: grupo 1 (mucosa bucal, gengiva queratinizada e palato duro) composto principalmente de *Firmicutes,* seguido em ordem decrescente de abundância relativa por *Proteobacteria, Bacteroidetes* e *Actinobacteria* ou *Fusobacteria*; grupo 2 (saliva, língua, amígdalas e garganta) teve uma abundância relativa diminuída de *Firmicutes* e níveis aumentados de quatro filos: *Bacteroidetes, Fusobacteria, Actinobacteria e TM7* (filo atualmente conhecido como *Saccharibacteria (22))*; e grupo 3 (placa subgengival e supragengival) teve uma diminuição de *Firmicutes* em comparação com os outros grupos e um aumento acentuado na abundância relativa de *Actinobacteria*. Além disso, os autores usando o sistema LEfSe (*LDA Effect Size*) foram capazes de identificar biomarcadores, ou seja, gêneros cuja alta ou baixa abundância era característica de um determinado grupo de sítios (21). Por exemplo, o filo *Actinobacteria* e táxons dentro da ordem *Actinomycetales* foram consistentemente mais abundantes nas superfícies dentárias do Grupo 3, no Grupo 1 *Streptococcus* (*Streptococcaceae*), com uma média de 18% de abundância, e *Gemella* (Staphylococcaceae), 5,1% de abundância se destacaram.

Os estudos acima relatados são exemplos de muitas pesquisas que revelaram padrões biogeográficos no microbioma oral. Contudo, para a análise real desses

padrões é preciso ir além das espécies já cultivadas e os sequenciamentos metagenômicos têm auxiliado nesse processo (23). Por exemplo, Zhou e cols. (24), ao analisarem padrões de distribuição bacteriana em 22 habitats humanos, destacam que todo sítio terá pelo menos um gênero dominante. Porém, um olhar atento às análises dos dados resultantes dos sequenciamentos de amostras é necessário, uma vez que esses gêneros de maior abundância são acompanhados, estatisticamente, por uma longa cauda de organismos menos abundantes e são essas assinaturas de baixa abundância que podem gerar importantes marcadores para compreensão da comunidade microbiana.

Esses organismos, em menores quantidades, geralmente são detectados após filtragem de qualidade e remoção de quimeras (24). O problema é que a filtragem é realizada de modo arbitrário e os estudos tendem a estabelecer o corte em <1% para um organismo ser considerado pouco abundante (3). Essa prática pode comprometer a identificação da verdadeira diversidade da microbiota humana. Domínios que não o *Bacteria* são os mais negligenciados ao se excluir unidades taxonômicas operacionais (OTUs) com abundâncias relativas inferiores a 1%.

O aumento da detecção e divulgação de resultados que incluam os organismos de baixa abundância, como as arqueias, gerará dados para a construção de padrões biogeográficos de microrganismos bucais mais próximos da realidade. Isso poderá permitir compreender melhor como as relações interespécies se desenvolvem (18).

1.3 *ARCHAEA*

1.3.1 Um Breve Histórico

As arqueias são microrganismos procariontes que foram inicialmente classificados como pertencentes ao mesmo grupo das bactérias (25). O primeiro relato sobre microrganismos com as características do grupo data de 1878, quando um microrganismo foi isolado de amostras de sal usado para salgar bacalhau nos EUA (26). As evidências da existência das arqueias que seguiram as associavam apenas a ambientes “extremos”.

A princípio, as mudanças fenotípicas observadas nas arqueias em relação aos demais procariotos, como o tipo incomum de membrana plasmática com cadeias

laterais de hidrocarbonetos ligados amoléculas de glicerol-1-fosfato por ligações do tipo éter, eram justificadas como sendo ocasionadas por adaptações evolutivas aos seus ambientes inóspitos (27). Outro fator relevante que dificultou a identificação das arqueias como um grupo único foi a crença de que as diferenças entre eucariotos e procariotos representem extremos filogenéticos (28).

O desenvolvimento de métodos moleculares permitiu uma análise filogenética baseada na caracterização da sequência do rRNA 16S. Usando tal abordagem, Carl Woese e cols. sequenciaram RNAs ribossomais denominados 16Sdas espécies de “bactérias” metanogênicas *Methanobacterium ruminantium* e *Methanobacterium thermoautotrophicum,* concluindo que se diferenciavam dos procariotos "típicos". (29). Essas constatações levaram Woese e seus colaboradores em 1977 a propor pela primeira vez uma divisão dos seres vivos em três linhagens (29): (i) as “eubactérias”, que incluíam todas as bactérias típicas; (ii) as “arqueobactérias”, que continham bactérias metanogênicas; e (iii) os “eucariotos” (30).

A proposta de separar os procariotos em *Eubacteria* e *Archaeabacteria* não foi inicialmente aceita pela comunidade acadêmica e Woese foi duramente criticado. Essa desaprovação se justificou pelo fato das metodologias de sequenciamento de ácidos nucléicos e o uso de análises comparativas destas sequências com finalidades filogenéticas ainda não estarem difundidas (28). Todavia, em 1978, Woese publicou o artigo “Archaebacteria” (30) que, baseado em evidências de pesquisas feitas por ele e outros autores, elencou as diferenças até então conhecidas destes organismos em relação às bactérias: (1) a presença de tRNAs e RNAs ribossômicos característicos; (2) a ausência de peptídeoglicano nas paredes celulares , com substituição em muitos casos por um revestimento amplamente proteico; (3) a presença de lipídeos de membrana compostos por cadeias laterais de hidrocarbonetos ligadas a moléculas de glicerol-1-fosfato por de ligações do tipo éter (4) ocorrência apenas em habitats incomuns(30).

Os avanços na caracterização a nível molecular dos diferentes organismos do planeta, revelou que as diferenças entre os três grupos primários da vida eram maiores do que as vistas entre os reinos anteriormente propostos. Assim, em 1990, Woese e cols. propuseram um novo sistema de classificação dos seres vivos, acrescentando uma nova categoria taxonômica, superior a reino, denominada

“domínio”. Foi proposto que os seres vivos compreenderiam três domínios, *Bacteria*, *Archaea* e *Eucarya*, (31). Nesse mesmo trabalho, os autores recomendam o abandono do termo "archaebacteria" para evidenciar as grandes diferenças filogenéticas entre estes organismos e as bactérias.

1.3.2 Diversidade Do Domínio *Archaea*

Filogeneticamente, *Archaea* foi inicialmente dividido, por Woese e cols. (31), em dois grupos distintos: *Euryarchaeota* e *Crenarchaeota.* O primeiro era fenotipicamente heterogêneo, abrangendo as linhagens das metanogênicas, halófilas, redutoras de sulfato e dois tipos de termofílicos. Já *Crenarchaeota* era homogêneo quanto a fisiologia de seus participantes e abrangia exclusivamente representantes termofílicos (31). Posteriormente, linhagens de arqueias foram agrupadas em quatro grandes clados: o filo *Euryarchaeota* e os super filos, *TACK (Thaumarchaeota, Aigarchaeota, Crenarchaeota, Korarchaeota*), *DPANN (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota)* e *Asgard* (32, 33).

Euryarchaeota, assim como inicialmente proposto, continua sendo um grupo fisiologicamente e morfologicamente heterogêneo e é um dos dois principais grupos de arqueias com representantes cultivados (21). Além das cinco classes metanogênicas (*Methanobacteria, Methanococci, Methanomicrobia, Methanonatronarchaeia e Methanopyri*), se destacam nesse filo as classes *Halobacteria,* halofílica extrema; as termofílicas *Thermococcus, Archaeoglobi e Methanopyri; e Thermoplasmata,* com representantes acidófilos (23).

Os avanços no sequenciamento genômico e abordagens computacionais estão contribuindo para ampliar a compreensão do domínio *Archaea*. Atualmente há cerca de 27 filos de arqueias propostos distribuídos entre os super filos supracitados (34). Contudo, apenas seis filos possuem representantes cultivados, o que destaca a relevância da biotecnologia para um melhor entendimento das arqueias (34). Porém, os bancos de dados genômicos crescentes fazem com que a árvore filogenética arqueal seja constantemente reavaliada (27, 35, 36).

A progressão do conhecimento sobre as arqueias, além de melhorar a construção de sua taxonomia, mostrou que nem todas as arqueias são extremófilas como se acreditava, e que na verdade as arqueias são universalmente distribuídas (32, 37, 38). Membros do domínio desempenham um papel fundamental na manutenção de importantes ciclos biogeoquímicos (27). Por exemplo, a metanogênese e a oxidação anaeróbica do metano são etapas importantes no ciclo do carbono e são realizados exclusivamente por arqueias (39). Outro ciclo em que as arqueias possuem destaque é o do nitrogênio, em que a oxidação da amônia em nitrito é realizada por espécies de *Thaumarchaeota* (40, 41).

Arqueias podem colonizar uma gama diversificada de hospedeiros (38) e estão envolvidas em associações simbióticas ou parasitárias com representantes de *Eukarya* (ruminantes, humanos, vegetais, artrópodes, esponjas marinhas), *Bacteria* e outras arqueias (42). Todavia, comparado às bactérias, nosso conhecimento das arqueias é limitado.

1.3.3 Principais características celulares e moleculares das arqueias

Apesar da grande maioria dos representantes do domínio *Archaea* se assemelharem morfologicamente aos de *Bacteria* (ultraestrutura unicelular sem núcleo) (43), alguns membros possuem morfologias geométricas únicas, tais quais as formas poligonais das arqueias halófilas, as formas de cocos muito irregulares de espécies termófilas, ou até mesmo espécies com formato quadrado ou triangular (44). Além das variações de forma, também há diferenças em tamanho. A maioria das células de arqueias possuem dimensões que variam de 0,5 a 3,0 µm de comprimento e cerca de 1,0 µm de largura, que está na faixa da maioria das bactérias (43). Todavia, algumas arqueias podem formar filamentos de até 100 µm, outras ocupam a posição de menores organismos vivos com 100–300 nm. Essa variedade de formas e tamanhos acontecem pelas características das membranas celulares, estruturas de superfície e maquinaria do cito esqueleto (45).

As arqueias não possuem peptidoglicano em suas paredes celulares e muitas apresentam uma camada de proteína cristalina em suas superfícies (46). Essa camada de superfície, chamada de camada S, pode contribuir para a forma e osmoproteção celular. A parede celular arqueal é extremamente variável, podendo ter

em sua composição pseudopeptideoglicano, metanocondroitiína, heteropolissacarídeos sulfatados, halomucina ou glutaminoglicano, com diferentes tipos de organização tridimensional (46, 47). Algumas espécies de arqueias sobrevivem sem essas estruturas, como alguns membros dos gêneros *Thermoplasma, Halococcus, Methanobrevibacter, Natronococcus* e *Methanosphaera.* A hipótese é que compostos como glicocálice, lipoglicanos ou glicoproteínas associadas à membrana substituem a função de uma parede celular para esses organismos (47).

Os envelopes celulares de arqueias ainda apresentam características singulares, como membranas plasmáticas com lipídios isoprenóides ligados a éter com uma cadeia principal de glicerol-1-fosfato, ou mesmo lipídios tetraéter e N-glicosilação de proteínas extracelulares (48) (27).

O cromossomo de arqueias é circular e a replicação ocorre de forma bidirecional, semelhante ao que acontece nas bactérias (49). Porém, as células das arqueias possuem proteínas envolvidas nas vias de processamento da informação genética (replicação, transcrição e tradução do DNA) que se assemelham às encontradas em células eucarióticas como organização dos ribossomos, a presença de histonas, RNA polimerases complexas e iniciação da tradução com metionina (45, 49) (50)

Não há um consenso em relação às características moleculares quando se comparam os diferentes filos de *Archaea*. Por exemplo, enquanto alguns filos, como *Euryarchaeota*, possuem um aparato de divisão semelhante ao observado em bactérias outros, como *Crenarchaeota*, possuem um sistema comumente observado em células eucarióticas. Mais interessante ainda é o fato de alguns filos, como *Thaumarchaeota*, possuírem genes relativos aos aparatos de divisão tanto de procariotos como eucariotos (51).

Os biofilmes são a forma mais comum em que os microrganismos existem e interagem entre si. Quanto a arqueias, poucos detalhes moleculares são conhecidos sobre a formação de biofilmes (52, 53). No entanto, sabe-se que as arqueias possuem estruturas de superfície celular que lhes permitem mover-se, aderir-se às superfícies e interagir com outras células (microbianas), como pili tipo IV e o archaellum (o flagelo

arqueal) (45, 54, 55). Apesar do conhecimento do papel de arqueias em microbiomas complexos ainda ser incipiente, estudos já demostraram que algumas cepas são capazes de formar biofilmes, produzindo quantidades relativamente baixas de polissacarídeos extracelulares, provavelmente contendo glicose, manose e galactose sobre diferentes substratos, incluindo mucosas (13, 56).

1.4 BIOGEOGRAFIA DO ARQUEOMA HUMANO

1.4.1 Padrões biogeográficos do Arqueoma Humano

A associação direta de espécies de arqueias com doenças até o momento não foi estabelecida (57). Entretanto, a identificação de espécies de arqueias em associação com infecções sugere fortemente um potencial infeccioso (58). Tal hipótese é reforçada devido arqueias compartilharem algumas características com espécies de microrganismos conhecidos por estarem envolvidos em situações de doença, como a capacidade de colonização e coexistência com a microbiota saudável em baixos níveis de abundância que aumentam em doença (14, 59).

A dificuldade de realizar uma associação clara entre arqueias e doenças humanas pode ser, em parte, explicada pelas limitações em detectar, identificar e isolar esses microrganismos (60). Os entraves relacionados ao cultivo e ao estudo da fisiologia das arqueias presentes no corpo são evidenciados ao analisarmos o histórico dos estudos desses microrganismos. Desde que a primeira arqueia foi caracterizada a partir de fezes humanas em 1982 até o ano de 2012, apenas três espécies de arqueias metanogênicas haviam sido cultivadas a partir de amostras coletadas em sítios do corpo humano: *Methanobrevibacter smithii (61), Methanosphaera stadtmanae (62)* e *Methanobrevibacter oralis (63).* E então, em 2012, a espécie *Methanomassiliicoccus luminyensis* foi cultivada (7).

Apesar da predominância de arqueias em humanos ser baixa quando comparada à de bactérias, é relevante conhecer como esses microrganismos colonizam os nichos humanos e, como isso pode influenciar o equilíbrio saúde/doença (52). Membros do domínio *Archaea* já foram identificados em diversos sítios do corpo, como vagina, pulmão, nariz, pele e, principalmente, em amostras do intestino e da boca (7, 11, 64).

1.4.1.1 Arqueias e o intestino

Ainda quando considerados como bactérias, organismos metanogênicos foram identificados em amostras humanas em uma investigação que tinha como objetivo analisar a prevalência de metano em amostras da respiração, flatulência e fezes (65). Já quando estabelecidos como um domínio a parte, a espécie *M. smithii* foi caracterizada a partir de análises fecais (61). Desde então, amostras do trato gastrointestinal são as que fornecem maior volume de informações sobre o arqueoma humano (57).

Em um estudo recente foram avaliados cerca de 1.167 genomas de arqueias recuperados de amostras do trato gastrointestinal em 24 países, incluindo populações rurais e urbanas. Dentre eles foram identificados três gêneros, 15 espécies e 52 cepas de táxons não descritos anteriormente. Ainda no mesmo estudo, Chibani e colaboradores (66), embasados em características genômicas específicas, propusera que *M. smithii* (a espécie mais frequentemente detectada), seja dividida em duas espécies distintas, sendo a nova representada por *Candidatus Methanobrevibacter intestini.*

Arqueias metanogênicas são consideradas elementos da microbiota intestinal anaeróbica madura saudável, representando 10% de todos os anaeróbios intestinais e parecem ajudar na eficiência da digestão (67). As metanogênicas mais prevalentes no intestino são *M*. *smithii* (até 97,5%) e *M, stadtmanae* (até 23%) (61, 62, 66, 68). A detecção do DNA de *M. smithii* durante os primeiros dias de vida em amostras de fezes indica que a aquisição de arqueias pode ser um evento precoce em recém-nascidos(13).

As arqueias também foram associadas ao processo de metabolização do trimetilamina (TMA) em metano. A TMA é um produto derivado do metabolismo de alguns nutrientes (lecitina, colina, TMAO, L-carnitina) que pode causar a trimetilaminúria (ou Síndrome do odor de peixe), além de doença cardiovascular. Por realizar o metabolismo da TMA ainda no intestino, as arqueias metanogênicas favorecem a diminuição do volume desse composto que chega até o fígado para a metabolização, diminuindo assim seus níveis plasmáticos (32, 33, 69, 70).

Bai e cols.(71) avaliaram o arqueoma do intestino humano em indivíduos saudáveis e sua associação com fatores ambientais e homeostase do hospedeiro. Os dados mostraram que fatores como geografia, urbanização, etnia e dietas habituais contribuem para que haja variações do arqueoma intestinal em indivíduos saudáveis e ressaltam o impacto da urbanização no arqueoma intestinal em associação com a saúde do hospedeiro na sociedade moderna.

Ao contrário dos trabalhos supracitados, alguns estudos associam a presença de arqueias a certas doenças do trato gastrointestinal, como a doença inflamatória intestinal, obesidade, distúrbios intestinais crônicos e ao câncer de colón (32, 70, 72, 73), sendo esse último correlacionado ao aumento de arqueias halófilas e a diminuição de metanogênicas no intestino (74). Com o objetivo de demonstrar uma relação significativa entre arqueias metanogênicas e o desenvolvimento de diverticulite, Yazici e cols. (75) analisaram 274 indivíduos e encontraram que indivíduos com diverticulite tinham maiores níveis de metano respiratório em comparação com aqueles sem diverticulite. Ao estudar pacientes que passaram por apendicectomia, Takakura e cols. (76) perceberam que o número de pacientes com CH_4 detectável ou em excesso foi diminuído nesse grupo. Essa diminuição pode ser explicada pela teoria de que o apêndice serve como um reservatório ativo de arqueias metanogênicas, com um biofilme espesso resistente à entrada de antibióticos (76). Apesar desses estudos relevantes, o arqueoma intestinal e suas variações de composição associadas com fatores do hospedeiro e suas implicações funcionais ainda são pouco claras.

1.4.1.2 Pele

Atualmente, não há consenso sobre a prevalência ou composição taxonômica de arqueias na pele de mamíferos (77). Apesar de detecções positivas para arqueias no microbioma da pele humana, os táxons detectados são variáveis, o que pode ser explicado pelas variações de fatores como idade e fisiologia da pele (78).

Arqueias oxidantes de amônia (AOA) do filo *Thaumarchaeota* são mais frequentemente detectadas e, por isso, consideradas como parte da microbiota da pele (78). A presença de AOA na pele é justificada por aspectos próprios do órgão, como a capacidade de difundir passivamente a amônia ou excretá-la no suor.

Ademais, xampus e sabonetes comuns geralmente contêm derivados de amônia que podem facilitar o crescimento de AOA (77, 79). Probst e cols (80) afirmam que arqueias compreendem até 4,2% do microbioma da pele, sendo parte da microbiota desse órgão, e defendem o potencial de renovação de amônia na pele humana pelas AOAs.

Outros táxons de arqueais frequentemente encontrados em amostras de pele humana são afiliados a haloarchaea (38). Até o momento, o papel das arqueias halofílicas em amostras humanas permanece incerto, contudo, devido a pele apresentar concentrações elevadas de sal sazonalmente, tal acontecimento poderia apoiar a existência de halófilas nesse orgão (78).

1.4.1.3 Trato Respiratório

Mackenzie e cols.([74]), ao avaliarem a prevalência, diversidade e abundância de arqueias em seios nasais humanos em saúde e em sinusite crônica, identificaram cepas de *Euryarchaeota* e *Thaumarchaeota*. No entanto, a diversidade e composição de arqueias nos seios apresentaram alta variação interpessoal, e não foi observado associação de *Archaea* com estado de doença (81).

Ao realizar técnicas de metagenômica, um estudo identificou membros da família *Methanobacteriaceae* dentre espécies sino-nasais, conseguindo identificar *Methanobacterium formicicum* a nível de espécie. De forma inesperada, essa foi uma das cinco espécies de arqueias com maior abundância no sítio (6).

Membros do grupo *Woesearchaeota* também foram identificados como o táxon arqueal dominante no pulmão humano (7). O conhecimento sobre essas arqueias é escasso, porém especulações sobre um potencial estilo de vida parasitário/simbiótico com outros grupos de arqueias já foi hipotetizado (7). Além disso, representantes do *Woesearchaeota* podem ter papéis em ciclos biogeoquímicos, funcionando com metanogênicos na ciclagem anaeróbica do carbono, e participando na ciclagem anaeróbica do nitrogênio (82).

As diferenças entre os exemplares de arqueias identificadas em estudos ligados ao trato respiratório, provavelmente se deve às divergências no local de

amostragem do trato respiratório superior (narinas superiores *versus* seios da face) ou diferenças geográficas na composição do arqueoma (7, 81).

1.4.1.4 Vagina e Trato urinário

Belay (5) (1990) identificou pela primeira vez a presença de *M. smithii* em amostras vaginais de pacientes com vaginose bacteriana. Mais recentemente. Grine e cols. (83) investigaram a presença de metanogênicas em espécimes de mulheres com e sem o diagnóstico de vaginose e, assim como no estudo de Belay e cols (5), nenhuma amostra do grupo controle foi positivo para arqueia, enquanto *M. smithii* foi identificada em 97% dos espécimes de vaginose bacteriana. Devido a sua alta taxa de detecção, foi sugerido considerar *M. smithii* como um possível biomarcador para o diagnóstico laboratorial de vaginose bacteriana.

Visando ampliar a visão sobre a composição geral do microbioma vagino-uterino Li e cols. (84) realizaram o sequenciamento shotgun de 52 amostras do canal cervical e do líquido peritoneal de mulheres chinesas em idade reprodutiva e identificou arqueias produtoras de metano. Nesse estudo, o gênero *Metanosaetaceae* teve uma abundância maior que 0,1% do número total de leituras identificadas nas amostras. Mais estudos sobre o arqueoma vaginal/uterino são necessários pois a presença de metanogênicos nessa região pode comprometer a microbiota de saúde, visto que esses microrganismos podem prevenir o acúmulo de ácido, aumentando do pH, o que implica na homeostase das bactéria acidúricas da região (85).

Sobre o trato urinário, *M. smithii* também foi a única espécie de arqueia identificada como parte da microbiota urinária de alguns indivíduos. Por ter sido detectada em amostras de pacientes com infecção urinária em conjunto com enterobactérias, conhecidas pela produção de hidrogênio, sugere-se que essa arqueia poderia desempenhar um papel na disbiose, por facilitar o crescimento de enterobactérias, reconhecidas como agentes causadores de infecções urinárias (83). Contudo, a identificação de arqueias em amostras de urina coletadas de 40 crianças saudáveis (86) ressalta a divergência do conhecimento atual e a necessidade de maiores investigações.

1.4.1.5 Olho

Aqueias tiveram sua presença e a diversidade documentadas em amostras de caixas de lentes de contato coletadas da superfície ocular de pacientes com ceratite microbiana (87). Nesse estudo, assinaturas de arqueias foram encontradas em todos os 30 pacientes, mas a abundância relativa para todos foi baixa (<1%). Os filos *Euryarcheota* e *Crenarchaeota* foram detectados em 96,7% dos casos, já o filo *Thaumarchaeota* foi detectado em apenas 3,3% dos casos (87).

Com o objetivo de estudar comunidades microbianas dos sacos lacrimais, espécimes foram isolados do saco lacrimal de 10 pacientes com obstrução primária adquirida do ducto nasolacrimal (PANDO) e arqueias representaram 0,04 (0,01%–0,14%) da distribuição taxonômica das amostras (88). Em outro estudo com amostras da conjuntiva e da córneas, membros *da Euryarchaeota* representaram <5% das leituras (89). Em oposição, Kang e cols. (90) identificaram arqueias apenas na córnea de pacientes controles sem conjuntivite. Portanto, conclui-se que, assim como em outros sítios, as funções de arqueias neste habitat quanto a defesa imunológica ou em distúrbios de doenças são desconhecidas (87).

1.4.1.6 O Arqueoma Oral

Assim como para o arqueoma intestinal, muitas questões com relação à prevalência, diversidade, potencial associação a disbioses bucais e interações do arqueoma oral com outras espécies microbianas permanecem indefinidas (12). A constatação da presença de arqueias em diferentes nichos bucais contribui para o avanço da compreensão de como as comunidades microbianas são estruturadas, não apenas espacialmente, mas também em termos de composição (4).

Em 1987, foi relatada a primeira detecção de metanogênicos na cavidade oral humana (95). Já em 1988, em amostras de sujeitos com algum grau de doença periodontal, arqueias que apresentaram morfologias características de *Methanobrevibacter spp*. foram identificadas e, em algumas amostras, foi possível chegar à identificação da espécie *M. smithii (64)*. Em 1994, Ferrari e colaboradores (63) isolaram uma nova espécie em forma de cocobacilo do biofilme subgengival humano, denominada *Methanobrevibacter oralis.*

As arqueias metanogênicas ainda são as mais comumente detectadas no microbioma oral. *Methanobrevibacter smithii* e *M. oralis* são os principais metanogênicos identificados na microbiota oral por meio de métodos moleculares e de cultivo, no entanto, diversos estudos relataram a presença de espécies "semelhantes a *M. oralis*", o que mantém a real diversidade do arqueoma oral ainda pouco compreendida (96). Mas além do gênero Methanobrevibacter, outras arqueias foram detectadas em amostras orais, como mostra a tabela 1. *E*studos mais recentes, baseados no sequenciamento parcial do gene 16S rRNA, identificaram uma maior diversidade em amostras orais (10, 37).

No microbioma periodontal, as interações entre arqueias e os demais membros ainda são pouco conhecidas e a presença concomitante com espécies específicas de bactérias, como ocorre com determinados microrganismos, ainda precisa ser melhor explorado. Sabe-se que a doença periodontal é uma desordem na qual determinadas espécies bacterianas exercem um papel de destaque e entender como determinadas arqueias se comportam em relação a elas é essencial. Uma correlação positiva entre os níveis de arqueia e *Porphyromonas gingivalis*, bem como *Tannerella forsythia,* foi observada em biofilmes subgengivais de indivíduos com periodontite crônica (97). A abundância de *M. oralis,* que é uma das espécies de arqueias mais frequentemente detectada na cavidade bucal, foi relatada como sendo pelo menos dez vezes maior em pacientes que também abrigavam espécies da bactéria *Prevotella intermedia* (98). Por outro lado, grandes proporções de populações de *Treponema* foram encontradas em sítios periodontais sem arqueias metanogênicas (99). Göhler et al. (2014) mostraram que abundâncias moderadas de arqueia na língua estavam associadas a um estado periodontal saudável, enquanto abundâncias maiores se correlacionavam com doença periodontal(100).

Lettiere e cols. (101), ao analisar o microbioma salivar de indivíduos com síndrome de *Papillon-Lefèvre*, encontraram sequências de arqueias em 9,84% das amostras de saliva de indivíduos com doença periodontal graves. Neste estudo, o domínio *Archaea* correspondeu a 10 táxons em nível de gênero, todos dos filos *Crenoarchaeota* e *Halobacteriota*, sendo os gêneros *Caldivirga* (família *Thermoproteaceae*) e *Sulfobococcus* (família *Desulfurococcaceae*) considerados parte do microbioma central de *Archaea* dos indivíduos. Já os estudos de Grine e cols.

(102) e Guindo e cols. (103) encontraram uma prevalência maior de metanogênicos na saliva de sujeitos fumantes.

O microbioma endodôntico possui especificidades para análise de seus representantes, devido às limitações da coleta de amostras em dentes vitais, o que limita o entendimento dos microrganismos associado às infecções endodônticas (104). Todavia, arqueias estão presentes em cerca de um quinto dos canais endodônticos e se mostraram mais prevalentes em pacientes com pulpite (105). Os representantes metanogênicos são os mais frequentemente identificados, entretanto representantes dos filos *Thaumarchaeota* e *Crenarchaeota* também já foram identificados (105).

Também já foi relatada a ocorrência de arqueias em tecido dentário cariado e em biofilmes supragengivais (10). Em cárie dentária, foram achadas sequências de DNA de *Thaumarchaeota* e *Methanocellales* (37). A presença de um clado relacionado ao gênero *Methanocella* pode ser justificado biologicamente pela alta presença de bactérias acidogênicas associadas a doença (106) (37). O resultado desses estudos mostra que a diversidade global de arqueias na cavidade bucal humana pode estar atualmente subestimada tanto quanto aos gêneros metanogênicos presentes, como quanto a outros filos não metanogênicos (107).

Tabela 1: Arqueias metonogênicas detectadas em amostras orais

Arqueia	**Método**	**Tipo/Local de amostra**	**Condições de doença/ saúde**	**Condições saúde**	**Hipótese de função**	**Referência (primeiro relato)**
Metanobrevibacter smithii	Cultura, sequenciamento por PCR de 16S rRNA	Biofilme dentário; saliva	Periodontite / Infecção endodôntica			Belay, 1988 (64); Bouzid, 2024 (108)
Metanobrevibacter oralis	Cultura, sequenciamento por PCR de 16S rRNA	Biofilme subgengival; saliva	lesões relacionadas à periodontite e bolsas peri-implantares / Infecção endodôntica	Sim	Doença periodontal por interações sintróficas com bactérias redutoras de sulfato	Ferrari, 1994 (63); Nguyen-Hieu, 2013 (109)
Methanosphaera stadtmanae	cultura	Biofilme dentário	periodontite			Belay, 1988 (64)
Metanobrevibacter genus	cultura	Biofilme dentário				Brusa, 1987 (95)
Methanosarcina vacuolata	pré-processamento de dados de sequenciamento	-	periodontite	Sim		Deng, 2017 (110)
Methanobrevibacter massiliense	*mcrA* e 16S rRNA PCR-sequencing	Biofilme subgengival; Polpa necrótica	Periodontitis severa	Não	vida simbiótica com a bactéria anaeróbica *Pyramidobacter piscolens*	Huynh, 2017 (111);
Methanosarcina mazeii.	*mcrA* e 16S rRNA PCR-sequencing	Biofilme subgengival; saliva	periodontite	Sim		Matarazzo, 2011 (112)
Methanosalsum zhilinae	pré-processamento de dados de sequenciamento	-	periodontite			Deng, 2017 (110)
Methanosarcina lacustris	pré-processamento de dados de sequenciamento	-	periodontite			Deng, 2017 (110)
Methanotorris igneus	pré-processamento de dados de sequenciamento	-	periodontite	Não		Deng, 2017 (110)
Methanosarcina barkeri	preprocessing of sequencing data	-	periodontite			Deng, 2017 (110)
Methanobacterium curvum/congolense	pré-processamento de dados de sequenciamento		Periodontite	Sim		Matarazzo, 2011 (112)
Methanocella genus	16S rRNA Sequanciamento de Sanger	Dentina cariada	Cárie	Não	Arqueias interagem com parceiros sintróficos, gerando nichos para a subsequente colonização de outros microrganismos.	Damé-Teixeira, 2020 (37)
Methanobrevibacter gottschalkii	sequenciamento por PCR de 16S rRNA	Canal dentário	Infecção endodôntica primária	Não		Efenberger, 2015 (113)

Candidatus Methanobrevibacter sp. N13	sequenciamento por PCR de 16S rRNA	Biofilme subgengival, calculo antigo	Lesões de periodontite severa	Não	Huynh, 2015 (114) ;

1.5 DESAFIOS NA IDENTIFICAÇÃO DE ARQUEIAS

A diversidade de espécies de arqueias no microbioma humano é baixa. As razões que justificam este fato podem estar ligadas aos problemas de métodos e técnicas utilizados na identificação desses microrganismos.

Sun e cols. (91) elencaram cinco causas que dificultam o cultivo de arqueias. São elas: a falta de troca de material interespecífico durante o cultivo *in vitro* (por exemplo, disponibilidade reduzida de substâncias metabólicas, produzidas por outros organismos *in situ*); competição interespecífica; taxa de crescimento intrinsecamente lenta e baixa abundância de arqueias, resultando em um rápido crescimento excessivo de bactérias durante o cultivo em laboratório; e condições de transporte impróprias ou parâmetros de cultivo *in vitro* sub-ótimos que diferem dos parâmetros ambientais *in situ*.

Além das dificuldades de cultivo, Pausa e cols (62) elencaram cinco razões principais que justificam a negligencia dos estudos para com o arqueoma humano: (I) a incompatibilidade de iniciadores (*primers*), já que os atualmente utilizados são desenvolvidos pensando em sequências de DNA bacteriano o que dificulta o anelamento destes com o DNA de outros microrganismos; (II) baixa abundância do DNA de arqueias nas amostras estudadas; (III) métodos inadequados de extração de DNA; (IV) escassez de informações sobre arqueias nos bancos de dados de referência do gene 16S rRNA, e; (V) a falta de interesse clínico em arqueias, ponto associado a pouca compreensão do papel patogênico das arqueias.

O gene rRNA 16S tem sido um dos pilares da análise bacteriana baseada em sequência de DNA por décadas. Entretanto, os aspectos relacionados à amplificação do gene rRNA 16S devem ser considerados tanto durante o planejamento experimental quanto na análise dos dados, pois a escolha da região molde pode resultar na amplificação preferencial de algumas sequências (92). Por exemplo, o par de *primers* procariótico predominantemente usado 515F/806R tem demonstrado baixo desempenho na identificação de sequencias arqueais (93).

Kim e cols. (94), ao tentarem identificar a região de sequência que permitisse uma análise filogenética mais precisa dos genes rRNA 16S, concluíram que nenhuma região de sequência parcial pode estimar a riqueza de OTUs ou defini-las de forma tão confiável quanto genes quase completos. Contudo as regiões V1–V3 e V4–V7 e o agrupamento de OTUs a nível de espécie a 0,03 e 0,02 distâncias, respectivamente, produziram melhores resultados para identificação de arqueias.

1.6 CONSIDERAÇÕES FINAIS:

Com base no disposto acima, nota-se que arqueias são uma parte do microbioma humano. Mais pesquisas são necessárias para entender completamente seu impacto na manutenção de um microbioma saudável, além de como podem estar envolvidas no crescimento excessivo de bactérias patogênicas, como ocorre no intestino, trato respiratório, pele e olho, doença periodontal e vaginose.

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2 CAPÍTULO II: The *Archaea* domain: exploring historical and contemporary perspectives with *in silico* primer coverage analysis for future research in Dentistry

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2.1 ABSTRACT

Objective: The complete picture of how the human microbiome interacts with its host is still largely unknown, particularly concerning microorganisms beyond bacteria. Although existing in very low abundance and not directly linked to causing diseases, archaea have been detected in various sites of the human body, including the gastrointestinal tract, oral cavity, skin, eyes, respiratory and urinary systems. But what exactly are these microorganisms? In the early 1990s, archaea were classified as a distinct domain of life, sharing a more recent common ancestor with eukaryotes than with bacteria. While archaea's presence and potential significance in Dentistry remain under-recognized, there are concerns that they may contribute to oral dysbiosis. However, detecting archaea in oral samples presents challenges, including difficulties in culturing, the selection of DNA extraction methods, primer design, bioinformatic analysis, and databases. Design: This is a comprehensive review on the oral archaeome, presenting an in-depth in silico analysis of various primers commonly used for detecting archaea in human body sites. Results: Among several primer pairs used for detecting archaea in human samples across the literature, only one specifically designed for detecting methanogenic archaea in stool samples, exhibited exceptional coverage levels for the domain and various archaea phyla. Conclusions: Our *in silico* analysis underscores the need for designing new primers targeting not only methanogenic archaea but also nanoarchaeal and thaumarchaeota groups to gain a comprehensive understanding of the archaeal oral community. By doing so, researchers can pave the way for further advancements in the field of oral archaeome research.

Keywords: *Archaea* domain, Oral Archaeome, Methanogens, Oral microbiome, *in silico* primer analysis, Review

2.2 INTRODUCTION

The interactions among the human body cells' and those of its resident microbiota give rise to various physiological processes. Humans are holobionts, multicellular eukaryotes that have developed in association with diverse microbial communities (1). However, several interactions that occur in various microenvironments of the human body still remain a mystery. The human microbiome exhibits significant compositional variation across and within diverse body sites. Although the bacteriome comprises the largest part of the human microbiome, low-abundance signatures of other microorganisms have been consistently detected in the last years (2-5). As research advances, the archaeome - the community of archaea residing within the human body - is gaining recognition as a crucial member of the human microbiome (1, 6-8).

Among human-archaeal interactions identified so far, those involving methanogenic archaea in the gastrointestinal tract and oral cavity have been found to have significant implications for both health and disease (1, 6, 8-14). Moreover, members of *Archaea* have been discovered in samples taken from the vagina, lung, nose and skin (7, 15-20). Nevertheless, the complete picture of their relationships with the human host is far from being fully understood. It remains unclear whether there is a parasitic-host interaction or if archaea possess their own virulence factors. Up to this point, there are limited studies linking members of *Archaea* with particular pathologies, and most of the research cases involve the gastrointestinal tract and periodontal diseases (21). The lack of confirmed archaeal human pathogens has been cited as a factor that has hindered the progress in human archaeome research (22, 23).

Presently, there is a growing tendency towards mapping archaeal species based on their biogeography, which refers to the spatial and temporal distribution patterns of these communities (7, 14, 24). Biogeography is crucial for understanding the ecology of microbial communities in the context of symbiosis and their impact on human health. The aim of this work was to comprehensively review the main aspects of the *Archaea* domain and the human archaeome, with a specific focus on the oral archaeome. We explore the reasons behind the limited interest in this research topic and propose potential areas for future studies. Additionally, we performed an *in silico*

analysis with different primers from previous research that could help detect archaea in oral samples.

2.3 A BRIEF HISTORY OF THE ARCHAEA DOMAIN

Since their cells are also of prokaryotic nature, archaea were initially classified as Monera, together with commonly known bacteria and cyanobacteria (25). The first report of microorganisms that now are known to be archaea can be traced back to 1878, when a red microbe was isolated from salt samples used for preserving cod in the USA (26, 27).

In the 1970's, studies with these "strange bacteria" revealed that one of their distinct phenotypic features, when compared to other prokaryotes, was their unusual plasma membrane, which is composed of long-chain alkyl groups linked to glycerol-1-phosphate molecules by ether-type bonds. This kind of membrane composition was initially considered an adaptation to their harsh environments, since the first organisms studied were retrieved from extreme environments, such as thermal or acidic ponds, as well as hypersaline lakes (28). The absence of other shared phenotypic traits among these extreme organisms posed a significant challenge in identifying archaea as a distinct group. Even within methanogenic archaea, the only commonality was the biochemistry of methanogenesis (29).

Comparative analyses of ribosomal RNA sequences of different organisms have played a crucial role in the identification of archaea as a separate group (30). Using this kind of approach, Carl Woese and colleagues were able to separate methanogenic "bacteria" in two different clades which share a mutual origin, but clearly distinct from that of commonly known bacteria (29, 31). These phylogenetical studies performed by Woese and colleagues culminated, in 1977, with the proposal of dividing the prokaryotes in two kingdoms, named: *Eubacteria* and *Archaebacteria*. According to their results, he eukaryotes would correspond to a separate group of living organisms (32).

This proposal was not well accepted by the academic community and Woese was heavily criticized. This disapproval was likely due to the fact that nucleic acid sequencing methodologies and the use of comparative analyses of these sequences for phylogenetic purposes were not yet widespread (29). In 1978, the article

"Archaebacteria" was published (33), which added halophiles and various thermoacidophiles as members of this group and listed the distinguishing characteristics of these organisms compared to bacteria based on research evidence. These characteristics included: (1) the presence of characteristic tRNAs and ribosomal RNAs; (2) the lack of peptidoglycan cell walls, which were replaced in many cases by a proteinaceous coat; (3) the occurrence of ether-linked lipids made up of phytanyl chains; and (4) their occurrence solely in unusual habitats in all cases studied up to that moment (33).

Subsequent research confirmed that the disparities observed among the three main phylogenetic groups of life were more significant than previously recognized. Consequently, in 1990, a novel classification system for living organisms, which introduced a new taxonomic category, superior to the kingdom, referred to as “domain” was proposed (34). The living world would, therefore, be divided into three domains: *Bacteria*, *Archaea*, and *Eukarya* (34). At this point, the authors suggested abandoning the term "archaebacteria" as it incorrectly implied a connection between *Archaea* and *Bacteria* (31, 34).

These first studies already classified archaea into two distinct groups, *Euryarchaeota* and *Crenarchaeota*, with the *Euryarchaeota* consisting of a heterogeneous group, with organisms presenting different phenotypes, such as methanogenic, halophilic, sulfate-reducing, and thermophilic. In contrast, the *Crenarchaeota* group was homogeneous in terms of the physiology of its members, consisting solely of thermophilic representatives (34).

The advances in genomic sequencing and computational methods have significantly contributed to expanding the knowledge about the *Archaea*, with the proposal of additional phyla, resulting in a constant and continuous reevaluation of their phylogenetic tree (28, 35, 36). Nowadays, there are approximately 27 proposed phyla of archaea (1, 8) and only six of these phyla have representatives successfully cultivated in laboratory so far (37).

The progress in the understanding of archaea has not only refined their taxonomy but has also revealed that not all archaea are extremophile organisms as previously assumed. It has become evident that archaea are ubiquitously distributed across various environments (1, 38, 39), and that they participate in symbiotic or

parasitic associations with representatives of *Eukarya* (such as ruminants, humans, plants, arthropods, and marine sponges), *Bacteria*, and other archaea (40). Furthermore, archaea play a crucial role in maintaining important biogeochemical cycles such as the carbon cycle, as they are responsible for processes such as methanogenesis and the anaerobic oxidation of methane, which are exclusively carried out by archaea (28, 41). Archaea also play a significant role in the nitrogen cycle, particularly in the oxidation of ammonia to nitrite, a process primarily carried out by *Thaumarchaeota* species (42, 43).

Phylogenetic analyses have revealed several similarities between archaea and eukaryotes. The *Asgard* superphylum, proposed in 2015, is considered the closest ancestor of eukaryotic cells. The presence of eukaryotic signature proteins in this group, such as the Ras superfamily of small guanosine triphosphatases, strengthens the evolutionary connection between archaea and eukaryotic cells. Recent metagenomic studies indicate the existence of multiple *Asgard* lineages that exhibit archaeal genetic and regulatory features also closely related to eukaryotes in molecular phylogenies. This raises questions about the origin of eukaryotes, but reinforces the idea that eukaryotes were originated from archaea (44-46).

2.3.1 Main Cellular and Molecular Characteristics

Although most of the *Archaea* representatives share similar morphologic types with *Bacteria* (47), some members possess distinct geometric shapes. These include the polygonal shapes of halophilic archaea, the highly irregular cocci shapes of thermophilic species, and even species with square or triangular shapes (48). The majority of archaeal cells range from 0.5 to 3.0 μm in length and about 1.0 μm in width, which is similar to the size range of most bacteria (48). Nevertheless, certain archaea are capable of producing filaments up to 100 μm in length, while others hold the distinction of being the smallest prokaryotes organisms, measuring 100–300 nm (49, 50). These differences in shape and size are attributed to the distinctive characteristics of their cell membranes, surface structures, and cytoskeletal machinery (51).

The cell walls of archaea do not contain peptidoglycan, and many species possess a surface layer of crystalline protein, called S layer (52). This proteinaceous layer has multiple functions such as maintaining cell shape and protecting against

osmotic stress (53). Other archaeal species present pseudopeptidoglycan, methanochondroitin, sulfated heteropolysaccharides, halomucin, or glutaminoglycan in their surfaces, with different three-dimensional structures (52). It has been suggested that certain compounds, such as glycocalyx, lipoglycans, or membrane-associated glycoproteins, may serve as substitutes for the cell wall in certain species of archaea that are able to survive without such structures (54).

As previously mentioned, the cell membranes of archaea are one of their distinctive characteristics, since they are composed of isoprenoid lipids with an ether linkage and a glycerol-1-phosphate backbone, as well as tetraether lipids (28, 55).

Like bacteria, the archaeal chromosome is circular, and replication occurs bidirectionally (56). However, archaeal cells have proteins involved in the processing of genetic information, such as DNA replication, transcription, and translation, that are similar to those found in eukaryotic cells. Other features shared with eukaryotic cells include the organization of ribosomes, the presence of histones in some phyla, complex RNA polymerases, and translation initiation with methionine (51, 56, 57).

In general, microorganisms exist and interact with other cell types through complex communities known as biofilms. While limited information is available on the molecular aspects of biofilm formation in archaea (58, 59), it is known that these organisms possess cell surface structures, such as type IV pili and the archaellum, that allow them to move, adhere to surfaces, and interact with other microbial cells (51, 60, 61). Although the role of archaea in complex microbiomes is still not well understood, some studies have shown that certain strains can form biofilms on different substrates, including mucous membranes, by producing relatively low amounts of extracellular polysaccharides containing glucose, mannose, and galactose (62, 63).

Like bacteria, archaea can also form biofilms in various environments, including human body sites. The process of archaeal biofilm formation involves multiple stages. Initially, microorganisms adhere to surfaces through physical forces, hydrophobic interactions, and the presence of extracellular polymeric substances (EPSs). This reversible adhesion is followed by irreversible adhesion mediated by extracellular surface structures. Afterward, cellular interactions lead to the formation of microcolonies. During the maturation phase, the biofilm develops a complex three-dimensional architecture with channels for nutrient and waste exchange. Finally,

biofilm dispersion becomes crucial for colonizing new sites, allowing cells to disperse and establish themselves in different locations (64). Understanding this process of formation and dispersion of archaeal biofilms in combination with bacteria could be the key for exploring the biogeographic patterns of the oral archaeome.

2.3.2 A Summary on The Biogeographic Patterns of The Human Archaeome

Since the first discovery of archaea in human feces in 1982, only three species of methanogenic archaea had been successfully cultured from human body samples, namely *Methanobrevibacter smithii* (65), *Methanosphaera stadtmanae* (66), and *Methanobrevibacter oralis* (67). It was not until 2012 that another species, *Methanomassiliicoccus lumyensis*, was successfully cultivated (7). Methanogenic organisms were studied in human samples since they were considered as bacteria (68). Further research has revealed the presence of other archaeal species from different phyla in various body sites. For instance, the lung has been found to harbor *Woesearchaeota*, and the skin hosts Haloarchaea and *Thaumarchaeota* (7, 69). These findings will be discussed in more detail below.

The impact of environmental factors and host homeostasis on the archaeome of healthy individuals in the human intestine was evaluated by Bai et al. (70). The results revealed that factors such as geography, urbanization, culture, and dietary habits influence the variation of the intestinal archaeome. The study emphasized the effect of urbanization on the intestinal archaeome and its association with host health in modern society. Samples from the gastrointestinal trait have provided the most significant amount of information about the human archaeome (9, 65). It is known that methanogenic archaea play a role in the healthy mature anaerobic intestinal microbiota, making up 10% of all intestinal anaerobes, and contribute to efficient digestion (71). *M. smithii* (up to 97.5%) and *M. stadtmanae* (up to 23%) are the most commonly found methanogens in the gut (65, 66, 72, 73). The detection of *M. smithii* DNA in stool samples during the first few days of life suggests that the acquisition of archaea may occur early in newborns (63).

Archaea have also been implicated in the metabolism of trimethylamine (TMA) to methane. TMA is a byproduct of the metabolism of certain nutrients such as lecithin, choline, TMAO, and L-carnitine, and its accumulation in the body can lead to

trimethylaminuria (or Fish Odor Syndrome) and cardiovascular disease. By metabolizing TMA in the gut, methanogenic archaea facilitate a reduction in the amount of this compound that reaches the liver for processing, thereby lowering its plasma levels (1, 8, 74, 75).

Some studies have linked the presence of archaea with certain gastrointestinal diseases, including inflammatory bowel disease, obesity, chronic intestinal disorders, and colon cancer (1, 75-77). Colon cancer has been associated with an increase in halophilic archaea and a decrease in methanogens in the intestine (78). Diverticulitis had higher levels of respiratory methane compared to those without the condition (79). Meanwhile, a reduction in the number of patients with detectable or excess CH4 among those who underwent appendectomy were detected (Takakura et al., 2020). It seems that the appendix could serve as an active reservoir of methanogenic archaea, with a thick biofilm that is resistant to antibiotics (80). Despite these significant findings, the composition of the intestinal archaeome and its variations in relation to host factors and functional implications remain unclear.

The skin archaeome was also studied. At present, there is no agreement on the proportions or classification of archaea in mammalian skin (81). Although archaea have been detected in the human skin microbiome, the taxa vary, probably due to differences in host factors such as age and skin physiology (17). Ammonia-oxidizing archaea (AOA) of the *Thaumarchaeota* phylum are frequently detected and considered a part of the skin microbiota (17). The presence of AOA on the skin is attributed to the organ’s ability to passively diffuse or excrete common shampoos and soaps can promote the growth of AOA (81, 82). According to Probst et al. (2013) (18), archaea constitute up to 4.2% of the skin microbiome, and they argue that AOAs have the potential to renew ammonia in human skin. Haloarchaea DNA has also been frequently detected in human skin samples (39). Although the role of halophilic archaea in human skin samples is uncertain, the fact that the skin has elevated salt concentrations during certain seasons suggests that it could support the existence of halophiles (17).

Variances in the archaeal taxa found in respiratory tract studies are likely attributed to disparities in the sampling location of the upper respiratory tract (e.g., upper nostrils versus sinuses) or regional dissimilarities in the archaeome composition (7, 16). *Euryarchaeota* (including *Methanobacteriaceae*) and *Thaumarchaeota* were

detected in both healthy and chronic sinusitis, with a high interpersonal variation in the diversity and composition of archaea in the sinuses, and no association between archaea and disease state was observed (15, 16). Nevertheless, it seems that the dominant archaeal taxon in the human lung consists of the *Woesearchaeota* group (7). It seems that they are a potential parasitic/ symbiotic lifestyle with other groups of archaea in those sites (7). Additionally, representatives of the *Woesearchaeota* may play a role in biogeochemical cycles, functioning as methanogens in anaerobic carbon cycling, and participating in anaerobic nitrogen cycling (83).

Archaea has also been detected in the vagina and the urinary tract. *M. smithii* was detected in vaginal specimens from women with and without a diagnosis of vaginosis (19, 20). Other methane-producing archaea were identified in vagino-uterine microbiome, with the genus *Metanosaetaceae* with an abundance greater than 0.1% of the total number of sequences detected (84). However, the presence of methanogens in this region could disrupt the healthy microbiota, as these microorganisms can inhibit acid accumulation, increasing the pH, which is necessary for the homeostasis of aciduric bacteria in the region (85). More research is needed to understand the vaginal/uterine archaeome.

M. smithii has been identified as the only archaeal species of some individuals' urinary microbiota. In patients with urinary tract infections, *M. smithii* was detected along with enterobacteria, which produce hydrogen. This suggests that *M. smithii* could facilitate the growth of enterobacteria, which are known to cause urinary tract infections and contribute to dysbiosis (20). However, a study of urine samples collected from 40 healthy children identified archaea (86), indicating a need for further investigation to reconcile the current divergent knowledge.

Archaea representatives have been identified in samples taken from contact lens cases of patients with microbial keratitis, demonstrating their existence and diversity in this context (87). The study showed that all 30 patients had archaeal signatures, but in low abundance (<1%). The phyla *Euryarchaeota* and *Crenarchaeota* were found in 96.7% of cases, while *Thaumarchaeota* was found in only 3.3% of cases (87). Samples were collected from the lacrimal sacs of ten patients with primary acquired nasolacrimal duct obstruction in order to study microbial communities, and archaea accounted for 0.04% (0.01–0.14%) of the taxonomic distribution of the

specimens (88). In another study involving conjunctival and corneal samples, members of *Euryarchaeota* represented < 5% of the total reads (89). In contrast, Kang et al. (90) exclusively detected archaea in the corneas of control patients who did not have conjunctivitis. Consequently, the function of archaea in this particular environment, regarding immune defense or potential disease disorders, remains undisclosed, much like in other sites (87).

2.4 THE ORAL ARCHAEOME: UN UPDATE

It is noteworthy that the oral cavity differs from all other human microbial habitats by the simultaneous presence of different surfaces and microniches for microbial colonization (91). The identification of archaea in distinctive oral niches contributes to our comprehension of how microbial communities are structured, both spatially and compositionally (6). The first report of methanogens in the human oral cavity dates back to 1987 (92). On year later, cells of *Methanobrevibacter spp*. with characteristic morphologies were detected in samples from individuals with various degrees of periodontal disease, with some samples even containing *M. smithii* (93). In 1994, a new coccobacillus species of the human subgingival biofilm was reclassified as *M. oralis* (67). However, it is very interesting that low proportions of DNA sequences from methanogenic archaea have been detected in the ancient dental calculus (94). A nearly complete genome of M. oralis was found in a neandertal with dental abscess sign. This demonstrates that archaea have been members of the human/humanoid oral microbiome throughout history.

In a previously published scoping review (6), we conducted an analysis of the existing evidence on the oral archaeome. To ensure a thorough examination, we conducted an extensive and inclusive systematic search that incorporated terms such as "archaeobacteria, " encompassing information predating the classification into a new domain. From 50 selected papers, 26 studies focused on sampling periodontal sites, while 10 studies targeted endodontic sites. Other oral sites, including tongue biofilm, supragingival biofilms, and caries, were also investigated. The initial studies that detected archaea utilized enrichment culture methods specifically designed for methanogenic "bacteria." However, most studies employed culture-independent techniques, utilizing primers targeting archaeal 16S rRNA genes, *mcrA* genes exclusive to methanogens, as well as *cnp60* genes specific to *M. oralis*, alongside other

molecular methods. This comprehensive analysis revealed the consistent presence of archaea in various oral sites, indicating that they are common constituents of subgingival biofilms. Through a cross-study analysis, we could infer that archaea may contribute to periodontal dysbiosis by creating conditions favorable for the growth of periodontal-related bacteria through cross-feeding interactions. This classical syntrophic behavior suggests the potential significance of archaea, even in cases where their abundance is relatively low (6).

To enhance the body of evidence in this field, we conducted a subsequent study focusing on meta-analyzing the overall prevalence of the periodontal archaeome and investigated differences between healthy and diseased individuals, as well as the impact of various treatments (14). Employing a systematic approach, we performed a meta-analysis using data from 30 studies, including 28 cross-sectional studies and 2 randomized clinical trials. These studies encompassed over 1300 individuals with varying degrees of periodontitis, alongside 400 periodontally healthy individuals. The findings revealed that the overall prevalence of archaea was 46% in individuals with periodontitis and 13% in those with healthy periodontal conditions. We conducted sensitivity analyses by excluding smaller studies or those utilizing different methods, yet the consistent trend persisted. Furthermore, individuals with periodontitis were found to be 7 to 9 times more likely to test positive for archaea when employing 16S rRNA or *cnp60* genes, compared to periodontally healthy individuals. These results consistently demonstrated the magnitude and direction of the effect. However, it is important to interpret the meta-analysis findings cautiously due to the low certainty of evidence indicated by the GRADE (Grading of Recommendations, Assessment, Development, and Evaluation) assessment. Most of the included studies were observational in nature and exhibited low to moderate methodological quality. Also, most studies focused on the detection of methanogens, revealing that the diversity of the periodontal archaeome is currently underestimated. During the methodological quality analysis, 22 studies were downgraded due to insufficient information regarding the classification of periodontitis and clinical data (14). It is worth noting that the GRADE system classified the evidence as uncertain due to imprecision. Nevertheless, our meta-analysis demonstrated that conventional periodontal therapy alone or in combination with antibiotics showed no significant difference in effectiveness. Both

approaches were equally successful in reducing the prevalence of archaea six months after treatment, likely due to the reduction in overall microbial load (14).

Nonetheless, considering the correlation between the presence of IgG antibodies responding to archaeal antigens in periodontitis (95, 96), we believe that archaea should be recognized as significant contributors to periodontitis. It is important to consider these findings in conjunction with the aforementioned evidence. The interactions between archaea and other members are still poorly understood, and their concurrent presence with specific bacterial species, as seen with certain microorganisms, needs further exploration. A positive correlation between archaeal levels and *Porphyromonas gingivalis*, as well as *Tannerella forsythia*, has been observed in subgingival biofilms of individuals with periodontitis (97). *M. oralis* abundance was reported to be at least ten times higher in patients who also harbored *Prevotella intermedia* (98). On the other hand, higher proportions of Treponema were found in periodontal sites without methanogenic signatures (99).

Although archaea were present in low abundance in most studies, the salivary microbiome of individuals with Papillon-Lefèvre syndrome (characterized by a very severe periodontitis phenotype) had archaeal sequences in 9.84% of the samples, comprising of 10 taxa at the genus level, all from the phyla *Crenoarchaeota* and *Halobacteriota*. *Caldivirga* (family *Thermoproteaceae*) and *Sulfobococcus* (family *Desulfurococcaceae*) were considered part of the core microbiome for these individuals (100).

In a recent systematic review, we conducted an evaluation of the overall prevalence of archaea in endodontic samples and examined its presence in relation to clinical parameters. Through a meticulous search of six databases and grey literature, we identified a total of 16 articles, out of which only three failed to detect archaea, likely due to the choice of primers used in those studies. Among the identified archaeal groups, methanogens were the most commonly observed, along with *Thaumarchaeota* and *Crenarchaeota* sequences. Archaea were found to be present in approximately 20% of individuals whose endodontic samples were analyzed. Interestingly, samples collected from individuals with primary infections were roughly twice as likely to test positive for archaea compared to those with secondary infections. Similarly, samples obtained from individuals who reported experiencing pain were also twice as likely to

exhibit archaeal presence compared to individuals with symptom-free infections. Considering these findings, we believe that the current challenge lies in understanding whether the decontamination process for infected root canals should incorporate the elimination or neutralization of archaea. Further research is needed to explore this aspect in order to enhance our understanding of archaeal involvement in endodontic infections and to develop effective decontamination strategies (101).

In a preliminary study, we observed the presence of *Thaumarchaeota* sequences in supragingival biofilms associated or not with caries, albeit in very low abundance, using universal primers and NGS (Next-Generation Sequencing) techniques. Additionally, we successfully cloned larger fragments of archaeal 16S from V1 to V5 in a representative sample, which once again revealed the presence of *Thaumarchaeota* sequences along with a methanogenic taxon. While we acknowledge the possibility of contamination, it is important to consider that the presence of ammonia oxidizing *Thaumarchaeota* could potentially disrupt a natural buffer system, thereby influencing the balance of the supragingival biofilm. Furthermore, the presence of methanogens in aerobic environments may initially seem like an anomaly. However, previous studies have demonstrated the ability of certain *Methanobrevibacter* species to thrive under micro-oxic conditions, thereby offering a plausible explanation for their presence in these particular samples (38). Therefore, further investigation into these findings is warranted to gain a deeper understanding of their implications.

Significant progress has recently been made in the oral archaeome research through the discovery of a novel nanoarchaeal species, *Nanopusillus massiliensis*, in dental biofilm samples. *Nanoarchaeota*, known for their smaller genomes, rely on other environmental archaea for their survival. Through specific PCR-based assays, the presence of *N. massiliensis* was detected using a combination of fluorescent in situ hybridization (FISH) and scanning electron microscopy. This analysis unveiled an intriguing interaction between *N. massiliensis* and *M. oralis* archaea within oral samples (102).

Figure 1 summarize the potential multiple roles that the oral archaeome plays in the oral cavity. Some archaea species can potentially contribute to pH maintenance due to their influence in ammonia oxidation and the nitrogen cycle. Others can act as secondary pathogens in a syntrophic partnership with bacteria, participate in methane

emission, enhance the efficiency of fermentation processes, increase the inflammatory characteristics of the microbiome, engage in hydrogen reduction, activate immune responses, produce glycosyltransferases, and contribute to the production of extracellular matrix. These diverse functions highlight the complex and dynamic interactions that archaea have within the oral microbiome, suggesting their significance in oral health and disease processes. Further research is necessary to fully understand the extent of these roles and their implications.

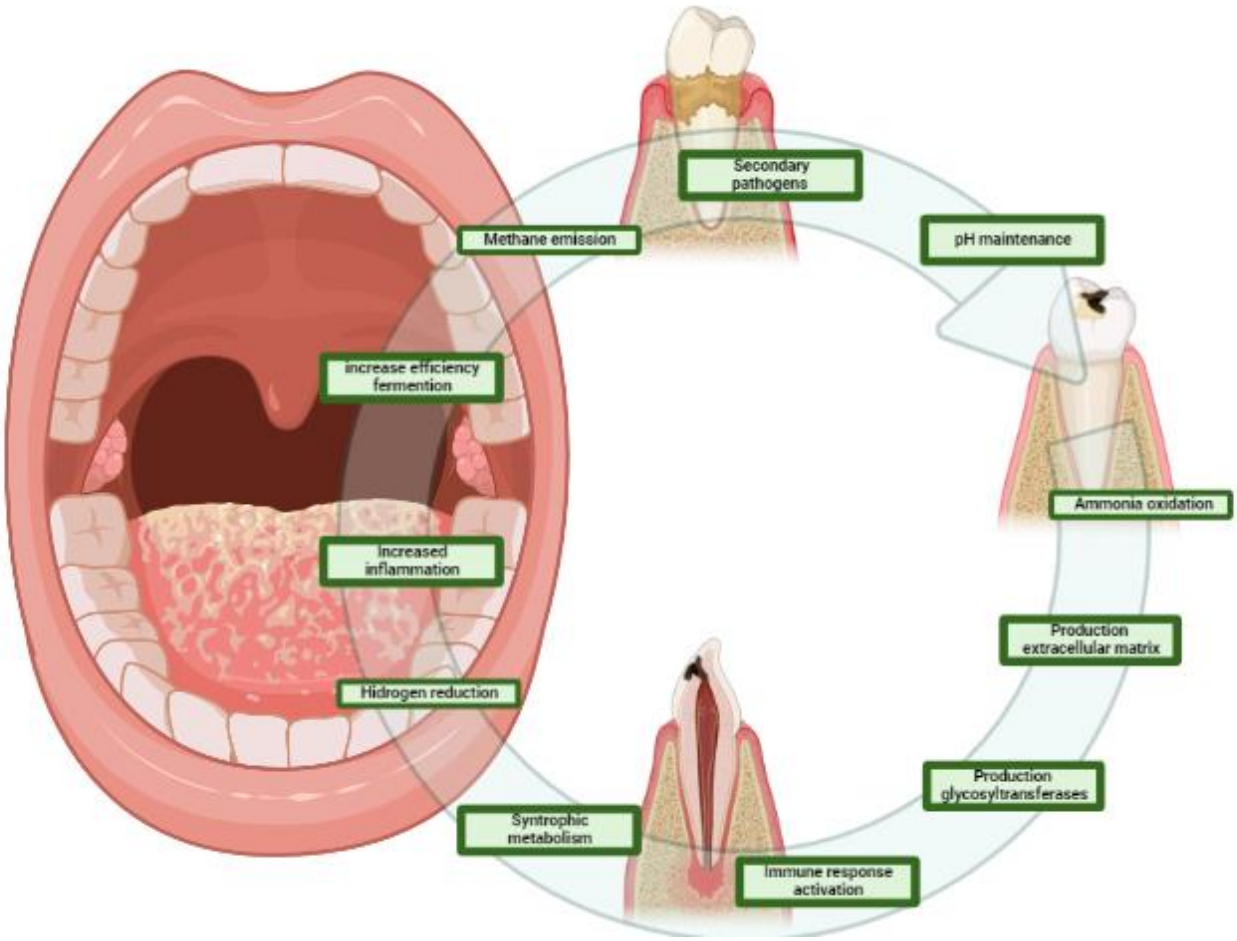

Fig. 1. Hypotheses of the role of archaea in the oral cavity.

2.4.1 Challenges in The Oral Archaeome Identification And Future Directions

The challenge of establishing a clear link between archaea and oral disease can be attributed, at least in part, to limitations in detecting, identifying, and isolating these microorganisms (23, 103-106). This limitation may also contribute to the relatively low

diversity of archaeal species documented in the oral microbiome. The hurdles associated with culturing and with studying the physiology of archaea present in the human body are evident when reviewing the history of research on these microorganisms. According to Sun et al. (107), there are five factors that make it difficult to cultivate archaea. These include: limited exchange of materials between different species during *in vitro* cultivation, leading to a reduced availability of metabolic substances produced by other organisms in the natural environment; interspecific competition; the inherently slow growth rate and low abundance of archaea, which can result in rapid bacterial overgrowth during laboratory cultivation; and suboptimal transport conditions or *in vitro* culture parameters that differ from the environmental conditions in which archaea thrive.

The exclusion of sequences with relative abundances lower than 1% is a common practice in studies using 16S rRNA NGS (Next-Generation Sequencing) to evaluate the human microbiome composition (108). This must also be considered to partly justify the non-detection of archaea in oral sites. Such practices can compromise the identification of the true diversity within the minority microbiota. Low-abundance sequences are likely to be artifacts or contamination rather than genuine microbial populations. To address this concern and avoid false positives, one potential solution is to analyze the prevalence of these sequences, considering the number of individuals who test positive for them, rather than solely focusing on their abundance.

Several other factors may contribute to the underrepresentation of archaea in studies focused on the oral microbiome. Firstly, the use of universal primers for 16S rRNA gene amplification, which may not effectively capture archaeal sequences due to their sensitivity to primer selection. Secondly, the DNA extraction methods currently employed, which have primarily been developed and optimized for bacterial cells, fail to adequately consider the unique characteristics of archaeal cell walls. Lastly, the databases used in these studies may lack comprehensive representation of archaeal sequences, further hindering their detection and identification (23). By acknowledging and addressing these factors, researchers can better elucidate the presence and role of archaea within the oral microbiome, ensuring a more accurate understanding of microbial diversity and function.

A better selection of DNA extraction protocols, primers, bioinformatic pipelines and use of appropriate databases can be also useful in the detection of archaeal cells on human mouth. The16 S rRNA gene has been a key tool for DNA sequence-based analysis of bacteria for many years. However, it is important to consider aspects related to the amplification of this gene during experimental planning and data analysis. This is because the choice of the template region can result in preferential amplification of certain sequences (109). For instance, the commonly used prokaryotic primer pair 515F/806R has been found to be inadequate for detecting archaeal sequences (110). For identifying archaea, the regions V1-V3 and V4-V7, as well as the grouping of OTUs (operational taxonomic units) at the species level at 0.03 and 0.02 distances, respectively, has been deemed to have better outcomes (111).

Nonetheless, perhaps the most important reason for misleading archaea from analysis is that not a single archaeal representative has been deemed to be a true pathogen. Although a direct link between archaeal species and diseases was not observed, the detection of archaeal species in infections strongly suggests their potential involvement in pathogenesis (9, 112). This theory is supported by the fact that archaea share some features with microorganisms known to be involved in disease, such as their ability to colonize and coexist with healthy microbiota at low levels of abundance, which tend to increase during pathological conditions (105, 113). Many questions regarding the oral archaeome acquisition, diversity, potential association with oral dysbiosis, and interactions with the bacteriome remain open. It is advisable for new studies to adhere to established scientific standards in order to reduce risk of bias and to enhance the certainty of evidence, ensuring the availability of higher quality clinical data particularly in the definition of health and disease to make better biological connections. Also, to increase the impact of the research, there is a need to promote research reproducibility, with better-quality study designs. Furthermore, collaboration between clinicians and microbiologists can be the key to improve the biological connections of microbiomes and diseases.

2.4.2 In Silico Coverage Analysis of Primers Employed in Previous Research

Amplification of target genes is the most widespread approach for the assessment of microorganisms' diversity, and it has been widely employed in studies

aiming to characterize archaea in the human body. Even though molecular techniques have improved over the years, primer choice remained as a critical step, since it determines how reliable sequence detection will be and, consequently, which conclusions about community composition and diversity will be drawn. The use of primers with nonspecific or restricted coverage has often impaired full assessment of microbial diversity, especially regarding the human archaeome (114). To evaluate which primers have been employed for archaeal detection in human samples and to what extent they cover currently known archaeal diversity, we conducted a comprehensive search in the PubMed database and thoroughly reviewed the reference lists of relevant studies to identify primers used for qPCR approaches for archaea detection in human body samples.

Our focus was specifically on primers used in qPCR assays due to the broad application of this technique in human archaeome studies over the years. Furthermore, protocol simplicity and reduced cost of this approach make it accessible to many microbiology laboratories, thus, information about qPCR primers could be valuable for future oral archaeome surveys. Whether these primers were employed for further sequencing analysis was also determined. Our comprehensive literature search included a total of 30 studies, yielding a collection of 44 primer pairs (Table 1 and Table 2). Among these, 28 primer pairs were found to be used only once. The predominant sample type examined in these studies was stool, with 16 studies focusing on this sample type. Subgingival plaque samples were the second most frequently analyzed, with 7 studies dedicated to their investigation. 16 S rRNA gene is the most investigated target, with 35 primers used for the detection of various regions of this gene (Table 1 and Fig. 2). To assess the specificity and coverage of the selected primer pairs, we employed the online tool TestPrime1.0 and performed in silico primer coverage analysis against the SILVA SSU r138.1 non-redundant database (104), enabling an evaluation of the performance of the primer pairs that have already been used to detected archaeal 16S rRNA gene sequences in human samples as a first step for selecting the most appropriate primer pair for specific applications. The coverages are accessible via taxonomy browser in the tool, facilitating the rapid identification of strengths and weaknesses associated with a specific primer pair. We analyzed the domain’s coverage proportion, with no mismatches and allowing one mismatch, and

for the primers targeting specific taxa, we also computed the coverage for those targets.

As previously mentioned, out of the 44 different primer pairs selected from the literature, 35 were specific to the 16S rRNA gene. Figure 2 summarizes the primers targeting each region of the 16S rRNA that have been used for qPCR studies. Except for V1 and V9, all other regions of this gene were screened. Additionally, 12 different primer pairs were utilized for pre-sequencing amplification, supporting both NGS and Sanger sequencing methods. Each of the methods used was successful in detecting archaea in human samples. However, it is essential to consider the potential presence of publication bias, as positive findings might have been selectively published, while negative results for archaea could have been left unreported.

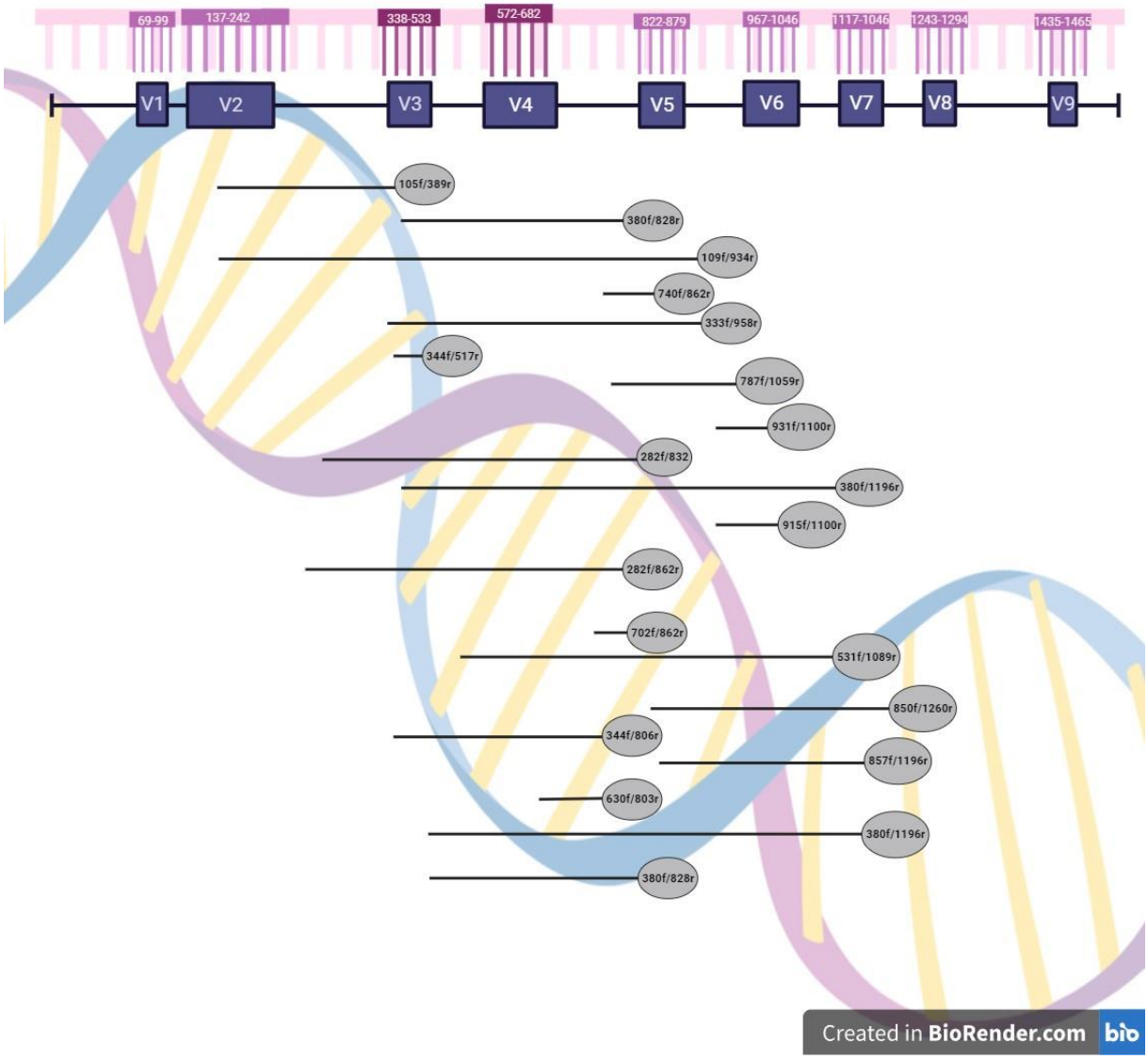

Figure 2. 16S rRNA primers that have been used to detect archaea by qPCR. Primer pairs which starting nucleotide positions were not specified were excluded from the figure.

The primer pair 740F/862R was the most frequently used in qPCR analyses, having been reported in seven studies (7, 73, 115-119). This primer pair was specifically designed for the *M. smithii* 16S rRNA gene (73), and the studies mostly analyzed stool samples, except for one that examined colostrum and milk. Despite being the most frequently used primer pair in our literature search on the human archaeome, our *in silico* primer coverage analysis yielded "no matches" for these primers. This outcome could be attributed to the search's inability to identify a match with only one species, the absence of the specific genome used as reference for the design of these primers in the database, or the discrepancy in stringency between the experimental conditions and the more rigorous in silico primer coverage analysis. Another set of primers, SDArch0333aS15f/SDArch0958aA19r, was employed in three studies, which examined samples from various human body sites such as dental plaque, tongue biofilm, endodontic samples, subgingival samples, stool, and amniotic fluid. Even though it presented only 17,1% of coverage for the whole domain *Archaea*, the intended taxonomic target in some studies (7, 99, 120), higher coverages were observed specifically for the genus *Methanobrevibacter* (82,8% with no mismatches and 94% with one mismatch allowed) (Table 1).

The primer pair Met630/Met803, originally designed for detecting 16S rRNA genes from methanogenic archaea in stool samples, demonstrated excellent domain coverage levels, with 95.8% match rate with one mismatch and 85.5% with no mismatches. Moreover, it also displayed favorable coverage for various archaea phyla, including *Euryarchaeota* (94.2%), *Thermoplasmatota* (94.9%), *Halobacteriota* (95%), and *Korarchaeota* (93.5%) (121). Among all the primers analyzed, Met630/Met803 exhibited the highest coverage. Since oral biofilm samples share similarities with stool samples in terms of DNA/RNA extraction methods, this primer pair could potentially be suitable for investigations in oral biofilm research.

A significant portion of the studies used primers directed to methanogenic archaea, especially those focused on gastrointestinal samples. As a result, the diversity of other archaeal groups was less explored. Obviously, this can influence the findings on predominance of methanogenic organisms in human samples and overlook the presence of other archaeal taxa in our microbiota.

Table 2. Primers targeting archaeal genes other than 16s rRNA described in studies involving human samples

. **Author**	**Year**	**Primer F**	**Primer R**	**Samples**	***Target***	***Coverage for the domain (1 mismatch) ****	***Coverage for the domain (0 mismatch) ****	***Coverage for specific targets (0 mismatch)***	***Used in Sequencing?***	***Used in qPCR?***
Koskinen	2017	344 a: ACGGGGYGCAGCAGGCGCGA	517 u: GWATTACCGCGGCKGCTG	Human skin	*Archaea*	*73.8*	*43*	*-*	*yes*	SYBR Green
Moissl-Eichinger	2017			Human skin					*yes*	SYBR Green
Probst	2013			Human skin					*yes*	SYBR Green
Koskinen	2017	519: CAGCCGCCGCGGTAATACC	AS2: AACAACTTCTCTCCGGCAC	Stool	*Methylotrophic; Methanogens*	*7.5*	*3.1*	*Methanomassiliicoccales (76%)*		SYBR Green
Koskinen	2017	740F: CCGGGTATCTAATCCGGTTC	862R: CTCCCAGGGTAGAGGTGAAA	Stool	*M. smithii*	*no matches found*	*no matches found*	*no matches found*	*yes*	Taqman probes
Bergstrom	2012			Stool					*yes*	SYBR Green
Drancourt	2021			Blood					*yes*	Taqman probes
Dridi	2009			Stool					*yes*	Taqman probes
Million	2012			Stool					*yes*	QuantiTect Probe PCR Kits
Miragoli	2017			Stool					*yes*	Taqman probes
Togo	2019			Colostrum and Milk					*yes*	QuantiTect Probe PCR Kits
Koskinen	**2017**	**850: GAGCACCACAACGCGU**	**1260: CTACGCATTCCAGCTTC**	**Stool**	***Methanogenes***	***14***	***8.6***	***Methanobacteria (82%)***		**SYBR Green**
Koskinen	**2017**	**915: AGGAATTGGCGGGGGAGCAC**	**1100A: TGGGTCTCGCTCGTTG**	**Stool**	***Methanobacteriales***	***48.9***	***18.8***	***Methanobacteriales (84.4%)***		**SYBR Green**
Patill	2012	931: AGG AAT TGG CGG GGG AGC A	m1100: BGG GTC TCG CTC GTT RCC	Stool	*Archaea*	*70.3*	*43*	*-*		Taqman probes
Koskinen	2017			Subgingival plaque						SYBR Green
Matarazzo	2011			Subgingival plaque						QuantiMix Easy SYG kit

Ramiro	2018			Subgingival plaque					SYBR Green
Koskinen	2017	A109F: ACKGCTCAGTAACACGT	A934R: GTGCTCCCCCGCCAATTCCT	Endodontic samples	*Archaea*	*68.2*	*37.4*	-	*yes*	SYBR Green
Vianna	2008; 2006			Endodontic samples; plaque					*yes*	SYBR Green
Brzezińska-Błaszczyk	2018			Endodontic samples	*Archaea*	*68.2*	*37.4*	-	*yes*	sqRT-PCR
Dridi	2009	AGGAGCGACAGCAGAATGAT	AGGAGCGACAGCAGAATGAT	Stool	*M. stadtmanae*	*0.2*	*0.2*	*Methanosphaera (24.5%)*	*yes*	Taqman probes
Mbakwa	2015	AGGAGCGACAGCAGAATGAT	CAGGACGCTTCACAGTACGA	Stool					*yes*	Taqman probes
Miragoli	2017			Stool					*yes*	Taqman probes
Pol	2017			Stool					*yes*	Taqman probes
Koskinen	2017	609:AGGTACTCCCAGGGTAGAGG	TCCCTCACCGTCAGAATCG	Stool	*M. smithii*	*3.3*	*0.3*	*Methanobrevibacter (6.2%)*		SYBR Green
Koskinen	**2017**	**ARC787F: ATTAG ATACC CSBGT AGTCC**	**ARC1059R: GCCAT GCACC WCCTC T**	**Stool**	***Archaea***	***92***	***72.9***	**-**		**Taqman probes**
Miragoli	**2017**			**Stool**				**-**		**Taqman probes**
Sagheddu	**2017**			**Stool**				**-**		**KAPA SYBR® FAST**
Koskinen	2017	Arch344: ACGGGGYGCAGCAGGCGCGA	Arch806: GGACTACCCGGGTATCTAAT	Stool	*Archaea*	*70.9*	*41.9*	-		SYBR Green
Koskinen	2017	AS1: CAGCAGTCGCGAAAACTTC	AS2: AACAACTTCTCTCCGGCAC	Stool	*Methanomassiliicoccales*	*4.9*	*1.8*	*Methanomassiliicoccales (71%)*	*yes*	SYBR Green
Koskinen	2017	B10-dir: GTTCGGATTGGGGGCTGTAA	B10-rev: CCCCAATAAGAAACATCCTC	Stool	*M. luminyensis*	*0*	*0*	*Methanomassiliicoccus (11.1%)*		Taqman probes
Mbakwa	2015	CCCGGTATCTAATCCGGTTC	CTCCCAGGGTAGAGGTGAAA	Stool	*M. smithii*	*no matches found*	*no matches found*	-		Taqman probes
Pol	2017			Stool				-		Taqman probes
Armougom	2011			Stool				-		Taqman probes
Gohler	2014; 2018	CGGTGAATAYGYCCCTGC	AAGGAGGTGATCCRGCCGCA	Tongue biofilm	*Archaea*	*0*	*0*	-		Taqman probes
Koskinen	2017	Cren771: ACGGTGAGGGATGAAAGCT	Cren957R: CGGCGTTGACTCCAATTG	Stool	*Crenarchaeota*	*22.4*	*4.5*	*Crenarchaeota (12.4%)*		SYBR Green

Vianna	2008; 2006	EuF: TCCTACGGGAGGCAGCAGT	EuR: GGACTACCAGGGTATCTAATCCTGTT	Endodontic samples; plaque	*Procarionts*	*77.5 for bacteria*	*68.4 for bacteria*	-	Sybr green
Miragoli	2017	fMbb1: CTC CGC AAT GTG AGA AAT CG	Arch r1386:GCG GTG TGT GCA AGG AGC	Stool	*Metanobrevibacter*	*7.6*	*0.6*	*Methanobrevibacter (6.8%)*	Taqman probes
Koskinen	2017	FP: CCGGGTATCTAATCCGGTTC	RP: CTCCCAGGGTAGAGGTGAAA	Stool	*M. smithii*	*no matches found*	*no matches found*	*no matches found*	SYBR Green
Drancourt	2017	ACCATAACyATCAGCAGCATTAT	AGTATTGGTGAAGGATTTaCTGT	Brain abscess	*M. smithii* and *M. oralis*	*no matches found*	*no matches found*	*no matches found*	Taqman probes
Koskinen	2017	Mbb-F1: CTCCGCAATGTGAGAAATCG	Arch-R1386: GCGGTGTGTGCAAGGAGC	Stool	*Methanobrevibacter*	*7.6*	*0.6*	*Methanobrevibacter (6.8%)*	SYBR Green
Sereme	2022	MBF: CGAACCGGATTAGATACCCG	MBR: CCCGCCAATTCCTTTAAGTT	Stool	Methanogenic archaea	*76.9*	*45.7*	*Euryarchaeota (76.5%) Halobacterota (67.7%)*	Taqman probes
Koskinen	**2017**	**MBT857f: CGWAGGGAAGCTGTTAAGT**	**MBT1196: TACCG TCGTC CACTC CTT**	**Stool**	***Methanobacteriales***	***10.2***	***9.3***	***Methanobacteriales (89.9%)***	**Taqman probes**
Lee	2011	MBT-NF: TCGCAAGACTGAAACTTAAAGGAA	MBT-NR: CGGCGTTGAATCCAATTAAAC	Stool	*Methanobacteriales.*	*24.8*	*9.4*	*Methanobacteriales (79.8%)*	Taqman probes
Koskinen	2017								Taqman probes
Koskinen	2017	MET-105: TGGGAAACTGGGGATAATACTG	MET-386:AATGAAAAGCCATCCCGTTAAG	Stool	*Methanobrevibacter*	*2.3*	*0.4*	*Methanobrevibacter (8.6%)*	SYBR Green
Mira-Pascual	2014								SYBR Green
Jalanka-Tuovinen	2011								SYBR Green
La-Ongkham	**2015**	**Met630: GGATTAGATACCCSGGTA**	**Met803: GTCACCGCTACACATG GAG**	**Stool**	**Methanogenic archaea**	***95.8***	***85.5***	***Euryarchaeota (94.2%) Thermoplasmatota (94.9%) Halobacterota (95%) Korarchaeota (93.5%)***	**SYBR Green**
Mira-Pascual	2014	MMB282F: ATCGRTACGGGTTGTGGG	MMB832R: CACCTAACGCRCATHGTTTAC	Stool	*Methanomicrobiales*	*4*	*3.2*	*Methanomicrobiales (74.6%)*	Taqman probes
Koskinen	2017								Taqman probes
Mira-Pascual	2014	Mnif-342: AACAGAAAACCCAGTGAAGAG	Mnif-363: ACGTAAAGGCACTGAAAAACC	Stool	*M. smithii*	*no matches found*	*no matches found*	*no matches found*	SYBR Green

Mira-Pascual	2014	Msc380: GAAAC CGYGA TAAGG GGA	MBT1196: TACCG TCGTC CACTC CTT	Stool	*Methanobacteriales*	*2.1*	*0*	*0*		Taqman probes
Mira-Pascual	2014	Msc380: GAAAC CGYGA TAAGG GGA	Msc828: TAGCG ARCAT CGTTT ACG	Stool	*Methanosarcinaceae*	*3.8*	*1.8*	*Methanosarcinaceae (56.8%)*		Taqman probes
Mira-Pascual	2014	MST702F: TAATCCTYGARGGACCACCA	MST862R: CCTACGGCACCRACMAC	Stool	*Methanosaetaceae*	*2.2*	*1.8*	*Methanosaetaceae (63.8%)*		Taqman probes
Koskinen	2017									Taqman probes
Koskinen	2017	SDArch0333aS15: TCCAGGCCCTACGGG	SDArch0958aA19: YCCGGCGTTGAMTCCAATT	Subgingival samples	*Archaea*	*52.3*	*17.1*	*Methanobrevibacter (82,8%)*	*yes*	SYBR Green
Lepp	2004			Dental plaque and Tongue biofilm					*yes*	SYBR Green, conventinal PCR
Jiang	2009			Endodontic samples					*yes*	RT-PCR
Togo	2019			Colostrum and Milk	*M. smithii*	*no matches found*	*no matches found*	*no matches found*	*yes*	QuantiTect Probe PCR Kits
Koskinen	2017			Stool; amniotic fluid	*Archaea*	*52.3*	*17.1*	*Methanobrevibacter (82,8%)*	*yes*	Taqman probes
Koskinen	2017	SDTher0531aS12: CGAGGAGGCTGC	SDTher1089aA16: TCAGATCCGGGGACCT	Subgingival samples	*Thermoplasmata*	*0*	*no matches found*	*no matches found*		SYBR Green
Koskinen	2017	Stadt_16S: AGGAGCGACAGCAGAATGAT	Stadt_16S: CAGGACGCTTCACAGTACGA	Stool	*M. stadtmanae*	*0.2*	*0.2*	*Methanosphaera (11.4%)*		Taqman probes

Despite the low overall domain coverage, the primer pair 850F/1260R, which targets *Methanobacteria* (82%), the primer pair 915F/1100A, targeting *Methanobacteriales* (84.4%), and the primer pair MBT857f/MBT1196, directed to *Methanobacteriales* (89.9%) (7), demonstrated favorable coverage for their specific 16s rRNA gene targets. These primers were previously employed for qPCR analysis of stool samples with SYBR Green, but we could not find documentation of their use in 16S rRNA gene sequencing. It is important to consider that primers targeting specific groups of archaea probably yielded low percentages for the whole domain, since the intended focus of each study was a specific subset of *Archaea*. In this context, caution should be taken when claiming that these primers are universal for the *Archaea* domain.

On the other hand, the primer pair ARC787F/ARC1059R presented 92% of coverage for the domain (1 mismatch). The use of this primer pair was reported in three papers using qPCR for stool samples. It is worth mentioning that we identified a low archaeal coverage for primers previously used for oral sample analysis. For instance, the primers targeting 16S rRNA that Göhler et al. (122) used to detect archaea in tongue biofilms (122) showed 0% coverage in our analysis.

It is important to mention that assessing primer coverage through *in silico* methods presents several noteworthy limitations (114). First, when assessing primers designed for the end of the 16S rRNA gene, it is important to consider that there is limited availability of sequences in these regions, resulting in reduced sequence diversity near the termini. Thus, results for primers targeting final regions of the 16S rRNA gene must be interpreted with caution. Secondly, considering mismatches in primer binding may lead to an overestimation of coverage since all primer nucleotide positions are treated uniformly and mismatches located at the 3’ region of the primer are known to greatly affect priming efficiency when compared to 5’ mismatches. Thirdly, the presence of primers in sequences deposited in public databases is a common issue and an *in silico* evaluation bias may arise if these primers replace existing genetic variations in the sequences, which could result in overestimations of primer coverage. The choice of the database used for in silico primer coverage analysis

can also impact the results, but minor effects in coverage results have been detected in previous comparisons (123, 124)

Primers designed for the 16S rRNA are more likely to have a universal application, especially those designed for the entire domain or for a broader taxonomic range. In contrast, other primers are frequently employed for the specific detection of particular taxa (Table 2). In our search, five primer pairs targeted the *mcrA* gene, three were directed to the *rpoB* gene, two were for the *cnp60* gene, and one was for the *amoA* gene. Due to its unique function in methanogenesis, the *mcrA* gene serves solely to detect methanogens and, even though cnp60 and *rpoB* genes are not exclusive to methanogens, they have also been frequently employed for the detection of this archaeal group (7, 115, 116, 125-128). This may also contribute to the predominance of these organisms in the archaeome analysis reports.

Due to its importance as a functional marker gene in ammonia oxidizing organisms (129), primers targeting the archaeal *amoA* gene could be useful to identify members of the *Thaumarchaeota* phylum in oral samples. Although strains of *Thaumarchaeota* have already been identified as common constituents of the human skin microbriota (81), their presence in different oral sites remain greatly unknown, and further studies aiming the specific detection of this archaeal group could help elucidate its role in the oral archaeome.

Challenges related to primer selection have been observed to significantly impact the accurate identification of archaea in human samples(7). Additionally, utilizing “universal” primers or primers specific for methanogenic groups could potentially compromise the accurate identification of a broader range of archaeal diversity. The repeated use of these primers has made it challenging to trace back to the original articles where they were first employed. Despite their longstanding usage, many of these primers were developed before the advances in sequencing techniques and databases. As a result, our *in silico* analysis underscores the need for designing new primers that could better encompass the currently known archaeal diversity. Depending on the specific objectives of each study and the samples being examined, it becomes crucial to design primers that focus on the particular group of interest, or even update the so-called "universal" primers accordingly. For new oral archaeome

Table 2. Primers targeting archaeal genes other than 16s rRNA described in studies involving human samples.

Author	Year	Reason for using the primer	Primer F	Primer R	Samples	*Target*	*Used in Sequencing*
cnp60 gene							
Togo	2019	*Previous of the qPCR methanogenic archaea were cultured from similar samples*	2F: GCTGGTGTAATCGAACCTAAACG	2R: CACCCATACCCGGATCCATA	Colostrum and Milk	*M . oralis*	*yes*
Belkacemi	2018	Specific quantitative amplification of a *M. oralis* strain	M. oralis-cnp602: GCTGGTGTAATCGAACCTAAACG	M. oralis-cnp602: CACCCATACCCGGATCCATA-3′)	Subgingival plaque	*M. oralis*	*yes*
***rpoB* gene**							
Drancourt	2021	*Previous of the qPCR methanogenic archaea were cultured from similar samples*	AAGGGATTTGCACCCAACAC	GACCACAGTTAGGACCCTCTGG	blood	*M. smithii*	*yes*
Dridi	2009	*mcrA* gene-derived primers failed to detect *M. stadtmanae* in previous studies. Therefore, authors decided using the rpoB gene.	AAGGGATTTGCACCCAACAC	GACCACAGTTAGGACCCTCTGG	stool	*M. smithii*	*yes*
Dridi	2009		TGCTTGGTATTTGTGCTGGA	TCCAAGAGCCTGTTTTGTCA	stool	*M. stadtmanae*	*yes*
amoA gene							
Probst	2013	Testing the metabolic potential for ammonia oxidation of the skin-associated archaea	amoA104F-1d (GCA GGA GAC TAC ATM TTC TA)	amoA616R (GCC ATC CAT CTG TAT GTC CA)	Human skin	*Archaea*	*yes*
***mcrA* gene**							
Vianna	2008 and 2006	*The analysis based on the mcrA gene, specific to methanogens, was done because the detection of this gene type raises the question whether it actually corresponds to an authentic methanogenic species*	LuF: GGTGGTGTMGGATTCACACART AYGCWACAGC	LuR: TTCATTGCRTAGTTWGGRTAGTT	endodontic sample, plaque	*Archaea*	*yes*
Miragoli	2017	*For the detection of hydrogenotrophic groups, primers targeting key functional genes, namely the methyl-coenzyme M reductase α-subunit (mcrA), was used to quantify methanogens*	LuF: GGTGGTGTMGGATTCACACARTAYGCWAC AGC	LuR: GGTGGTGTMGGATTCACACARTAYGCWACA GC	stool	*Methanogens*	*yes*
Belkacemi	2018	*To broad-spectrum screening of the methanogen DNA was used the mcrA gene*	M. massiliensis-mcrA: ACTCACTTTGGCGGATCTCA	M. massiliensis-mcrA: GTACATGGACAAGTACCATGC	Subgingival plaque	*M. massiliense*	*yes*
Teigen	2021	*The gene was used for specific detections of the methanogenic archaea*	mlas-mod-F: GGYGGTGTMGGDTTCACMCARTA	mcrA-rev-R: CGTTCATBGCGTAGTTVGGRTAGT	stool	*Euryarchaeota*	
Sagheddu,	2017	*The gene was used for specific detections of the methanogenic archaea*	qmcrA: TTCGGTGGATCDCARAGRGC	qmcrA: GBARGTCGWAWCCGTAGAATCC	stool	*Archaea*	

studies, it appears essential to design primers targeting not only methanogenic archaea but also nanoarchaea, thaumarchaeota as well as other archaeal groups to gain a comprehensive understanding of the archaeal oral community. The employment of approaches that do not depend on primer amplification and, thus, avoid its potential biases could also help to expand the knowledge about the human oral archaeome.

2.5 CONCLUSION

This comprehensive review investigated the possible reasons behind the limited interest on the oral archaeome. The challenges in studying *Archaea*, such as their low abundance, difficulty in culture and detection through molecular methods typically targeting bacteria, have hindered their comprehensive exploration. The *in silico* primer coverage analysis conducted during this review revealed that several primer pairs used in previous researches aiming the detection of *Archaea* in human samples may no longer be suitable, since they demonstrated low coverage for the domain, or specific groups. Considering the detection of archaea in oral samples, the development of novel strategies as well as the design of new primers pairs directed to other groups, besides methanogenic archaea, are crucial to better understand the oral microbial community. By doing so, researchers can pave the way for further advancements in the field of oral archaeome research.

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3 CAPÍTULO III: META-ANALYSES ON THE PERIODONTAL ARCHAEOME

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3.1 ABSTRACT

Recently, we have published a scoping review on the oral archaeome, showing that these microorganisms inhabit various oral niches, including periodontal sites. In order to reinforce the importance of this group and alert the scientific community about the importance of inter-domain relationships in oral dysbiosis, we have performed meta-analyses evaluating the prevalence of archaea in periodontal diseases (PROSPERO protocol: CRD42020213109). A systematic search in the literature was conducted in several databases and in grey literature, retrieving 30 reports on periodontal archaeome, published from 1980-2020. The methodological quality of included studies and the certainty of evidence were evaluated by using validated tools. Most studies focused on the detection of methanogens, revealing that the diversity of the periodontal archaeome is currently underestimated. Two meta-analyses concluded that individuals with periodontitis are prone to have archaeal-positive subgingival biofilms when compared to periodontally healthy individuals (OR 6.68, 95% CI 4.74-9.41 for 16S rRNA gene analysis and OR 9.42, 95% CI 2.54-34.91 for *mcrA* gene analysis). Despite the archaeal enrichment in sites with periodontitis, less than half of the individuals with periodontitis tested positive for archaeal DNA (general estimative of 46%; 95% CI 36%-56%). Conventional treatment for periodontitis reduced the archaeal population, but systemic antibiotics used as adjunctive therapy did not increase its effectiveness. Hence, it could conceivably be hypothesised that archaea are secondary colonizers of areas with dysbiosis, probably flourishing in the inflammatory environment. Due to their lower prevalence, archaeal cells are probably underestimated by the current detection protocols. It may also be speculated that archaea do not have a single central role in the infection, with bacterial cells directly involved in that role. New studies are necessary, with different methodological approaches, to explore the underestimated diversity of the oral archaeome.

Keywords: Oral Microbiology; *Archaea* domain; Periodontal diseases

3.2 INTRODUCTION

Over the past few years, a putative contribution of *Archaea* in periodontal disorders has been suggested, even though these microorganisms were shown to represent a small fraction of the total prokaryotic load in oral samples (1). The proposal of the *Archaea* domain is relatively recent and was based on the unique phylogenetic, structural, physiological, and molecular features of these prokaryotes, which clearly distinguish them from bacteria (2).

Although initially associated to inhospitable habitats, since the first recognized *Archaea* members mostly thrived in extremely hot, salty, or acidic conditions (2, 3), the increased use of culture-independent methods, particularly the detection of markers genes such as the 16S rDNA directly from environmental and clinical samples, revealed the ubiquity of these organisms (4). The presence of *Archaea* in the human microbiome has been increasingly reported, with members already detected in gastrointestinal and respiratory tracts, skin, and oral cavity samples (5-8).

Archaea were first identified in the human oral cavity in 1987, through the enrichment of methanogens from subgingival plaque samples (9). Since then, the knowledge about the oral archaeome has been significantly expanded. A scoping review recently published by our group revealed that *Archaea* members have been described on various oral niches, including saliva, different portions of the tongue, periodontal and endodontic sites (10). Furthermore, the putative involvement of archaea in oral diseases, especially periodontitis, is being increasingly pointed out, with studies suggesting that, although these organisms are part of the indigenous oral microbiota, their abundance rises along with the increase of periodontitis severity (10-13)

The contribution of archaea to periodontal dysbiosis may be related to their ability to provide conditions for the growth of pathogenic bacteria in subgingival sites (14). Methanogens are the most representative archaeal group in periodontal samples, where they may play a central role in the removal of hydrogen excess from different electron donors, such as methanol, methylamine, acetate, ethanol or formate. Methanogens metabolize the H_2 generated during the fermentation of carbohydrates in methane gas, raising the local pH and rendering the microenvironment favourable

to the anaerobic subgingival bacterial population, including opportunistic pathogens. This kind of event represents a classical syntrophic behaviour, with cross-feeding among prokaryotic species within the anaerobic microniches in a mature biofilm (15). Thus, it can be hypothesized that the increase in methanogenic activity leads to a reduction in the availability/concentration of acidic products, acting as "acid sinks", and consequently help the homeostasis maintenance for non-aciduric organisms growth, also allowing the enrichment of the proteolytic organisms (16).

Due to this environmental change mediated by inter-domain interactions, some methanogenic species have been considered putative keystone pathogens of periodontal diseases (5, 17). However, even though the role of methanogens in oral health is becoming increasingly clear, recent molecular findings suggest that the diversity of archaea inhabiting human oral sites may be currently underestimated. DNA sequences affiliated to the non-methanogenic class *Thermoplasmata* have been detected in patients with different stages of periodontitis (11, 18, 19). Moreover, metatranscriptomic analyses performed in periodontal pocket samples from individuals with chronic periodontitis and periodontally healthy subjects revealed that only five out of ten archaeal reads were classified in methanogenic groups, with transcripts associated to non-methanogenic *Euryarchaeota*, and ammonia-oxidizing *Thaumarchaeota* taxa (20). Recently, 16S rDNA sequences of ammonia-oxidizing thaumarchaeal groups have also been identified in both supragingival and carious biofilms (21).

Understanding the context of archaea within oral biofilms and how their presence could modulate oral conditions is imperative. Although oral archaeome is an increasingly discussed research topic, to date no consensus regarding their role in oral diseases has been reached (Belmok et al., 2020). To shed some light on the periodontal archaeome comprehension, we performed meta-analyses on the prevalence of these organisms in periodontitis sites comparing it with healthy sites, as well as after periodontitis treatments. Some hypotheses on the role of archaeal species that comprise the periodontal archaeome were also discussed.

3.3 METHODS

3.3.1 Studies eligibility and search strategy

Meta-analyses were performed to answer two questions: 1) what is the prevalence of archaea-positive subjects at subgingival periodontal sites in health and disease? 2) Do periodontal treatments reduce the prevalence of archaea in periodontal sites?

Details on the search strategy and studies selection were described elsewhere (Belmok et al., 2020). Briefly, a systematic search was conducted in five databases (MEDLINE via PubMed, Cochrane Library, Scopus, LILACS, and Livivo), as well as the grey literature (Google Scholar, OpenGrey) and hand search of included studies references lists. General controlled vocabulary (MeSH Terms) and keywords (Archaea AND Cavity oral AND Periodontitis) were chosen and the searches had no language, year or publication type restriction. Here, the search strategy was updated to gather any new study published in 2020. New databases (Embase, Proquest) were included at the updated search.

Eligibility criteria were also described by Belmok *et al*. (10). Only observational/clinical studies where the target population consisted of humans of any age who were donors of periodontal samples (biofilms, crevicular fluid) from oral cavity were included. Studies could evaluate archaea using methanogenic cultures, methods of PCR amplification and sequencing, DNA-DNA hybridization, next-generation sequencing, etc. Either animals, *in vitro*, or not original studies were excluded. Two reviewers independently screened the eligibility in two steps (1: reading titles and abstract; and 2: reading the full-text). A subsample of identified studies on periodontal sites was used to these meta-analyses. The full screening of the articles was revised in the updated search.

3.3.2 Quality assessment of individual studies

Two reviewers independently assessed the methodological quality of individual studies, using validated tools according to the type of study: the revised Cochrane tool

for randomised controlled trials (RoB2) (22) and the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Analytical Cross-Sectional Studies (23).

For JBI, due to the design of included articles, besides all eight questions are considered important, four of them were considered critical domains to this systematic review (criteria 1-4). Criteria related to the outcome were considered non-critical (criteria numbers 5-8). At least one "no" and one "unclear" or two “unclear” in critical domains, or two "unclear" and one or more "no" in non-critical domains represented low methodological quality. Decision on critical and non-critical domains and classification system was discussed with research team before the application of the instrument, as described at JBI Reviewer’s Manual (23).

The risk of bias of included randomized clinical trials was assessed through RoB2 tool independently by two calibrated authors, that scored each item as "yes", "probably yes", "probably no", "no" and "no information". A third reviewer solved disagreements, and overall risk of bias was calculated using the RoB2 tool algorithm.

3.3.3 Data extraction and qualitative analysis

Data from the subsample of studies including subgingival samples was orderly in an Excel file to analyse the periodontal archaeome. The following information was collected: author, year; country; sample size; characteristics of periodontal sites (index used for periodontal diseases diagnosis); type of sample collection (paper point, manual instrument); DNA extraction method; methods of archaea detection; sets of primers used; the proportion of individuals with archaea detection in each group; and the relative abundance of archaea in each group.

A combination of quantitative and qualitative approaches was used in the data analysis. A narrative synthesis of the findings regarding the molecular techniques and the clinical sampling and periodontal diagnosis was performed, as well as a qualitative synthesis was performed.

3.3.4 Meta-analyses and certainty of evidence

Continuous variables were compared through the DerSimonian & Laird random-effects meta-analytic model and presented as mean differences and 95% confidence interval.

Dichotomous variables (prevalence of archaea comparison between periodontal health and periodontitis) were compared through Peto Odds Ratio, with fixed effect model and 95% confidence interval. Peto Odds Ratio was chosen due to prevalence of archaea in healthy individuals was rare and some studies returned zero event.(24) Archaea global prevalence was meta-analysed through Restricted Maximum Likelihood model for Raw Proportions with 95% CI (jamovi software version 1.6 retrieved from https://www.jamovi.org/). Heterogeneity between studies was estimated by Cochran's Q test and the inconsistency by I^2 statistic. Study characteristics considered as potential sources of heterogeneity were analysed through sensitivity analysis (removing on study at a time – one-by-one approach, and removing small sample studies) and subgroup analysis regarding the type of primers used to archaea detection (meta-analysis comparing periodontally diseased and healthy individuals). Publication bias was evaluated through funnel plot analysis. The certainty of evidence was evaluated by using GRADE (Grading of Recommendations, Assessment, Development and Evaluation) approach, through the analysis of risk of bias, inconsistency, imprecision, indirectness and publication bias (25).

3.4 RESULTS

3.4.1 Narrative synthesis and the quality assessment of individual studies

The inclusion of other databases revealed three new studies, resulting in a total of 30 studies that were used in the meta-analyses of the periodontal archaeome (28 cross-sectional; 2 randomized clinical trials). Those studies comprised 1312 individuals with periodontitis, and 441 periodontally healthy individuals sampled for archaeal detection.

Table 1 shows the results of the quality assessment of individual studies, type and method of sample collection, and DNA extraction method of the selected reports. Low methodological approaches were detected in seven studies. However, eight were classified with a high quality and fifteen with a moderate quality.

In most studies, samples of subgingival biofilms were collected using sterile curettes, while in others the crevicular fluid was collected using endodontic paper points, corresponding to pooled or specific sites samples. Unquestionably, this

Table 1. The quality of individual study, sampling, and DNA extraction method of the periodontal archaeome studies.

Author, year	Quality assessment of individual studies	Type of sample (sample collection method)	DNA extraction method
Aleksandrowicz, 2020	+	Subgingival biofilm samples (curettes)	Genomic Mini kit (A&A Biotechnology)
Ashok, 2013	+	Subgingival biofilm samples: periodontitis (curette); healthy controls = gingival crevices.	Chemical lysis with Tween 20, Nonidet and proteinase K protocol.
Belay, 1988	-	Subgingival plaque (curette).	**NA**
Bringuier, 2013	+	Subgingival plaque from 3- to 12-mm periodontal pockets.	FastPrep-24 incubation with acid-washed beads, followed by extraction with Qiagen EZ1 DNA tissue kit (Qiagen, France).
Brusa, 1987	+	Pool of subgingival plaque from gingival crevice (curettes).	**NA**
Brusa, 1993	-	Dental biofilm pools from pockets 3 to 7 mm deep, one in each quadrant (curettes).	
Dabdoub, 2016	+	Healthy subjects = pools from 15 mesial sites on teeth (paper-points). Periodontitis = pools of subgingival plaque from four nonadjacent proximal sites were collected using 15 paper points.	Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA, USA)
Deng, 2017	-	Several sites (two paper points per site).	According to Szafranski (2015).
Fermiano, 2011	++	Periodontitis = subgingival biofilm from 9 interproximal sites (curette); Healthy = 9 sites (curette).	Manual extraction.
Ferrari, 1994	-	Subgingival samples (curettes), according to Socransky et al.	**NA**
Göhler, 2014	++	Tongue biofilm from the middle third of the tongue dorsum with a sterile spatula.	MagNA Pure LC platform.
Göhler, 2018	++	Tongue biofilm from the middle third of the tongue dorsum with a sterile spatula; Subgingival plaque from the mesiobuccal pocket of the most distally located, clinically examined, upper tooth in the periodontally examined quadrants (paper points were inserted until the pocket base for 10 seconds).	
Horz, 2012	-	Pool of subgingival plaque from the four deepest periodontal pockets (paper points).	QIAamp DNA Mini kit ("tissue protocol", Qiagen, Germany).
Horz, 2015	-	According to Horz (2012).	
Huynh, 2015a	+	**NA**	FastPrep-24 incubation with acid-washed beads, followed by extraction with Qiagen EZ1 DNA tissue kit (Qiagen, France).
Huynh, 2015b	+	Subgingival dental plaque = all periodontal pockets (curette).	**NA**
Huynh, 2017	++	Subgingival dental plaque = all periodontal pockets (curette).	
Kulik, 2001	+	Pool of plaque samples.	Phenol-chlorophorm.
Lepp, 2004	+	Subgingival plaque samples from 6–12 periodontal pockets (curettes).	

Li, 2009	+	Subgingival samples from periodontal pockets with a probing depth >4 mm (curettes).	Genomic DNA Extraction Kit (Tiangen, Beijing, China).
Li, 2014	+	Subgingival plaque sample from the bottom of the deepest pocket (curette).	
Lira, 2013	++; **	Subgingival plaque (curettes).	
Matarazzo, 2011	+	Subgingival samples (curette), after removing supragingival biofilm.	Qiamp DNA minikit (Qiagen, Germany).
Ramiro, 2018	++; **	Subgingival plaque (curettes) collected after supragingival plaque removal -- baseline and at 6 months post-SRP from 6 non-contiguous interproximal sites per subject.	Masterpure RNA & DNA purification kit (Epicentre).
Robichaux, 2003a	-	Subgingival plaque from interproximal sites with pocket depths of 1-4 mm, after a removal of all supragingival plaque and isolation with cotton wool (curette).	**NA**
Robichaux, 2003b	-	Tissue samples (curette).	**NA**
Sogodogo, 2019	+	Subgingival plaque samples (curettes). Swabs from the abscesses.	Tissue DNA Kit.
Vianna, 2008	++	Pools of subgingival plaque samples from the four deepest periodontal pockets (paper points). Healthy subjects = pools of plaque from vestibular sulcus of first molars from all quadrants (paper points).	Qiamp DNA Mini kit (with modifications: addition of zirconia-silica beads and FastPrep before proteinase K).
Vianna, 2009	+	Pools of subgingival plaque from the four deepest periodontal pockets (paper points).	Qiamp DNA minikit (Qiagen, Germany).
Yamabe, 2008	+	Plaque samples from the periodontal pockets (paper points).	InstaGene Matrix (Bio-Rad).

(-) low quality of individual study; (+) moderate quality; **(++)**: high quality; **(++)**: high quality. (**) RCT studies analyzed with RoB2

approach may not represent the actual microbial content, as this sampling technique collects mainly the fluids surrounding the biofilm, while scraping the subgingival biofilm with a curette displaces the sticky biofilm from both, the periodontal pocket and the tooth. Since several microbial species, mostly the anaerobic ones, grow inside the biofilm, the paper point sampling method will retrieve preferentially the organisms present in the biofilm surface, leaving behind important members of the subgingival microbiome.

Various DNA extraction kits and protocols were employed in the different studies. The use of commercial kits is considered a faster and easier alternative when high quality nucleic acids are desired, especially when samples containing a low number of organisms are processed (26). It is worth mentioning that this step may hamper the analyses of archaeal DNA (27, 28), since the current DNA extraction protocols have been established and optimized for bacterial cells, and do not consider the peculiarities of the archaeal cell wall (29). Therefore, a more realistic view of the

diversity and quantity of archaea associated with the human microbiome would greatly benefit from improvements and standardizations of archaeal nucleic acids extraction methods.

In this review, only one study (30) adopted the current classification established by the American Academy of Periodontology and the European Federation of Periodontology in 2018, which proposes a multi-dimensional periodontitis staging and grading system (31). It is important to point out that most of the included studies were performed from 1999 to 2018, and adopted the classification proposed by Armitage (32) that assessed, at the time, two different conditions: chronic (11, 14, 15, 17-20, 33-43) and aggressive periodontitis (16, 36, 43, 44). New insights about these conditions have proposed to be part of the same disease group. This is justified, among other factors, by the common end result presented from a pathophysiologic point of view and the similar metabolic end-products stemming from microbial complex (45). On this basis, this review coupled those results in the meta-analyses regardless the periodontitis classification (shown below). Besides, another study followed the classification proposed by the World Workshop in Clinical Periodontics in 1989 (AAP, 1989) and classified the participants as juvenile, adult, rapidly progressing and refractory periodontitis (46), conditions which were not differentiated in this study as well. Lastly, a glossary from American Dental Association (AAP, 1977) was referred as the criteria for diagnosis in three studies (47-49) (**Table 2**).

The first studies describing the detection of methanogenic archaea in periodontal sites employed culture-based methods. Afterwards, culture-independent studies based on PCR employed several pairs of primers directed to the archaeal 16S rRNA genes, as well as primers targeting other specific housekeeping genes, such as *mcrA* (Methyl-Coenzyme M reductase, found in methanogens) and *cnp60* (heat shock chaperone 60) from *Methanobrevibacter oralis* have become more frequent. Recurrently, more than one target gene was used to assess the archaeal diversity in the same sample. Also, other approaches, such as fluorescence in situ hybridization (FISH), real-time quantitative PCR (RT-qPCR), and less frequently, metagenomics and metatranscriptomics, were used to estimate the proportion of archaeal-positive subgingival biofilms from individuals with and without periodontitis (Table 3).

Table 2. Periodontal diseases classification across the selected studies (n=30).

Author, year	Periodontitis classification
Aleksandrowicz, 2020	New Classification of the American Academy of Periodontology.
Huynh, 2015a; Lira, 2013; Ramiro, 2018	American Academy of Periodontology.
Ashok, 2013	Healthy sites: sulcus (PD ≤ 3 mm), shallow pocket (4 mm ≤ PD ≤ 5 mm) and deep pocket (PD ≥ 6 mm).
Belay, 1988; Robichaux, 2003a; Robichaux, 2003b	According to the criteria of the American Dental Association.
Bringuier, 2013	Chandra RV. 2007, using BOP, PD, plaque index, and radiographs.
Brusa, 1987	According to Socransky et al. (1963).
Dabdoub, 2016	Attachment loss ≥ 5, PD ≥ 5, gingival index > 1 in 30% or more sites.
Deng, 2017	According to Szafranski et al. (2015).
Fermiano, 2011	PCrG Group: have at least 20 teeth, excluding third molars, 30% of the sites with PS and NCI ≥ 5mm and age ≥ 30 years PAgG Group: have at least 20 teeth, excluding third molars, have at least 6 teeth with at least 1 interproximal site presenting non-contiguous PD and NCI ≥ 5mm, located in the region of incisors and molars and another 6 teeth with the same clinical characteristics located in other groups of teeth and age <30 years SP Group: have at least 20 teeth, excluding third molars; not presenting sites with OS and / or NCI ≥ 4mm; no clinical signs of generalized gingivitis. Do not present more than 10% of the sites with BOP and age ≥ 18 years
Göhler, 2014	Mean clinical attachment levels and mean probing depths ranged within the highest quartile calculated separately within sex and 5-year-age categories.
Göhler, 2018	PD and clinical attachment levels = four sites per tooth (disto/mid/mesiobuccal and midlingual) according to the half-mouth method, alternating on the left or right side, excluding third molars.
Horz, 2012	Different stages of severity of chronic periodontitis
Horz, 2015	PD and clinical attachment level recorded at six sites per tooth.
Huynh, 2015b	According to Chandra RV et al. (2007)
Huynh, 2017	Generalized dental calculus, generalized BOP and pockets with a depth of 7 mm in tooth 38, 6 mm in teeth 16 and 27 and 5 mm in teeth 16, 15, 13, 12, 25, 26, 38, 37, 44 and 47. Radiography showed bone loss along the apex of 16 and up to the third center of 15 and 13–27.
Kulik, 2001	Followed the classification proposed by the World Workshop in Clinical Periodontics in 1989 (AAP, 1989) and classified the participants as juvenile, adult, rapidly progressing and refractory periodontitis.
Lepp, 2004	Clinical attachment loss to the nearest millimeter at the mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual sites around each tooth.
Li, 2009	Based on radiographic evidence, with an absence of periapical periodontitis and other oral soft tissue diseases.
Li, 2014	Presence of BOP, suppuration and visible plaques, PD, and clinical attachment loss.
Matarazzo, 2011	According to Faveri et al. (2011) = presence to disc-shaped bone defects of> 3 mm, a PD of >5 mm and an inflamed mucosa showing BOP and / or suppuration.
Sogodogo, 2019	According to Armitage et al.
Vianna, 2008; Vianna, 2009; Yamabe, 2008	Healthy sites= PD<3 mm and no BOP; Periodontitis= PD≥4mm.
Brusa, 1993; Ferrari, 1994	**NA**

PD = probing depth; NCI= clinical insertion level; BOP= Bleeding on probing.

Table 3. Sample size, methods of archaea detection, and results of the selected studies (n=30).

Author, year	N periodontal diseases group / type of periodontal disease	N control group	Methods of archaea detection	Proportion of individuals with archaea periodontal diseases group / or Relative Abundance (RA)	Proportion of individuals with archaea control group / or Relative Abundance (RA)
Aleksandrowicz, 2020	61 = periodontitis	NA	Sequencing of the 16S rRNA gene.	Mild periodontitis = 53%; Moderate/advanced periodontitis = 64%.	None.
Ashok, 2013	34 = chronic periodontitis	16	PCR for 16S rRNA gene.	Chronic periodontitis = 29.41%.	12.50% (2/12).
Belay, 1988	36= some degree of periodontal disease	**NA**	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis, transmission electron microscopy and antigenic fingerprinting.	Methanogenic = 9 patients; Moderate = 26%; advanced-periodontitis = 76%; Early periodontitis (7%).	7%.
Bringuier, 2013	22 = periodontitis	10	RT-qPCR for *cnp60* gene with *M. oralis*-specific and probe.	*M. oralis* = 54% individuals, *M. oralis*-negative = 100% individuals with a clinical score of <20; and positive = 63% patients with a clinical score of >20.	*M. oralis* = 30%.
Brusa, 1987	10 = periodontitis	**NA**	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis; fluorescence microscopy.	Methane production and observation of methanogens in 3 of the 10 cultures.	**NA**
Brusa, 1993	20 = periodontitis	**NA**	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis; fluorescence microscopy; methanogenic archaea counts.	45% individuals.	**NA**
Dabdoub, 2016	25 = generalized moderate to severe chronic periodontitis	25	Illumina MiSeq.	RA ≤0.003	**NA**

Deng, 2017	4 = chronic periodontitis	10	Metatranscriptome.	RA 0.11% = periodontitis; Transcripts of archaea = 0.22% of total putative mRNA reads in the metatranscriptome. The 10 most abundant archaea accounted for more than 72% of archaea reads on average. *Methanosarcina vacuolata* was the most abundant archaeal species in periodontitis for 21.5% of total archaeal sequences.	RA = 0.27%; *M. vacuolata* was the most abundant archaeal species = 62.5% of total archaeal sequences.
Fermiano, 2011	15 = generalized chronic periodontitis (GCrP); 15 = generalized aggressive periodontitis (GAgP)	15	PCR for 16S rRNA gene with euryarchaeal primers.	SP Group: 12 (80%).	PCrG Group: 11 (73.3%); PAgG Group: 9 (60%).
Ferrari, 1994	**NA**	10	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis; fluorescence microscopy; physiological tests and DNA dot plot hybridization.	**NA**	Methane production = 40%.
Göhler, 2014	88 = periodontitis	88	RT-qPCR for 16S rRNA.	23 (26.1%).	17 (19.3%).
Göhler, 2018	237 samples	**NA**	RT-qPCR for 16S rRNA.	26 (65.0%) / RA: 2.63%.	**NA**
Horz, 2012	30 = different stages of severity of chronic periodontitis	**NA**	PCR for *mcrA* gene with two primer sets for all methanogens; three Thermoplamatales-clade; PCR for 16S rRNA gene with three Thermoplasmatales-clade; RT-qPCR for *mcrA* gene.	16S rRNA and MM_Mx3Fw_/MM_Mx3_Rv = 3/30 samples. RT-qPCR: RA 0.5% of Thermoplamatales; 2/3 samples with *M. oris*.	**NA**
Horz, 2015	125 = various stages of chronic periodontitis	25	PCR pre-amplification for 16S rRNA gene with universal archaeal primers; RT-qPCR for *M. oralis*; sequencing.	56 (45%) positive for methanogens; Methanogens RA = 5.1%.	None.
Huynh, 2015a	7= chronic periodontitis	**NA**	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis; detection of *M. oralis* in methane positive cultures by	MST4 was the most frequent genotype, which was found in 4/17 isolates in two individuals with generalised severe periodontitis; 17 clinical isolates exhibited different genotypes from that of MST1 of the reference *M. oralis* DSM 7256; MST9 was found in 1/17 isolates	**NA**

			RT-qPCR for *cnp60* gene with *M. oralis*; multispacer sequence typing - PCR for intergenic spacers of *M. oralis*.	and was obtained from a patient with generalized severe periodontitis; two individuals harbored three different *M. oralis* genotypes.	
Huynh, 2015b	65 = periodontitis	15	Enrichment cultures in anaerobic conditions; methane detection by gas-chromatographic analysis; detection of *M. oralis* in methane positive cultures by RT-qPCR for *cnp60* gene with *M. oralis*; multispacer sequence typing - PCR for intergenic spacers of *M. oralis*.	36/65 (55.38%) positive for methane; Positive PCR amplification in 31/36 (86.11%) methane-producing; Cultures = *M. smithii* (n=2 subjects); *Methanobrevibacter* sp. strain N13 (n=3 subjects with severe periodontitis.	Control group methane detection = 1/15 (6.67%). Cultures = *M. oralis* (n=1 subject).
Huynh, 2017	1 = chronic, severe generalized periodontitis	**NA**	Same as Huynh, 2015b.	An 18-month culture yielded a co-culture between a new archaea species (propesed to be called "Methanobrevibacter massiliense"), identified by 16S rRNA and *mcrA* gene sequencng, and Pyramidobacter piscolens, identified by 16S rRNA gene sequencing	**NA**
Kulik, 2001	48 (37 with periodontitis; 8 with rapidly progressing periodontitis; 1 with localized juvenil periodontitis; 1 with refractory periodontitis; 1 with epilepsy and periodontitis)	**NA**	PCR for 16S rRNA gene with euryarchaeal primers; Hybridization of PCR products in agarose gels with probe ARCH 915; cloning, Sanger sequencing.	Positive amplification and probe hybridization = 37 (77%).	**NA**
Lepp, 2004	50 = periodontitis	8	PCR for 16S rRNA gene and RT-PCR for 16S rRNA gene; Cloning, Sanger sequencing; FISH (cloned amplified 16S rDNA).	Archaeal SSU rDNA: 36% = periodontitis patients (76.6% sites); RA in relation to total prokaryotic SSU rDNA > severe and moderate periodontitis; < RA post vs. before treatment; FISH = [18.5% (≥6 mm); 7.2% (4–5 mm); 0.4% (2–3 mm)].	None.
Li, 2009	41 = chronic periodontitis	15	PCR for 16S rRNA gene with 3 different primers; Cloning, Sanger sequencing.	Primer set 1 = 31 (73.2%); Primer set 2 = (70.7%).	None.

Li, 2014	49 = chronic periodontitis	45	PCR for 16S rRNA gene with universal archaeal primers; Nested PCR for 16S rRNA (including Thermoplasmata specific primers); RT-PCR for 16S rRNA.	Universal archaeal primers: 34 samples; 49 subjects. Prevalence of archaea phylotypes = 69.4%. **Nested PCR**: Thermoplasmata in 9 samples (prevalence = 18.4%); **RT-PCR**: 87.8%; **RA** = 0.01% to 7.53%, with a median value of 0.459% and an average value of 0.91%; Thermoplasmata ranged from 0.83% to 32.22%, with a median value of 3.05% and an average value of 8.21%.	**Nested PCR** : No Thermoplasmata DNA. **RT-PCR**: 3 samples; Thermoplasmata RA = 0.20%, 0.11%, and 0.07%.
Lira, 2013	15 = Test group SRP + systemic MTZ at the dosage of 400 mg and AMX at the dosage of 500 mg.	15 = Control group SRP + Placebo	PCR for 16S rRNA gene with euryarchaeal primers.	TEST GROUP: baseline: 9; 6mo: 3; Proportion of sites colonized by archaea: Baseline: 53.3 ± 22.5; 6 mo.: 19.3 ± 10.5.	CONTROL GROUP: baseline: 9; 6mo: 4; Proportion of sites colonized by archaea baseline: 59.2 ± 18.4; 6 mo.: 22.6 ± 12.4
Matarazzo, 2011	30 = generalized aggressive periodontitis (4 samples each)	30	PCR for 16S rRNA gene with euryarchaeal primers; RT-qPCR for 16S rRNA; cloning, sequencing.	GAgP = 27 (68% of the sites); **qPCR**: 11.2 _ 104 _ 6.6 _ 104 copies of the 16S rRNA gene; **Sequencing:** *M. oralis* = 82% of the clones identified in the samples from the GAgP group; *M. curvum*/congolense phylotype, = 7.2% clones; *Methanosarcina mazeii* = 10.8% clones; RA in relation to the total prokaryotes = 0.08%.	26 (58.3% of the sites); **qPCR:** lower levels (0.6 _ 104 _ 0.2 _ 104) copies of the 16S rRNA gene; **Sequencing:** *M. oralis* = 70.1%; M. curvum/congolense = 17.9%; Methanosarcina mazeii = 12%; RA in relation to the total prokaryotes = 0.02%.
Ramiro, 2018	TEST 1 = 20 patients (SRP + MTZ) ; TEST 2 = 19 patients (SPP + TZ + AMX)	40 = SRP (control)	PCR for 16S rRNA gene with primers 931f and m1100r.	TEST GROUPS: Baseline: 25 (adding the two test groups) 6 mo: 10 (adding the two test groups); Proportion of sites colonized by archaea: TEST 1: Baseline: 36.2 ± 31.7; 6 mo.: 11.2 ± 16.1. TEST 2: Baseline: 39.9 ± 28.3; 6 mo.: 12.3 ± 13.4	CONTROL GROUP: Baseline: 14 6 mo: 7. Proportion of sites colonized by archaea: Baseline: 42.5 ± 31.7; 6 mo.: 28.3 ± 32.0
Robichaux, 2003a	8	**NA**	Methane detection by gas chromatographic analysis; enrichment	6 subjects, type III periodontal disease = > methanogens.	NA.

			cultures of sulfate-reducing bacteria and methanogenic.		
Robichaux, 2003b	17 = varying degree of periodontitis	**NA**	Enrichment cultures in anaerobic conditions; isolation in pure culture and characterization of a methanogenic by microscopic techniques and physiological test.	Detection of microbial growth after five transfers in only one culture from type IV periodontal patient's samples; detection of methanogenic archaea in pure culture.	**NA**
Sogodogo, 2019	2= abcesses; 29 = periodontitis	NA	16S rRNA gene PCR-sequencing-based detection of methanogens; fluorescent in situ hybridization detection of methanogens.	Methanogens in 26% samples (*M. oralis*, *M. smithii*, *M. massiliense*; 1 co-infection with *M. oralis, M. massiliense*)	**NA**
Vianna, 2008	102 samples = different stages of severity of chronic periodontitis	65	RT-qPCR for *mcrA* gene; direct sequencing of qPCR amplicons.	Methanogens = 43.1% periodontitis. Absolute abundance of methanogens = 0.26% [0.5% (≥6 mm) and 0.1% (<6 mm)].	None.
Vianna, 2009	44 = periodontitis	**NA**	T-RFLP analysis of methanogens (*mcrA* gene); 16S rRNA PCR; cloning and Sanger sequencing.	*M. oralis* = the sole methanogenic organism in 39 samples; *mcrA* gene = 3 samples; Coexistence of *M. oralis* and *mcrA* gene = 2 samples.	**NA**
Yamabe, 2008	49 = periodontitis (17 aggressive periodontitis)	17	16S rRNA PCR; cloning, Sanger sequencing; Western immunoblotting - detection of humoral immune response to the archaeal components. *M. oralis* and *M. smithii* used as antigens.	11 patients (22.4%); 20.6% in plaque samples from pocket depth>or=6 mm; 29.4% = aggressive periodontitis.	None.

3.4.2 The taxonomy of the Archaea domain in periodontal sites

As described before, several approaches were applied to detect archaeal cells or DNA in periodontal niches. However, most investigations are focused on protocols which were directed preferably to the detection of methanogenic archaea. Consequently, the most frequently reported archaeal taxa in human periodontal samples are *Methanobrevibacter* species, particularly *M. oralis* (Yamabe et al., 2008, Vianna et al., 2009; Matarazzo et al., 2011). However, sequences affiliated to *Thermoplasmata*, a class of *Euryarchaeota*, were also found when species-specific primers were used (Li et al., 2009; Horz et al. 2012). Interestingly, previously described organisms belonging to this class are usually acidophiles, growing optimally at pH below 2 and lacking a cell wall (50). In this case, it is more reasonable to assume that this organism could thrive on supragingival biofilms, where an acidic environment is habitual. Thus, physiological characteristics associated with *Thermoplamata* phylotypes encountered in subgingival niches must be further explored to confirm this taxonomy.

3.4.3 The prevalence of archaea in periodontal sites and its association with periodontitis

Although the differences in both sampling and methodologies employed for the detection of archaea led to divergent data, it was possible to meta-analyse the results from these studies, and, not surprisingly, all revealed higher proportions of individuals with periodontitis positive for archaea than periodontally healthy ones. The data of approximately 1000 individuals with different degrees of periodontitis from 24 studies reporting the number of periodontally-diseased individuals with subgingival biofilms positive for archaea were grouped, regardless the method to detect archaea, reaching a general estimative of 46% of individuals positive for archaea (95%CI 36%-56%; I^2=94%) (Fig.1). Sensitivity analysis was performed, removing the smaller samples studies (14, 16, 33, 34, 36, 43), but the same tendency persisted (pooled prevalence 39%, 95% CI 0.28-0.52, I^2=94%). Data from the baseline of the longitudinal studies were used.

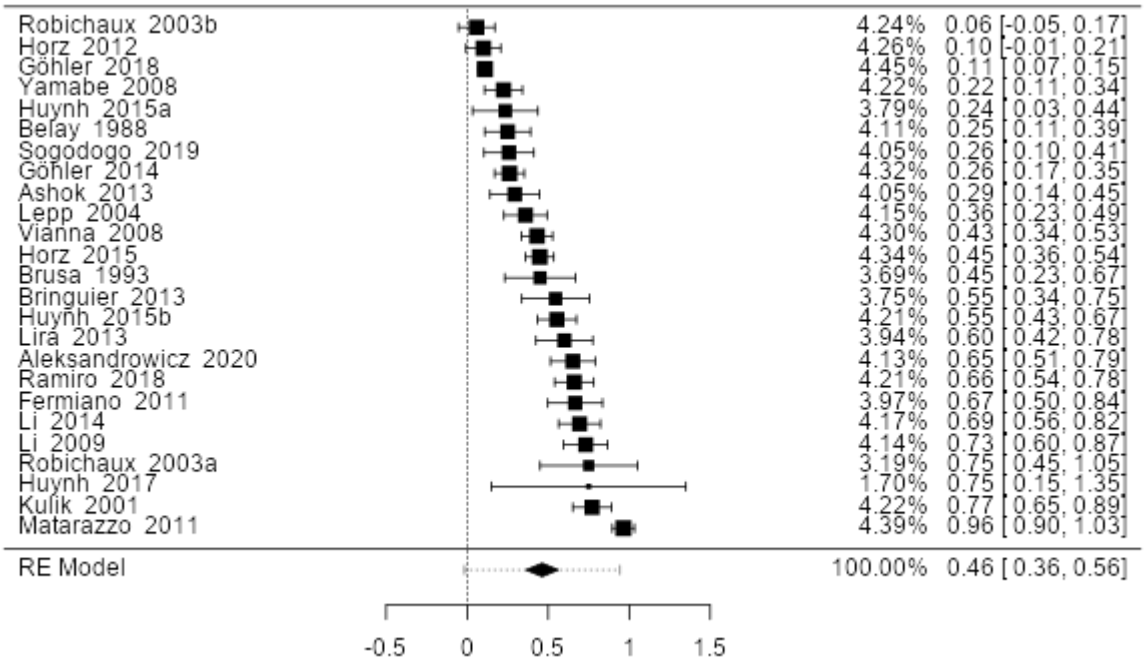

Fig. 1. Prevalence of the *Archaea* domain detection in individuals with periodontitis across 24 studies on periodontal archaeome.

In healthy sites, some divergences have been noticed. While some studies have not detected archaeal DNA in subgingival biofilm samples from periodontally healthy subjects (11, 18, 37, 43), others described archaea as a common member of oral samples from individuals without periodontitis (12, 33, 51, 52). Prevalence of archaea in periodontally health individuals was 13% (95%CI 0.02-0.28, I^2=92%). When periodontally healthy and diseased individuals were compared, a significant positive association between periodontitis and the detection of archaea in subgingival biofilms was found. For that, two meta-analyses were performed including studies based on culture-independent methods (Fig. 2A= 16S rRNA gene – 10 studies; 2B= *cnp*60 gene – 2 studies). The first meta-analysis was sub grouped according to the pair of primers used to amplify the 16S rRNA gene. Individuals with periodontitis were 6.68 fold (95% CI = 4.74-9.41) and 9.42 fold (95% CI = 2.54-34.91) more likely to test positive for archaea than the periodontally healthy individuals, as shown in Figures 2A and 2B, respectively. The results were consistent in terms of magnitude and direction, despite the high heterogeneity in both cases (Fig. 2C). Sensitivity analysis, removing one study at a time (one-by-one), revealed that no study could significantly modify these results. Studies using culture-based methods were not meta-analysed due to the absence of a control group.

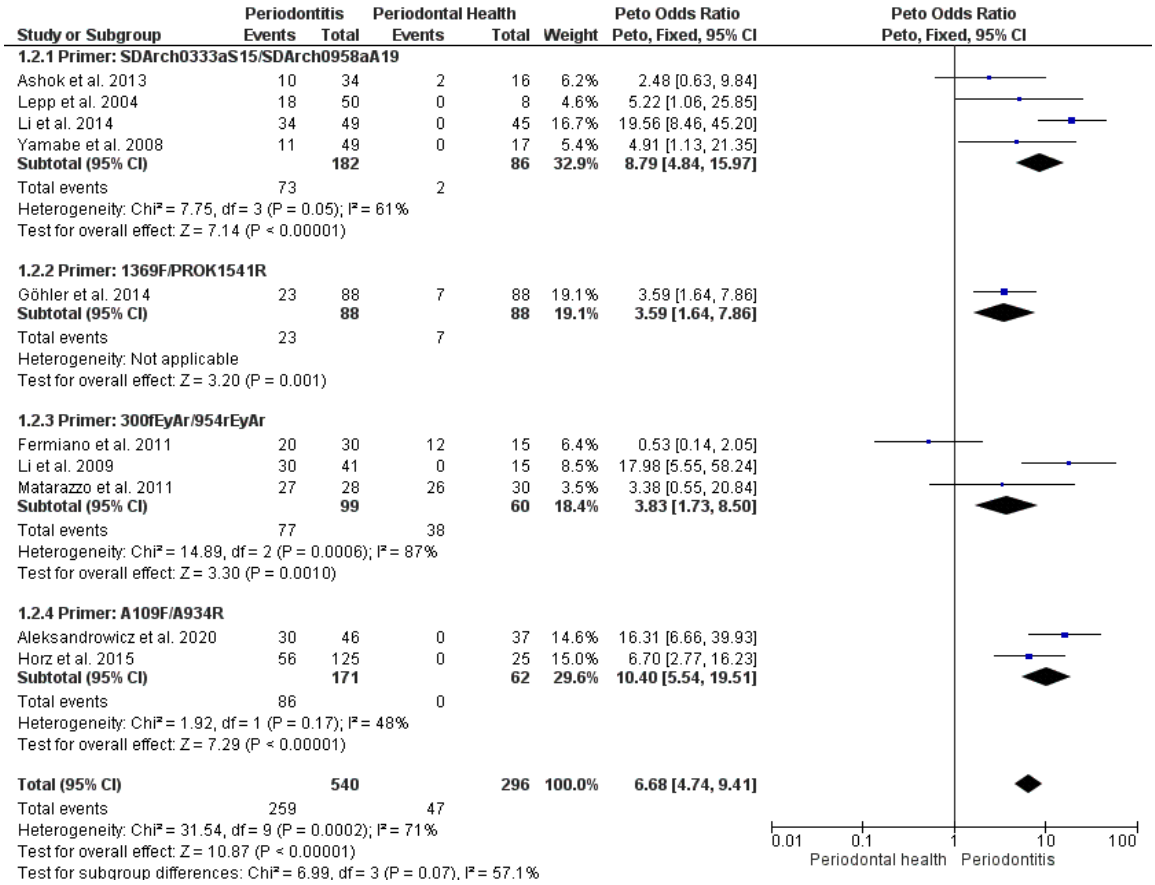

Fig. 2. Forest plot of the prevalence of archaea-positive periodontitis vs. periodontally healthy individuals. A: 16S rRNA gene with different pairs of primers; B PCR of the cnp60 gene. C: Funnel plot representing publication bias for studies of the meta-analysis.

3.4.4 Periodontal treatment

Two studies evaluated samples from individuals positive for archaea, before and after different treatments for periodontal diseases (40, 44). Two meta-analyses were performed, comparing the scaling and root planning (SRP) (conventional non-surgical periodontal treatment) alone or in combination with systemic antibiotics (amoxicillin and metronidazole association) (Fig. 3A and 3B). The articles showed a low heterogeneity (I^2 = 0%), however, just as in the primary studies, the meta-analyses found no significant differences between treatments, suggesting that the addition of systemic antibiotics as adjunct to conventional periodontal treatment does not significantly contribute to reduce archaeal cells. Since archaeal cell walls do not possess peptidoglycan, antibiotics directed against the process of cell wall synthesis are ineffective against *Archaea* (53). However, it has been shown that methanogens can be sensitive to antibiotics that interfere with nucleic acids synthesis (54) and, perhaps this could explain the small tendency of reduction in archaea numbers in subjects using metronidazole.

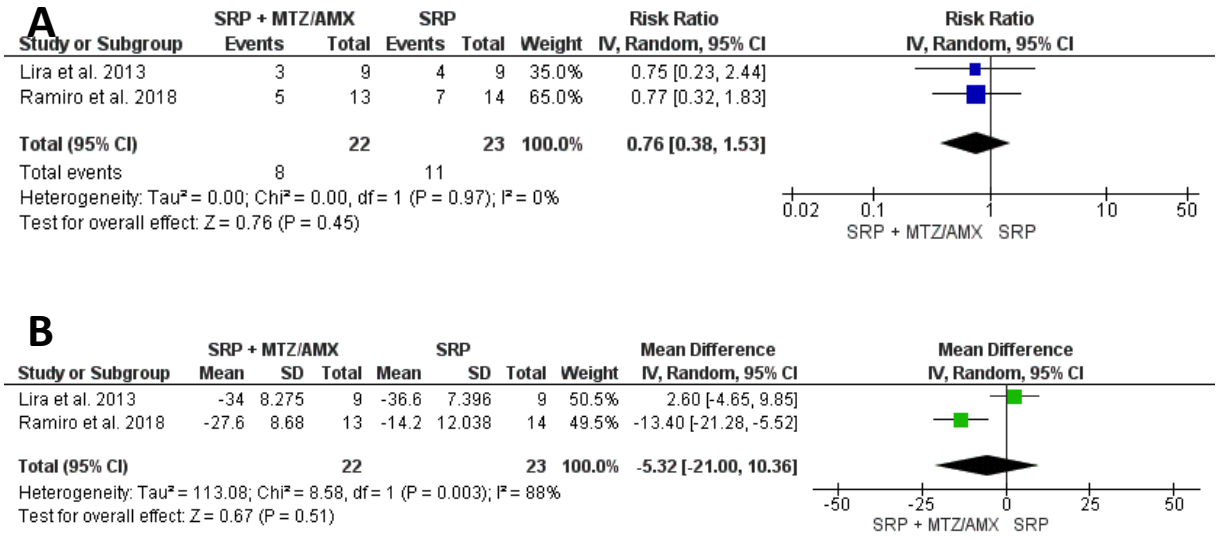

Fig. 3. Forest plot of the prevalence (**A**) and relative abundance average (**B**) of *Archaea* domain in treatments for periodontal disease with scaling and root planing (SRP) + metronidazole and amoxicillin (MTZ/AMX) vs. SRP alone.

3.4.5 Certainty of evidence

Although the same trend was observed in all included studies, the certainty of evidence from outcomes assessed by the GRADE system was low for the prevalence of archaea in patients with and without periodontal disease, since most of the included studies were of observational design and were evaluated as moderate methodological quality. Also, a low certainty of evidence was graded to the reduction of archaea prevalence when the treatments SRP and SRP associated with systemic antibiotics were compared (Table 4), mainly due to imprecision (low number of treated individuals in included studies). In this sense, we believe that more studies, employing more stringent protocols are necessary to increase the level of evidence in this field.

Table 4. Certainty of the evidence evaluated by the GRADE system.

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	intervention	control	Relative (95% CI)	Absolute (95% CI)		
Archaea prevalence Periodontitis versus Healthy individuals												
12	Observational	Serious [b]	Not serious	Not serious	Not serious	Strong association	298/612 (48.7%)	50/321 (15.6%)	OR 6.93 (5.02 to 9.57)	**405 more per 1.000** (from 325 more to 483 more)	⊕⊕◯◯ LOW	IMPORTANT
Archaea Prevalence after periodontitis treatment (AMX + MTZ + SRP versus SRP) (follow up: median 3 months)												
2	RCT	Not serious	Not serious	Not serious	Very serious [a]	None	8/22 (36.4%)	11/23 (47.8%)	RR 0.76 (0.38 to 1.53)	**115 fewer per 1.000** (from 297 fewer to 253 more)	⊕⊕◯◯ LOW	IMPORTANT

CI: Confidence interval; RR: Risk ratio; OR: Odds ratio; Explanations: a. Optimal Information Size (OIS) not achieved, IC reaching significant risk (1.53); b. Included studies with moderate methodological quality. AMX + MTZ + SRP= scaling and root planing + metronidazole and amoxicillin; SRP= scaling and root planning.

3.5 DISCUSSION

There are still many unanswered questions about the roles of archaea in periodontitis, even after nearly 30 years of the first archaea detection on periodontal sites. In this study we compiled the available data on periodontal archaeome in health and disease conditions, as well as after different treatment protocols, and the ultimate conclusion is clear: the urgent need for more research on this topic. The detection of archaea in periodontal sites has frequently been related to disease, leading to the proposition by Horz and Conrads (1) that the presence of archaea in the oral cavity

would be a predictive factor for this pathological condition. The meta-analyses performed in this study confirmed that *Archaea* is enriched in periodontitis, and 46% of individuals with periodontitis tested positive for archaea in subgingival biofilms.

Other aspect worth mentioning is the probable underestimation of archaeal diversity in oral niches since most of the studies focused on methanogenic organisms. The methods used to detect archaeal DNA on oral samples are also questionable, especially in those articles that adopt conventional PCR assays to estimate the archaeal diversity. Primer bias is a recognized problem with PCR-based approaches, rending the reliability of the species identification and level of diversity questionable. Furthermore, the detection of archaea is strongly sensitive to methodological issues such as DNA extraction protocols, primer choice and sequence data processing pipelines (Koskinen et al., 2017) that can lead to underestimated values, or result in erroneous classification as artefacts or contamination (Lazarevic et al., 2016). These issues may contribute to the relatively low prevalence of archaea-positive individuals (46%).

Another hypothesis to that would explain the global archaeal prevalence is the fact that archaea are not officially recognized as ‘keystone’ pathogens, with their enrichment more associated to inflammatory sites, in accordance to the ecological hypothesis proposed by Marsh (55). For instance, *P. gingivalis* widely recognized as a keystone pathogen in periodontitis has also been found in low prevalence, identified in only 23 out of 73 periodontitis samples in combination with the other ‘red complex’ bacteria in a study using NGS approach (Dabdoub et al., 2016), undoubtedly less sensitive to bias than the techniques used to detect archaea thus far. The diversity and complexity of periodontal biofilms represent an unique component of pathogenicity, in addition to bacterial partners that colonize that surfaces. Specific associations among species in dental biofilms are dependent of the biofilm complexity. As an example, cells of the genera *Prevotella* are among those showing the most interspecies associations, suggesting a central role in establishing and maintaining biofilm complexity. A significant positive correlation between methanogens abundance and the amount of *P. intermedia* suggested that, if the metabolic activity of methanogens promotes the bacterial species from the red and orange complex growth in periodontitis, this role is probably mediated through direct or indirect interactions with *P. intermedia* (37). As

oral biofilms become more complex and mature, they are joined or replaced by other species (56), and different colonizing organisms will produce biofilms with varying pathogenic potential. In addition, the low global archaeal prevalence is expected, since these organisms comprehend a small fraction of the human microbiome, as described recently by Koskinen (6).

3.5.1 Archaea detection in periodontitis and healthy subgingival sites

Despite the expressive differences observed between periodontally healthy and diseased individuals, these were not considered statistically significant in some primary studies. Such findings were previously linked to ethnic and dietary aspects of the subjects (52). However, in studies employing small sample sizes of healthy groups (see table 3), caution must be taken to interpret this hypothesis, as the absence of archaea can be related to the poor discriminating power of the study. On the other hand, sensitivity analyses that excluded small sample studies (less than 25 periodontally healthy individuals) returned even more marked results (OR: 8.52, CI = 95% 5.64-12.87). The meta-analyses performed in the present study reinforce the conclusions of most studies on periodontal archaeome and represent a breakthrough in questions regarding the ecological potential, function, and structural interactions with the host and other microorganisms.

To better contextualize our analyses, we present a brief timeline of the periodontal archaeome discoveries. In the past few years, archaea were described as common inhabitants of the oral cavity, and these findings add another variable to be taken into account in studies of the etiopathogenesis of oral diseases (Grine et al., 2018). As described above, the first reports describing the periodontal archaeome were based on the cultivation of methanogens from subgingival plaque samples (9, 52, 57). Only after 14 years, the first article describing the detection of archaea in patients with periodontitis using molecular techniques was published (46). The genus *Methanobrevibacter* was the first methanogen identified and characterized in the oral cavity (47) and until now is the genus most related to oral dysbiosis (35, 58). Although the predominance of methanogens in oral sites has been shown, these results should be interpreted with caution, since other archaeal phyla have been described in the last years (Deng et al., 2017; Koskinen et al., 2017).

3.5.2 The methanogens in subgingival biofilms

Currently, methanogens are the only archaeal group which putative role on oral diseases is relatively known and debated. These organisms thrive in anaerobic microniches, which are frequently found on biofilms, or formed as a result of oxygen consumption by facultative anaerobic microorganisms (Dridi et al., 2011), probably playing important roles in the carbon cycle (Thauer and Shima, 2008). The co-occurrence of methanogenic archaea and sulfate-reducing bacteria (SRB), as well as other microorganisms in periodontal samples (Vianna et al., 2008), and a positive correlation between archaea and *Porphyromonas gingivalis* and *Tannerella forsythia* levels in subgingival biofilms from subjects with chronic periodontitis (Matarazzo et al., 2012) suggest a syntrophic metabolism in these oral biofilms. Theoretically, this role of methanogens can be replaced by SRB and reductive acetogens in subgingival biofilms negative for archaea, since both groups are able to grow in H_2 with H_2S and acetate as final metabolic products. In this context, the antibiotic support therapy could be valuable, since it may reduce the bacterial population, but not the archaeal one. Conventional treatment for periodontitis with SRP would reduce the whole biofilm and, consequently, the archaeal content.

The syntrophic interactions of methanogens and fermentative bacteria are beneficial to both, since they sustain their growth. Depending on the bacterial species involved in those interactions, methanogenic archaea can be viewed as secondary pathogens of the human microbiota (Horz and Conrads, 2010). When interacting with some bacterial species, methanogens collaboratively degrade organic substances, such as acetate, propionate, and butyrate, producing methane under anaerobic conditions (Horz and Conrads, 2011). In addition to the co-occurrence of archaea with bacterial periodontal pathogens, their physical interaction with other organisms may result in relevant outcomes. A curious interaction between *Methanothermobacter thermautotrophicus*, a non-oral methanogenic archaea and the bacterium *Pelotomaculum thermopropionicum*, results in the stimulation of methanogenesis. When the bacterial flagellar tip touches the surface of the archaeal cell, the propionate produced by the bacterium is converted to methane, restoring the environmental pH, which is positive to both organisms (59). It is tempting to speculate that this kind of interaction can also happen in other complex microenvironments. The discovery of

such kind of detoxifying mechanism in subgingival biofilms would not be surprising since the removal of the H^+ produced by many bacterial species would also restore the microenvironment pH, favouring the colonization of bacteria linked to periodontitis.

The well known oral methanogens, *Methanobrevibacter* and *Methanosphaera* species can also produce diverse glycosyltransferases, enzymes that play a major role in maintaining the integrity of oral biofilms by producing the biofilm's extracellular matrix (60). Furthermore, experimental evidence has shown that methanoarchaeal species commonly associated with human mucosa are able to form biofilms on different surfaces (29), which could indicate archaeal participation in subgingival biofilm structures, conferring beneficial properties in this microenvironment as well as another manner of network with bacterial cells (61).

The occurrence of interactions between archaea and humans is still under debate, as well as if archaea have their own virulence factors, since studies associating these organisms with specific pathogenesis are scarce. Probably, the better explored cases linked them to periodontal diseases (Aminov, 2013). Subsequently, we will discuss some hypotheses for the enrichment of archaea other than methanogens in periodontitis.

3.5.3 Putative roles of other archaeal groups in periodontitis

Although previous studies have concluded that archaea constitute only a minor component of the oral microbiome and that their diversity is restricted to methanogens (62), other species can also compose this microbiota, as recently determined by molecular surveys and metagenomic analyses (11, 18-20).

Some archaeal groups which characteristics would allow them to be part of the periodontal microbiota are discussed below. However, it is important to highlight that the information about these groups are still extremely limited and further investigations are needed to test our hypotheses in the context of the periodontal archaeome.

a) Thermoplasmatales

These heterotrophic and thermoacidophilic organisms are phylogenetically grouped with methanogenic and halophilic archaea in the Euryarchaeota phylum. They

were first reported in subgingival samples in 2009 (18) and further detected in other studies investigating archaeal presence in similar samples (11, 19), as well as in ancient calculus specimens (38). Since these non-methanogenic organisms were identified by PCR studies using primers directed to the *mcrA* genes, it was speculated the existence of a new sister group of Thermoplasmatales present in the oral cavity and intestine, probably able to produce methane (1).

b) Ammonia-oxidizing archaea

Besides euryarchaeotes, other archaeal phyla have been shown to play crucial roles in environmental nutrients cycling. Members of Thaumarchaeota have been found to greatly contribute to nitrogen cycling, performing oxidation of ammonia to nitrite, and subsequently to nitrate in numerous marine and terrestrial environments (63). Ammonia oxidizing thaumarchaeotes of subgroup I.1b were found to be the most abundant archaea in human skin, with active physiological status and potential for ammonia oxidation further suggested by FISH visualization and *amoA* genes detection (Probst et al., 2013). Although the clinical relevance of Thaumarchaeota in the human skin remains unclear, it has been speculated that they might impact it by lowering the pH and removing certain nitrogen compounds (7). Interestingly, DNA sequences affiliated to I.1b thaumarchaeotes have recently been identified in supragingival and carious biofilms, suggesting that ammonia-oxidizing archaea may inhabit oral niches (21).We can speculate that the nitrate produced by these could be used as the final electron acceptor by oral anaerobic bacteria during their respiration.

c) Halophilic archaea

Halophilic archaea have already been identified in the human digestive tract (64), but their impact on the host is still unclear. Since haloarchaea have mostly been detected by DNA sequencing approaches (64), has been hypothesized that these archaea transitorily pass the human gut as a result of the ingestion of salty foods, which can be heavily colonized by these organisms, (64). However, recent comprehensive studies based on genomics, culturomics and FISH analyses of fecal samples suggest that halophilic archaea may be residents of the human gastrointestinal tract and not only transient passengers(65, 66). Considering the close association of the gastrointestinal tract and the oral cavity, the ability of these archaeal groups to colonize

oral niches has been previously hypothesized (1) and further reinforced by the recent detection of haloarchaea sequences in a metatranscriptomic analysis of periodontitis samples (Deng et al., 2017). Despite salinity requirements, haloarchaea present very diverse physiological and metabolic features, thriving in aerobic and anaerobic conditions and being able to catabolize numerous compounds (Falb et al., 2008). Furthermore, the formation of biofilms by different haloarchaeal species has already been described, evidencing that these archaea are able to strongly attach surfaces and produce varied biofilm structures (67).

3.6 CONCLUSION AND PERSPECTIVES

It could be hypothesised that archaea may act as secondary pathogens in areas in dysbiosis and be favoured in the inflammatory environment. Their higher detection in different pathological conditions, when compared to healthy sites suggest a putative inter-domain interaction between different archaeal and bacterial species. This kind of speculation is reinforced since until now no virulence factor has been identified in archaeal cells. Although some halophiles could secrete some bacteriocins, no true virulence factors for human cells have been detected.

The archaeal diversity on the oral cavity must be better characterized, since recent works have described new genera and classes, previously not described, such as halophiles and thermoplasmas.

There is an urgent need to better characterize the physiology of archaeal species that are members of the human microbiome, since only after this kind of studies, their actual role in the oral cavity may be clarified. An interesting perspective would be the development of a worldwide network of researchers interested in pioneering the oral archaeome. This kind of project could provide important data concerning the diversity of oral archaeas in subjects of different ages, diets, and ethnicities. The studies could also provide a worldwide picture of the different oral pathologies in which archaea could be isolated.

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4 CAPÍTULO IV: Unraveling the endodontic archaeome: A systematic review with meta-analysis

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4.1 ABSTRACT

Aim: Introduction: The controversial issue of whether the Archaea domain plays a role in endodontic infections is the focus of this systematic review with meta-analysis. The aim is to emphasize the significance of minority microbial domains in oral dysbiosis by evaluating the prevalence of archaea in root canals and its association with clinical parameters such as symptomatology and type of endodontic infection.

Methods: The search strategy involved researching six databases and the gray literature. Publications were accepted in any year or language that identified archaea in samples from endodontic canals. A 2-step selection process narrowed the final choice to 16 articles. The methodological quality of the studies was evaluated using tools from the Joanna Briggs Institute, and the certainty of evidence was assessed using the GRADE approach.

Results: The results showed that archaea were present in 20% (95%CI=8%-32%) of individuals with endodontic samples analyzed. The samples were about twice as likely to be archaeal-positive if collected from individuals with primary versus persistent/secondary infection (OR=2.33; 95%CI=1.31-4.14; I^2=0%), or individuals with self-reported versus symptom-free infections (OR=2.67; 95%CI=1.47-4.85; I^2=0%). Methanogenic archaea were reported in 66% of the included studies. Representative members of phyla Thaumarchaeota and Crenarchaeota were also identified.

Conclusions: Archaea are present in about one-fifth of the infected root canals. Recognized biases in experimental approaches for researching archaea must be addressed to understand the prevalence and roles of archaea in endodontic infections, and to determine whether the decontamination process should include the elimination or neutralization of archaea from root canals (PROSPERO protocol=CRD42021264308).

Keywords: Oral Microbiology; Archaea domain; Endodontic diseases; Low-abundance microorganisms; Endodontic microbiota, Endodontic infectionmicrobiology

4.2 INTRODUCTION

Comprising the third domain of life, as per Woese and Fox's classification (1), archaea are prokaryotic microorganisms classified initially as belonging to the same group of bacteria. Relatively recent phylogenetic analyses based on nucleic acids suggest that archaea differed from bacteria, and presented more biochemical and genetic similarities to eukaryotic organisms, as well as unique features (2). Most representatives of the *Archaea* domain are morphologically similar to *Bacteria* (unicellular ultrastructure without a nucleus), with dimensions ranging from 0.5 to 3.0 µm long to about 1.0 µm wide, and a coccoid or bacillary morphology (3). However, some members have unique morphologies, such as polygonal or very irregular coccoid shapes (4).

It has long been postulated that archaea can thrive in habitats of elevated temperature, low pH, high salinity, or strict anoxia (5), leading to the initial belief that members of this domain were strictly extremophiles. This was credited to the good chemical stability and low porosity of their cell membrane (6, 7). However, the later detection of archaea in a variety of environments, including soils, marine and freshwater, and lake sediments, among others, revealed that they were not restricted to extreme environments (8). Indeed, the isolation of these microorganisms in human feces has helped to support the hypothesis that archaea are not exclusively extremophile microorganisms (9).

Since its initial identification, the human archaeome biogeography has been explored increasingly, and has revealed a site-specific taxonomic pattern (10, 11). Studies have shown that the occurrence and diversity of archaea are greater in two body sites: the gut and the oral cavity (12). In the oral cavity, members of the *Archaea* domain were identified in different microniches, such as saliva (13), tongue (14), dental caries (15), and biofilms (16). Low proportions of DNA sequences from methanogenic archaea have been detected in the ancient dental calculus of the *Homo sapiens* (17),

and a Neanderthal specimen (18). This suggests that archaea have been members of the human/humanoid oral microbiome for a long time. However, the relationship of these microorganisms with oral diseases is not yet fully understood. Archaea can be found in both healthy and dysbiotic sites (19). Its prevalence is higher in sites of periodontal diseases (11), and is likely associated with endodontic infections (20). However, when its identification in oral samples is limited, the reason could be linked to the uneventful dismissal of data related to low-abundance microorganisms in studies on 16S rRNA sequencing, in which case it may be misinterpreted as a contamination or an artifact, a misconception that could generate a false-negative result (21).

Diseases that affect the root canals, resulting in apical periodontitis, usually develop as a low-intensity inflammatory response to microorganisms and their products (22). Most bacteria involved in the etiology of apical lesions are strictly anaerobic, such as the genera *Peptostreptococcus, Prevotella, Porphyromonas, Fusobacterium, Eubacterium*, and some facultative anaerobes such as *Actinomyces* and *Streptococcus* (23). However, innovations in the methods of analyzing the endodontic microbiome have recently identified new genera, such as *Lachnospiraceae, TM7, Synergistetes, Dialister invisus* and *Olsenella* (24), thus bringing to light a greater diversity of microorganisms than what was found in classical studies (23). Furthermore, intracanal polymicrobial communities may change under different endodontic conditions (25). No consensus on the true pathogenic nature of microbial biofilm communities in root canals has yet been reached in the literature. However, current results warn us that species not yet cultivated or poorly identified in the etiology of apical periodontitis require caution (23, 26).

In this scenario, the possible relationship of archaea with endodontic infections, their virulence, and their direct role in inflammatory processes should be better explored (27-29). The association between other members of the microbiota and methanogenic archaea could influence the survival of the bacterial species found inside the root canal, since this process occurs through syntrophy with anaerobic bacteria in specific niches within the natural environment, and likely also within niches in the oral cavity (30-32). It could be assumed that archaea participate in the microbial community of the root canal, however, it is prudent to exercise caution called archaeas as endodontic pathogens, solely based on its presence in infected root canals (33).

Understanding the components of archaea and their role in endodontic infections holds significant importance, particularly concerning their resistance to existing endodontic antibiotics and disinfectants(34). Archaea have been found to be insensitive to many antibiotics that are effective against eubacteria and eukaryotes(35). In a study conducted by Dridi (36), the antibiotic resistance characteristics of eight methanogenic archaea were determined using the macrodilution method in Hungate tubes, along with light microscope observation and methane production monitoring. The study revealed that human methanogenic archaea display high resistance to antibiotics(36). The primary cause of this resistance is attributed to the characteristics of archaeal cell walls, which lack peptidoglycan. Instead, some archaea may possess pseudopeptidoglycan, methanochondroitin, sulfated heteropolysaccharides, halomucin, or glutaminoglycan, each with unique three-dimensional structures (7, 37). Additionally, archaeal cell envelopes display distinctive features, such as plasma membranes containing isoprenoid lipids with an ether linkage and a glycerol-1-phosphate backbone. Furthermore, they possess tetraether lipids and N-glycosylation of extracellular proteins(37).

This systematic review was devised to emphasize the significance of interdomain relationships, and the minority oral microbiome. It analyzed the prevalence of members of the *Archaea* domain in root canal systems across the currently available data, thus contributing to further the development of studies on the oral archaeome and the treatment of endodontic infections.

4.3 METHODS

4.3.1 Study design

The acronym PEOS (38) was used to build the following research question: “What is the prevalence of members of the *Archaea* domain inside the root canals?”, where P (Population)= human root canal samples; E (Exposure)= organisms belonging to the *Archaea domain;* O (Outcome)= presence/relative abundance; and S (Study design)= clinical/observational studies.

4.3.2 Eligibility criteria and search strategy

Publications were included when the target population consisted of individuals at any age that donated endodontic samples. No year or language limits were applied. The exclusion criteria comprised: (1) animal studies; (2) clinical results without microbiological analysis (3) *in vitro* studies, (4) book chapters, conference abstracts, opinions, letters, study protocols without results, or reviews.

The PEOs was also used to set up the search strategy. After selecting keywords and MeSH terms, a detailed search was developed for each of the following bibliographic databases: PubMed/MEDLINE, EMBASE, LILACS, Web of Science, Scopus, and gray literature (Google Scholar, Livivo and ProQuest Dissertations & Theses Global), (Supplementary material, Appendix Table S1). Duplicate studies were removed using a reference manager software (EndNote®, Thomson Reuters).

The selection of studies was carried out by two independent reviewers in a two-step process using the Rayyan data manager (Rayyan QCRI®; Qatar Computer Research Institute, Qatar), as following: 1) titles and abstracts selection; 2) full-text reading and final inclusion. Discordant decisions were resolved by consensus. A complementary hand search was also performed in the reference lists of all included articles.

4.3.3 Data extraction

The reviewers collected the information from the selected articles. The information was checked for its accuracy by a third reviewer. The following data were recorded: year and country of study, sample number, demographic characteristics of participants in each study, the prevalence of archaea, characteristics of endodontic sites, type of sample, DNA extraction method, method for archaea detection, pair of primers used in PCR-dependent methods and the taxonomy of the identified archaea (at the taxonomic level referenced by the authors). For analysis, it was considered as primary endodontic infections were those without any previous endodontic treatment. Persistent/secondary infections or persistent infections were those which occurred after endodontic treatment.

4.3.4 Methodological quality assessment

Methodological quality assessment was performed using the Joanna Briggs Institute (JBI) Checklist for Analytical Cross-Sectional studies tool (39). The instrument has eight questions, from which four were considered critical domains for this systematic review (criteria 1-4). Outcome-related criteria were considered non-critical (criteria numbers 5-8). At least one “no” and one or two “not clear” in critical domains, or two “not clear” and one or more “no” in non-critical domains represented low methodological quality. The decision on critical and non-critical domains and classification system was discussed with the research team prior to the application of the instrument, as described in the JBI Reviewer's Manual(39). Two authors (JAC, JAV) independently assessed the methodological quality and scored each item as “yes”, “no” or “uncertain”. Disagreements were resolved by reaching a consensus.

4.3.5 Meta-analyses and certainty of the evidence

Continuous variables were compared using the DerSimonian & Laird meta-analytic random effects model and presented as mean differences and 95% confidence intervals. Dichotomous variables (archaea prevalence) were compared using Peto Odds Ratio (OR), with a fixed effect model and a 95% confidence interval. Peto odds Ratio was chosen as the prevalence of archaea is low and some studies returned zero events (40). The overall prevalence of archaea was meta-analyzed using the restricted likelihood model for crude proportions with 95% CI (Jamovi software version 1.6 obtained from https://www.jamovi.org/). The certainty of the evidence was evaluated by using the GRADE (Grading of Recommendations, Assessment, Development, and Evaluation) approach, through the analysis of the risk of bias, inconsistency, imprecision, indirectness, and publication bias.

4.4 RESULTS

The search recovered 1884 titles, from which 1142 remained after removing the duplicates. After titles and abstracts analyses, 22 entries were selected for a full-text reading. Six studies were then excluded: five systematic reviews and one

conference abstract (Supplementary material, Appendix Table S2), for a total of 16 reports of 15 included studies. Figure 1 corresponds to the PRISMA flowchart detailing the complete selection process, inclusion, and exclusion of studies. Studies were developed in seven different countries; while Brazil had the largest number of studies, China had the highest prevalence of archaea-positive samples.

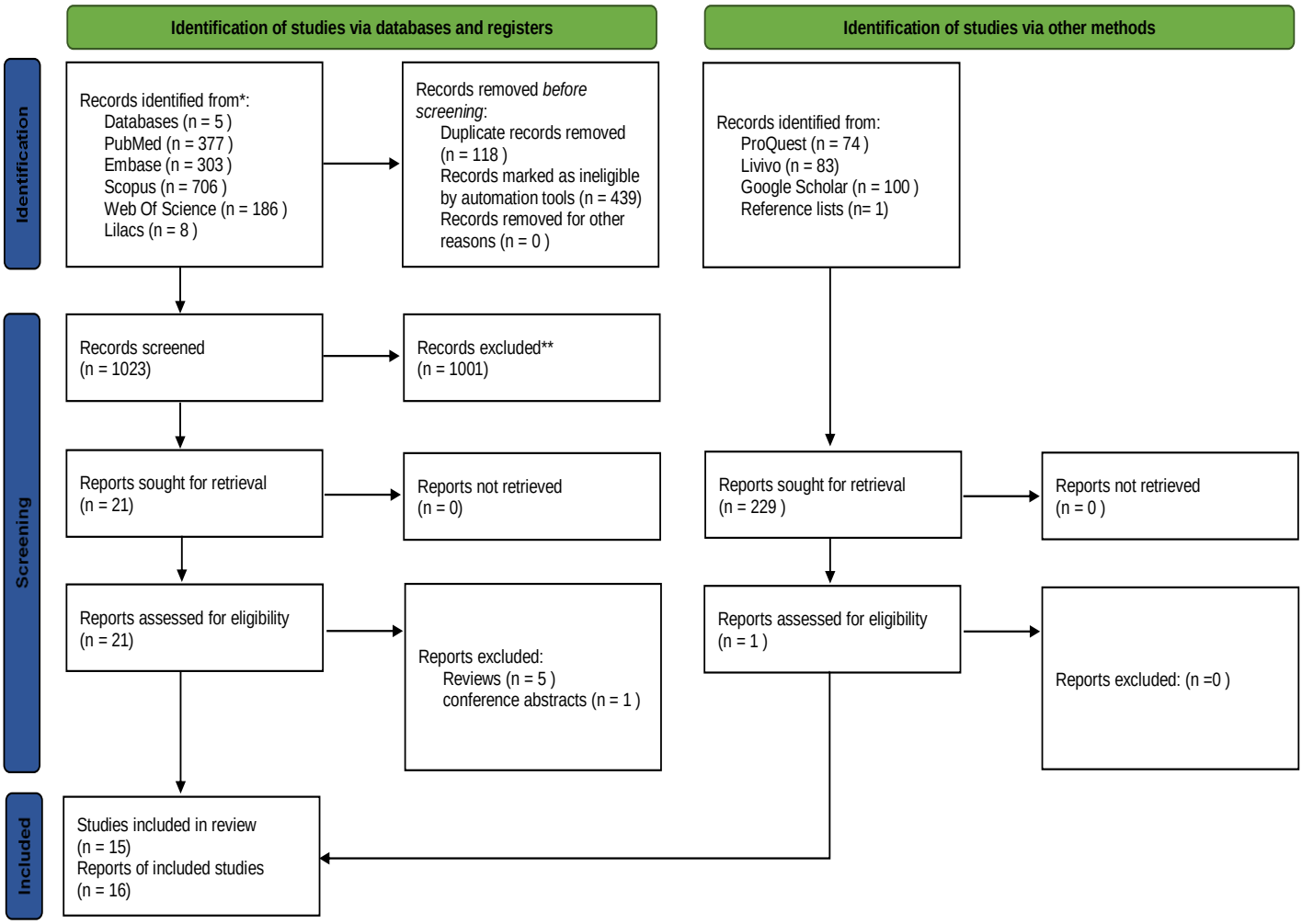

Figure 1. Flow diagram for study selection according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines.

Table 1 summarizes the main characteristics of the articles, including essential steps in molecular analysis that can potentially bias archaeal species identification.

Sample collections were most commonly performed with paper points (27-29, 33, 41-46). Other methods used for sampling were pulverization after tooth extraction (47, 48), aspiration (28, 49, 50), and curettage (51, 52). Most studies employed commercial kits for DNA extraction. In studies using nucleic acid amplification dependent techniques, the most commonly used pair of primers for amplification was the universal A109F/A934R alternative. Eight out of the 10 studies that performed a taxonomic analysis reported the use of GenBank (27, 41-43, 45, 51, 53), while one study used SILVA (47), and one study did not report the database (49).

Archaea were not detected in 3 studies (27-29) (Table 1, Supplementary Material, Appendix Table S3). In contrast, 80% of the articles detected archaea in their samples. The taxonomy of the *Archaea* domain and the associated bacteriome in archaeal-positive endodontic sites across the included studies is shown in Table 2. The most commonly detected archaeon was the *Methanobrevibacter oralis,* interestingly sharing the same site with some bacterial species such as *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythia, Treponema denticola,* and *Synergistetes* sp. (33, 45, 48). Sequences belonging to the phyla *Thaumarchaeota* (48) and *Crenarchaeota* (49) were also detected (Table 2).

Table 1. Characteristics of the included studies (n= 16 studies), including their sample size, clinical characteristics, methods of sampling and archaea detection. Search date: 09/01/2023.

Author, year	N	Clinical characteristics of endodontic sites sampled	Type of sample	DNA/RNA extraction method	Primers	Method of archaea detection	Database	Archaea-positive individuals (N)	Funding
Anderson, 2012	21	Secondary/persistent endodontic infections	Paper points	Genomic mini kit (Qiagen)	A109F/A934R	Culture 16S rRNA amplification + cloning Sanger Sequencing	Genbank, EMBL and DDBJ	0	Public funding from German Research Foundation.
Brzezińska-Błaszczyk, 2018	64	Primary or secondary endodontic infections Asymptomatic/symptomatic periradicular lesions	Paper points	Genomic Mini kit (A&A Biotechnology)	109f/934r	16S rRNA amplification, semi-quantitative reverse transcription PCR (sqRT-PCR) Sanger Sequencing	GenBank	31	Public funding from Medical University of Lodz
Efenberger, 2015	20	Primary endodontic infections	Paper points	Genomic mini kit	A109F/A934R	16S rRNA amplification Sanger Sequencing	GenBank	17	Public funding from Medical University of Łódź
He, 2017	69	Primary endodontic infections + asymptomatic periradicular lesions	Sterile nerve broach with cotton	NA	NA	Culture	NA	16	National Natural Science Foundation of China

Jiang, 2009	77	Secondary/persistent endodontic infections + asymptomatic periradicular lesions	Paper points	TRIzol Reagent for RNA extraction	SDArch0333aS15/SDArch0958aA19	Reverse Transcription PCR for 16S rRNA gene	NA	22	Science and Technology Commission of Shanghai Shanghai Leading Academic Discipline Project National Natural Science Foundation of China
Keskin, 2017	40	Primary endodontic infections + asymptomatic periradicular lesions	Pulverization	Norgen bacterial DNA extraction kit	F515/R806	16S rRNA amplification 454 Pyrosequencing	NCBI BLAST	4	Public funding from The Scientific and Technological Research Council of Turkey-TUBITAK
Özok, 2012	23	Asymptomatic periradicular lesions	Cryo-pulverization	AGOWA mag Mini DNA Isolation Kit	785F/ 1061R	16S rRNA amplification 454 Pyrosequencing	SILVA Ribosomal Database Project Classifier	2	Supported by a subcontract to Mitchell Sogin from the NIH Human Microbiome
Paiva, 2012	30	Primary endodontic infections + asymptomatic periradicular lesions	Paper point	QIAamp DNA Mini Kit	333f/934r	16S rRNA gene amplification	NA	1	Public funding from FAPERJ and CNPq
Provenzano, 2013	14	Primary endodontic infections + asymptomatic periradicular lesions + acute apical abscess	Aspiration	QIAamp DNA Mini Kit	8f/519r	16S rRNA gene amplification Nanoflow liquid chromatography + LTQ Velos Orbitrap and LC-QTOF	NA	1	Public funding from FAPERJ and CNPq
Rôças, 2011	27	Primary endodontic infections + asymptomatic periradicular lesions	Paper points	QIAamp DNA Mini Kit	333f/934r	16S rRNA amplification Reverse-capture Checkerboard Assay	.	0	Public funding from FAPERJ and CNPq.

Siqueira, 2005	35	Primary endodontic infections + asymptomatic periradicular lesions + acute periradicular abscesses Secondary/persistent endodontic infections + asymptomatic periradicular lesions	Paper points + aspiration	QIAamp DNA mini kit	Arch21F/Arch 958R	PCR	NA	0	not declared
Slaton, 2017	9	Primary endodontic infections + asymptomatic periradicular lesions	Aspiration	Zymo Research kit		Culture using RAST medium 16S rRNA amplification MiSeq NextGen Sequencing	NA	6	not declared
Subramania n, 2009	34	Secondary/persistent endodontic infections + asymptomatic periradicular lesions.	Curettage	Chemo-mechanical lysis without kit	785; 422; 1512r; 1400r; A17; 317	qRT-PCR 16S rRNA amplification + cloning Sanger Sequencing	GenBank Ribosomal Database Project II	1	not declared
Vianna, 2006	20	Primary endodontic infections + asymptomatic periradicular lesions	Paper points	Qiamp DNA minikit	LuF; LuR; A109F; A934R; PF1; PR1; EuF; EuR	Real time quantitative PCR and Direct sequencing of qPCR amplicons	GenBank	5	Public funding from The Brazilian grant agencies CAPES and FAPESP, and the START program of the Faculty of Medicine, RWTH, Aachen, Germany
Vianna, 2009	32	Primary endodontic infections (chronic)	Paper points	Qiamp DNA minikit	ME1f/LuR; LuF/LuR and A109F/A934R	T-RFLP analysis of methanogens - *mcrA* gene + amplification + cloning	GenBank	8	Public funding from START program of the Faculty of Medicine, RWTH, Aachen, Germany.

						Sanger Sequencing of archaeal 16S rRNA			
Vickerman, 2007	34	Primary endodontic infections + asymptomatic periradicular lesions	Paper points	Chemo-mechanical lysis without kit	SDArch0333aS15/SDArch0958aA19	16S rRNA amplification + cloning Sanger Sequencing	GenBank	2	United States Public Health Service (USPHS) and the University at Buffalo Division of Endodontics Funds

Table 2. The taxonomy of the Archaea domain and the associated bacteriome in archaeal-positive endodontic sites across the included studies.

Author, year	Type archaea found	Prevalent bacteriome in endodontic samples	Bacterial taxa more prevalent in sample archaea-positive
Anderson, 2012	No archaeal DNA	*Enterococcus faecalis, Streptococcus spp., Propionibacterium acnes* **Detected for the first time in filled root canals**: *Neisseria elongata, Actinomyces oris, Corynebacterium minutissimum, Proteus hauseri/vulgaris* and the genus *Rummeliibacillus*	NA
Brzezińska-Błaszczyk, 2018	*Methanobrevibacter oralis*	*Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*, and *Synergistetes sp.*	*Significant negative association in coexistence between archaea and *Treponema denticola* in the cases of secondary/post-treatment infections

Efenberger, 2015	*M. oralis (ZR), Methanobrevibacter smithii (PS), Methanobrevibacter thaueri (CW), Methenobrevibacter millerae (ZA-10), Methanobrevibacter gottschalkii (HO), Methanobrevibacter woesei (GS 97), Methanobrevibacter arboriphilus (DH-1), Methanobrevibacter acididurans (ATM), Methanobrevibacter boviskoreani (JH1), Methanobrevibacter ruminantium (M1), Methanobrevibacter wolinii (SH), Methanobrevibacter cuticularis (RFM-1), Methanobrevibacter olleyae (KM1H5-1P), Methanobrevibacter curvatus (RFM-2), Methanobacterium flexile (GH), Methanobrevibacter filiformis (RFM-3),*	*T. denticola* (70%) *T. forsythia* (45%) *P. gingivalis* (40%) *P. intermedia* (20%)	NA
Hey, 2017	NA	*A. viscosus, A. naeslundii, A. israelii* and *E. faecalis*	NA
Jiang, 2009	NA	NA	NA
Keskin, 2017	Euryarchaeota - *Methanobrevibacter oralis* Thaumarchaeota - *Candidatus Nitrosoarchaeum limnia*	Phyla - Proteobacteria (33.4%), Firmicutes (32.3%), Bacteroidetes (26.3%), Fusobacteria (4.2%), and Actinobacteria(2.9%) Genera - *Prevotella* (19.6%), *Porphyromonas* (16.5%), *Neisseria* (13.2%), *Lactobacillus* (11.7%), *Parvimonas* (11.1%), *Streptococcus* (10.7%), *Enterococcus* (3.5%), *Camplyobacter* (1.1%), and *Granulicatella* (1%)	NA
Özok, 2012	Phylum Euryarchaeota, genus *Metanoregula*	*Lactobacillus, Actinomyces, Streptococcus* and *Prevotella*	NA
Provenzano, 2013	Identification of the enzyme methyl coenzyme M reductase	NA	NA
Rôças, 2011	No archaeal DNA	*P. acnes* (75%), *Bacteroidetes oral (clone X083)* (63%), *Selenomonas sputigena* (63%), *P. endodontalis* (58%), and *P. acidifaciens* (54%).	NA
Siqueira, 2005	In the case yielded archaeal DNA		Spirochetal DNA was detected in 32 out of 40 cases

Slaton, 2017	Archaea-Crenarchaeota Archaea-Euryarchaeota 5	1	Phyla - Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Genera - *Streptococcus*	NA
Subramanian, 2009	*Methanobrevibacter oralis*		*E. faecalis, Burkholderia cepacia* *Campylobacter gracilis* and *S. gordonii* were associated with root ends, while *Atopobiumrimae, Peptostreptococcus micros, S. genomo species C8, Dialister sp E2_20E1, and Eubacterium strain A35MT* were associated with periradicular lesions	NA
Vianna, 2006	*M.* oralis *Methanobrevibacter ruminantium* *Methanobrevibacter arboriphilus*		NA	NA
Vianna, 2009	*M. oralis* Methanogenic archaea (eight positive cases)		*Treponema pallidum*, (18 positive cases), *T. forsythia* (13 positive cases), *P. gingivalis* (9 positive cases), *Synergistetes spp*. (7 positive cases) and *P. intermedia* (5 positive cases)	Significant positive association between methanogenic archaea and *Synergistetes spp*. was found with an odds ratio of 7 (95%CI-1.11 to 44.06).
Vickerman, 2007	*M. oralis*		*Eubacterium, Fusobacterium sp., Granulicatella adiacens, Lactobacillus paracasei, Lactobacillus salivarius, Mogibacterium neglectum, Neisseria bacilliformis, Prevotella nigrescens, Rothia dentocariosa, Sphingomonas sp. (P2), S. gordonii, S. mutans, S. sanguinis, Veillonella sp. (oral clone), Desulfovibrio sp.,* E*ubacterium nodatum*	*T. forsythia*, *P. endodontalis, P. gingivalis, P. micros, F. nucleatum, P.intermedia, Enterococcus sp.*

It was possible to meta-analyze the data collected from 15 studies. The overall prevalence of archaea was 20% (95%CI=8%-32%) of the individuals whose endodontic samples were analyzed (Figure 2). A sensitivity analysis was performed, removing studies with the smallest sample sizes (27-29), but the same trend persisted (Overall prevalence=29%; 95%CI=0.13-0.44).

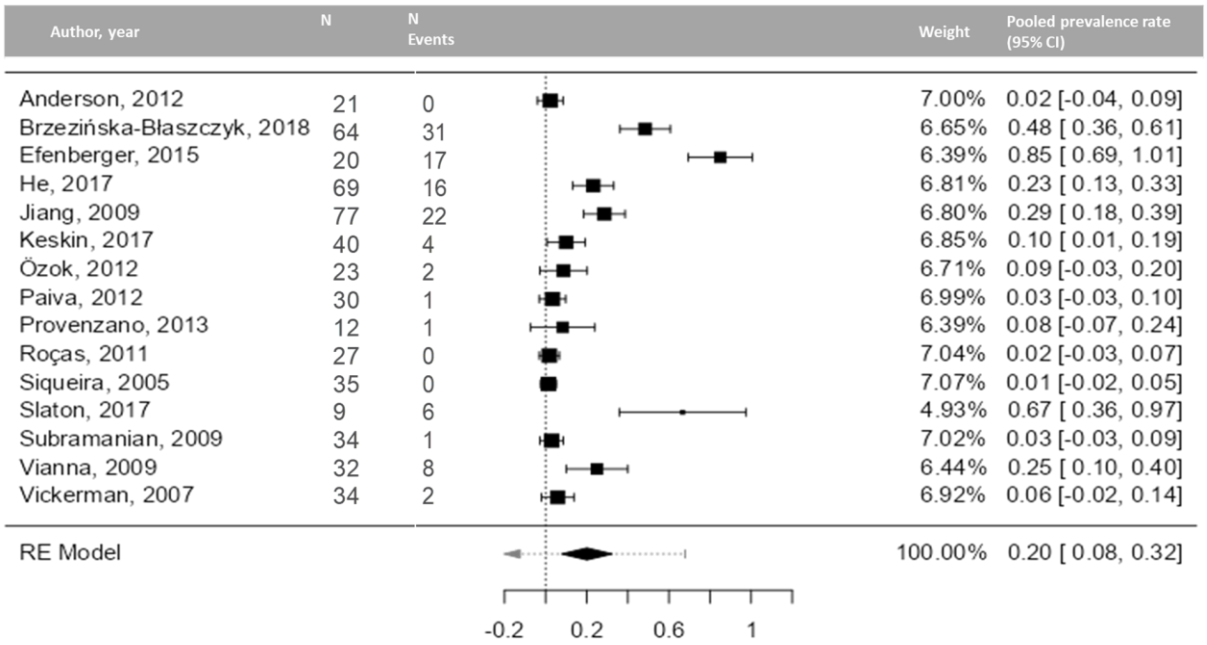

Figure 2. Overall prevalence of the Archaea domain in endodontic canals of individuals in 15 studies on the endodontic archaeome.

When the painful symptomatology was considered (with versus without pain symptoms), as well as the type of endodontic infection (primary versus secondary/persistent), significant positive associations were found after developing two meta-analyses. Samples were about twice as likely to be archaeal-positive if they were collected from individuals with self-reported symptoms versus symptom-free infections (OR=2.67; 95%CI=1.47-4.85; I^2=0%) (Figure 3A). Samples of individuals with primary endodontic infection were also about twice as likely to be archaeal-positive versus individuals with secondary/persistent infections post-treatment (OR=2.33; 95%CI=1.31-4.14; I^2=0%) (Figure 3B). In both meta-analyses the articles showed a low heterogeneity (I^2 = 0%)

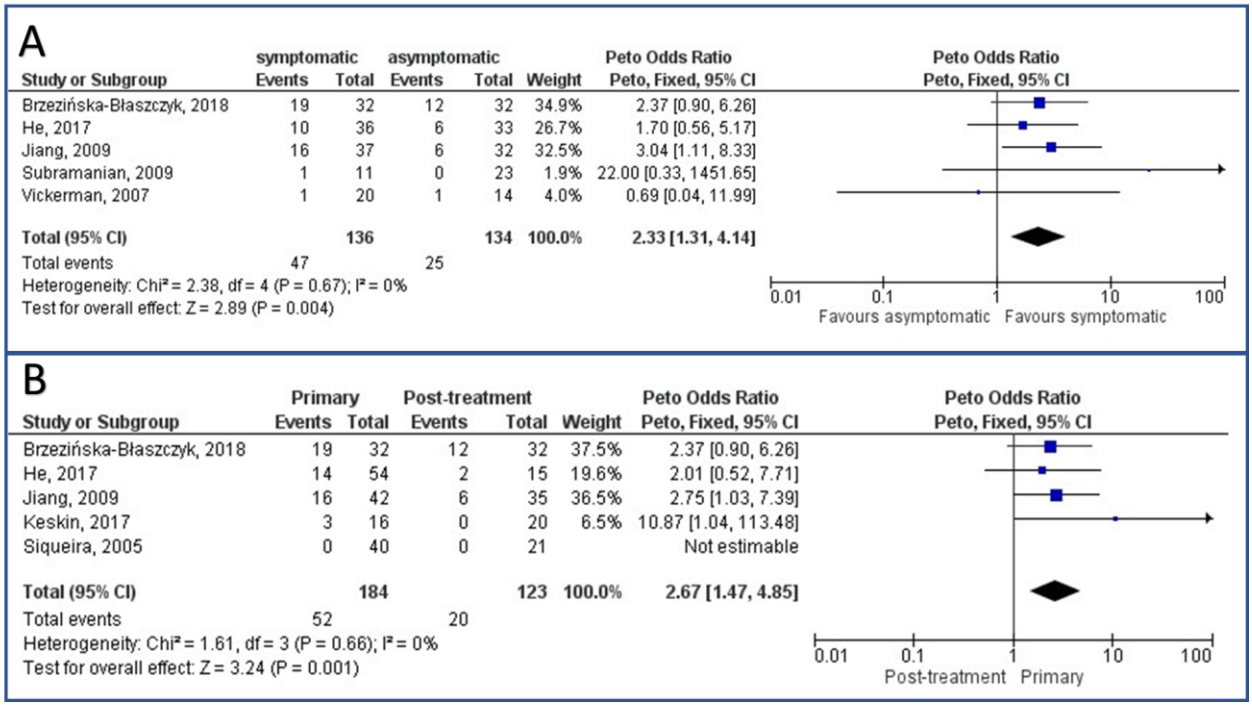

Figure 3. Forest plot of the prevalence of archaea-positive endodontic sample according to the clinical characteristics of the endodontic disease. A - symptomatic versus asymptomatic samples. B - Primary infections versus secondary/persistent endodontic infections post-treatment.

The certainty of the evidence was moderate for both meta-analyses. As per the GRADE approach recommendation, the analysis started with low certainty of evidence due to the design of the studies (observational). Despite the inclusion of studies with low to moderate methodological quality (Supplementary Material, Appendix table S3) and with relatively small sample sizes (Table 1), a large magnitude of the effect was found and this was the reason for the upgrade. Furthermore, the sensitivity analysis did not change the effect direction (Supplementary Material, Appendix Table S4).

4.5 DISCUSSION

Some authors hypothesized that archaea could invade endodontic sites naturally and participate in polymicrobial infections (19, 43). The results of our meta-analyzes corroborate with the hypothesis that archaea are members of the endodontic

microbiome, and more likely to be present in symptomatic and primary infections. The presence of archaea was reported in 13 out of 16 articles. Archaea probably increase in primary endodontic infection when compared to persistent/secondary endodontic infections, as well as in symptomatic versus asymptomatic endodontic infection (moderate certainty of evidence). Nonetheless, we believe that the archaea abundance and diversity were underestimated in the studies due to biases in the methods of identification. Methodological aspects should doubtless be further scrutinized.

It is unknown how archaea respond to classical endodontic disinfectants (Horz and Conrads 2011). Their presence in persisting/secondary endodontic infections could increase the risk of worsen the symptoms of the infection. The determination of archaea components and their roles in endodontic infections is of utmost importance, especially in terms of their resistance to antibiotics and currently available endodontic disinfectants. As archaeal cell walls differ from bacterial peptidoglycan walls, antibiotics directed against the bacterial wall synthesis process are ineffective and do not inhibit archaeal (54). However, some methanogens may be sensitive to antibiotics that interfere with nucleic acid synthesis (36).

So far, no attempt was made to quantify the association between the prevalence of archaea and clinical characteristics, as symptomatology. Same goes for the association between the prevalence of archaea in primary versus persistent/secondary apical periodontitis. Our meta-analysis showed a significant positive association between the prevalence of archaea and primary root canal infections. Importantly, root canals previously treated are expected to have a less diverse microbiota, especially those with apparently adequate root fillings (26). Therefore, the number of archaea can be further reduced after the chemomechanical procedure in cases of persistent/secondary apical periodontitis (46, 55, 56). The greater load of microorganisms in primary endodontic infections could justify the increased prevalence rate of archaea (26).

Another finding was the potential association between the presence of symptoms and the archaea content. Literature has shown that the structure of the microbial community differs when comparing symptomatic and asymptomatic infections (42, 57, 58). Some taxa have been associated with the manifestation of symptoms, such as members of the genera *Prevotella, Peptostreptococcus,*

Porphyromonas, and *Lactobacillus* (59) and they are usually more abundant in archaea-positive samples (33, 45, 48)(Table 2). Despite not having a consensus regarding the pathogenicity of archaea, their association with symptoms may be due to inflammatory mechanisms (34). Archaea can influence the local microbiota, leading to the proliferation of microorganisms that generate a greater inflammatory response even if the methanogenic archaea are not virulent themselves. In addition, some members of archaea may have components in their lipid membrane that can provoke an inflammatory response (32).

In this context, understanding the intra and interdomain archaeal relationships seem to be essential (15, 28, 44). It has been suggested that the interdomain positive relation may be associated with a significantly higher prevalence of clinical symptoms compared with the sole presence of bacteria in root canals (44). A negative interdomain relationship has also been theorized: studies that did not detect archaea in their experiments justified their findings due to competition with hydrogen-metabolizing or sulfate-reducing bacteria, and suggesting that archaea are not associated with endodontic infections (28). This hypothesis was also defended by others, that also found no or few archaeal sequences in endodontic samples (29, 46) (27).

It could conceivably be hypothesized that the real archaea prevalence is even higher than we detected in this cross-study analysis. The lack of standardization methods to explore the presence of archaea may hamper their detection. In the included studies, we could identify some factors that may have influenced the non-detection of archaea. First, the low rates of sequences recorded in databases could be the reason for the low diversity of archaea currently detected (21). Another factor may be an obstacle was a culture-step prior to PCR amplifications in some studies, as archaea are well-known difficult-to-culture organisms (60, 61). The potential causes that hinder the cultivation of archaea has been listed elsewhere: 1) lack of exchange of interspecific material during *in vitro* culture, for example, growth factors, and signaling molecules produced by other organisms *in situ*; 2) interspecific competition; 3) intrinsically slow growth rate and low abundance of archaea, resulting in rapid bacterial overgrowth during laboratory cultivation; and 4) improper transport conditions or sub-optimal *in vitro* culture parameters that differ from *in situ* environmental parameters (60).

The rather contradictory results of no archaea detection in endodontic samples from three out of 16 studies may also be due to the pair of primers used for PCR amplification. After retesting the archaeal primers used by Siqueira et al. (2005) (28), Vianna, 2006 (41) observed that *M. oralis* and *Methanobrevibacter smithii* were not amplifiable by using these primers. Few positive results were described using the same pair of primers (46), possibly due to their larger sample size. The primers used by Anderson (27) and Rôças (29) were applied in other studies with positive results, although using different combinations. This fact reinforces the importance of choosing the primer pairs according to their specificities.

The arbitrary filtering of low abundance sequences is also a weakness of the studies that must be considered to justify the non-detection of archaea, as well as their low diversity. For instance, Keskin *et al*. (2017) detected for the first time in the root canals a novel archaeal species, namely *Candidatus Nitrosoarchaeum limnia,* from the *Thaumarchaeota* phylum. The authors hypothesized that could result in the pH regulation of the root canal environment or it could compete with ammonia-oxidizing bacteria. Even though most studies establish the cutoff at <1% of relative abundance for an organism to be considered present (21), this practice may compromise the identification of the true diversity of the microbiota.

Caution must be applied regarding the external validity in our cross-study comparison, as studies had a small overall sample size (less than 300 endodontic samples searched for archaeal content). Moreover, it's important to note that the included studies are of an observational nature. Although we assessed the methodological quality of these studies to gauge how well they addressed potential biases in their design, conduct, and analysis, caution is still warranted.

Another source of uncertainty is the geographical bias, as only seven countries contributed to the studies on the endodontic archaeome. While conducting the evidence synthesis, low-quality studies were taken into account. Nonetheless, a sensitivity analysis was performed, which involved the removal of these low-quality studies, yet the observed trend persisted. Furthermore, during the certainty analysis of the evidence generated, the quality of the studies was fully considered. We found a higher prevalence of archaea in studies from China and Poland. Microbial diversity can vary due to subjects' geographical origins and habits, such as diet, nutrition habits, and

food resources, exerting selective pressure on the oral microbiome (62) and justifying the difference in distribution of positive archaea individuals (63). Through a systematic review, we can gather and analyze all available evidence on the emerging area of studying the oral archaeome, identifying areas where further research is needed. This process may enhance the understanding in the field and informs future research directions.

In conclusion, members of the *Archaea* domain are present in about one-fifth of endodontic canals and might be considered a minor part of the endodontic microbiome. These microorganisms are more likely to be present in symptomatic and primary infections, but the certainty of the evidence is moderate. Recognized biases in experimental approaches with archaea need to be addressed to understand the prevalence and roles of these microorganisms in endodontic infections and whether the decontamination process should include their elimination or neutralization from root canals.

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5 CAPÍTULO V: Biogeografia do Arqueoma Oral: uma análise da distribuição de arqueias metanogênicas em sítios orais

5.1 INTRODUÇÃO

O microbioma oral humano é muito diverso e apresenta uma ecologia complexa, incluindo bactérias, microeucariotos, arqueias e vírus que coexistem e interagem entre si e com o hospedeiro humano. Esses microrganismos vivem em comunidades com distribuição organizada espacialmente, onde há diversas trocas metabólicas e de informação genética (1). A forma como os microrganismos orais se organizam tem sido observada por décadas, mas tem sido analisada de forma mais abrangente e precisa nos últimos anos usando métodos independentes de cultivo (2).

Fatores que influenciam a composição das comunidades microbianas em diferentes regiões incluem as características das superfícies onde elas se fixam. Na cavidade oral, há uma variedade de habitats distintos, como as superfícies duras do esmalte e dentina, os sulcos, as áreas queratinizadas do palato, gengiva e papilas da língua, a mucosa bucal, entre outros. Esses ambientes variados criam gradientes de oxigênio, nutrientes e pH, que determinam quais espécies conseguem colonizar, crescer e se estabelecer como componentes principais ou secundários da microbiota local (3).

Contudo, apesar dessas diferenças entre sítios, estudos mostram que podemos encontrar um conjunto diverso de mais de 700 espécies bacterianas (4), além de uma grande diversidade já conhecida de vírus e fungos (5). Trata-se do microbioma central generalista, com representantes que possuem uma grande adaptabilidade e que podem se ajustar a uma variedade de condições (6). Essa adaptabilidade permite que a formação de biofilmes ocorra nas superfícies dentárias através de sucessivas etapas de colonização microbiana, durante as quais a diversidade e a quantidade de microrganismos aumentam gradualmente ao longo do tempo. Nesse sentido, ocorrem assim interações inter-reinos, promovendo a expansão do biofilme e aumentam sua complexidade funcional e taxonômica (7).

Estudos voltados para a descrição do bacterioma central generalista e sítio-específico da cavidade bucal são abundantes (8-10). Sabe-se que as espécies bacterianas que compõem a maior parte do microbioma oral são geralmente conservadas entre os indivíduos. No entanto, há escassez de conhecimento sobre as possibilidades de distribuição espacial de organismos de baixa abundância, tais quais as arqueias. Já há algum relato de sua presença em alguns sítios orais, como saliva, biofilme dentário subgengival e língua (2). Embora ainda existam muitas lacunas sobre a prevalência, diversidade e interações das arqueias na cavidade oral, sua presença já é documentada desde 1987 (11), com a identificação da espécie *Methanobrevibacter oralis*.

As arqueias metanogênicas são as mais comumente detectadas no microbioma oral. Constituem um grupo diversificado de microrganismos presentes em diversos microbiomas ambientais e no hospedeiro, desempenhando um papel no ciclo global do carbono, devido à sua capacidade de produzir metano (12). A metanogênese, um processo evolutivamente antigo exclusivo das arqueias, é uma forma complexa de respiração anóxica, que envolve a biossíntese de várias coenzimas e cofatores. Dependendo do substrato utilizado, diferentes etapas levam à liberação de metano pela enzima metil-coenzima M redutase (MCR), essencial para o processo. Embora a produção de energia seja baixa (até 1 ATP por molécula de metano), alguns metanogênicos otimizam esse rendimento por meio de sistemas de citocromo e bifurcação de elétrons, permitindo crescimento rápido (13).

As metanogênicas já foram encontradas em biofilmes sub (14-16) e supragengivais (17), tecido dentário cariado (17) e microbiomas endodônticos (18). Porém, sua distribuição na cavidade oral em diferentes sítios ainda não foi descrita. Apesar de incipientes, por meio dos dados obtidos por nosso grupo através de revisões sistemáticas e análises *in sílico,* vemos que distintos sítios orais, terão condições diferentes de arqueias. Considerando isso, esse estudo teve como objetivo avaliar a distribuição de arqueias metanogênicas em 5 sítios/tipos de amostras da cavidade oral: saliva, biofilme lingual, dentina cariada, biofilme sub e supragengival.

5.2 MATERIAIS E MÉTODOS

5.2.1 Desenho do estudo:

Este estudo transversal foi conduzido a partir de coletas de amostras de adultos, selecionados por conveniência, e analisadas por métodos moleculares para traçar perfil biogeográfico de arqueias metanogênicas. A coleta de dados foi realizada entre abril de 2022 a junho de 2024. O relato desse trabalho seguiu *STORMS Microbiome Reporting Checklist* (19).

5.2.2 Participantes:

Os participantes, doadores de amostras, foram selecionados entre os pacientes atendidos na Unidade de Saúde Bucal do Hospital Universitário de Brasília (USBUC/HUB/Ebserh). Este estudo foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Ciências da Saúde da Universidade de Brasília (processo número 54947422.2.0000.0030). Todos os doadores de amostras foram informados dos objetivos do estudo e assinaram um consentimento formal para participar. Os doadores responderam à um questionário sobre a presença de condições sistêmicas como hipertensão e diabetes.

Os critérios de inclusão exigiam que os doadores tivessem 18 anos ou mais. Foram excluídos fumantes, gestantes ou lactantes, aqueles com histórico de radioterapia na região de cabeça e pescoço, ou que tivessem utilizado antibióticos no último mês antes da coleta, além de indivíduos que faziam uso regular de enxaguantes bucais antissépticos. Para a coleta de dentina cariada e biofilme subgengival, os doadores deveriam apresentar, respectivamente, lesão de cárie cavitada ativa (20), com indicação de tratamento restaurador, e diagnóstico de doença periodontal conforme a classificação de 2018 da *American Academy of Periodontology e da European Federation of Periodontology* (21).

5.2.3 Cálculo amostral

De acordo com a revisão sistemática sobre a prevalência de arqueias em doença periodontal (22), a proporção estimada de indivíduos arqueia positivos em

situação de doença é 46%. Já entre os indivíduos saudáveis essa proporção é 13%. Utilizou-se esses dados no cálculo amostral para diferença entre 2 proporções (teste do qui-quadrado), considerando erro alfa de 5% e o erro beta de 20% e chegando ao resultado de N= 29 participantes por grupo. Uma taxa de 10% de perda foi incluída, no caso de perdas de amostras por degradação do DNA, totalizando 32 amostras por grupo.

5.2.4 Coleta das amostras

Saliva

A coleta de saliva foi realizada no período da manhã (8h-10h) para minimizar os efeitos dos ritmos circadianos. O tempo de coleta da saliva total estimulada foi de 5 minutos, sendo feita por expectoração em um recipiente de plástico. Para estimular a produção de saliva, os doadores mastigaram um pedaço de Parafilm® (5 cm) durante todo o processo de coleta. Após a coleta, as amostras foram alíquotadas em volumes de 500 µL em microtubos livres de DNAse e RNAse e armazenadas imediatamente em gelo para transporte ao laboratório. O restante do volume salivar foi destinado à aferição do pH e da capacidade tampão, para caracterização da amostra.

A aferição do pH das amostras foi realizada utilizando um pHmetro de bancada microprocessado, previamente calibrado com soluções de referência de pH 7,0 e pH 4,0, com precisão de ± 0,01 pH. A análise da capacidade tampão foi conduzida nas amostras que apresentaram volume residual de pelo menos 1 mL após a alíquota de 500 µL. Para isso, foram adicionados 3 mL de ácido clorídrico 0,005M a 1 mL de saliva, e, após 2 minutos, o eletrodo do pHmetro foi imerso na solução para medir o pH correspondente à capacidade de tamponamento. As proporções utilizadas foram adaptadas de acordo com a disponibilidade de amostras. Valores inferiores a pH 4,0 foram considerados indicativos de baixa capacidade tampão. O fluxo salivar estimulado foi calculado dividindo-se o volume total de saliva coletada pelo tempo de coleta, expressando o resultado em mL/min.

Dentina cariada

Coletas de amostras de dentina cariada foram realizadas usando uma cureta estéril e armazenadas em microtubos livres de DNAse e RNAse. Após a coleta das amostras, todos os indivíduos foram submetidos a tratamento convencional para cárie e acompanhamento.

Biofilmes

As amostras de biofilme subgengival em doadores com doença periodontal foram coletadas após o exame periodontal para diagnóstico, utilizando uma cureta periodontal minifive Gracey estéril (número 5/6) (foi selecionado o dente com a bolsa de maior profundidade). Antes da coleta das amostras subgengivais, o dente selecionado era seco e limpo com rolos de algodão para remover qualquer biofilme supragengival e um único movimento de raspagem no sentido ápico-cervical era realizado para minimizar a presença de sangue na amostra.

Os dados referentes à presença ou ausência de placa visível, sangramento à sondagem (presença ou ausência) e profundidade de sondagem (em milímetros) foram coletados de forma independente por diversos examinadores treinados. Para verificar a consistência das análises e a confiabilidade entre os examinadores, foi calculado o coeficiente de concordância kappa com pesos lineares (*Equal/Linear Weighted*), obtendo um valor de 0,845 ($p < 0,001$).

Já para coleta do biofilme supragengival, era solicitado ao doador não se alimentar pelo menos 2 horas antes da coleta. O local a ser amostrado era isolado com rolos de algodão e seco com um jato suave de ar. Com uma cureta Gracey 5/6, toda a placa supragengival era removida da superfície vestibular dos dentes mais distais com placa visível, em seguida, as amostras coletadas eram armazenadas da mesma foram que supracitado.

O pseudobiofilme lingual foi coletado do centro do dorso lingual seguindo as orientações do *NIH Human Microbiome Project (23)*. Foi solicitado que o doador abrisse a boca e estendesse a língua levemente e um *swab* estéril foi passado por 10 segundos na parte posterior da língua em movimentos de um lado para o outro, seguindo um padrão imbricado para maximizar a coleta. Imediatamente após o esfregaço, o *swab* era pressionado contra a parede do tubo microtubos várias vezes durante 20 segundos para garantir a transferência dos microrganismos. O *swab* foi

cortado com tesoura estéril e mantido no microtubo até o momento da extração do DNA (24).

5.2.5 Extração do DNA genômico

A extração do DNA dos microrganismos encontrados nas amostras se deu seguindo o protocolo estabelecido por Smalla et al. (1993)(25), que já vem sendo utilizado com sucesso para extração de DNA de procariotos. O protocolo consistiu nas amostras serem suspensas em 472µL de tampão TE contendo 3 µL de Proteinase K (20 mg/mL) e 5µL de RNAse A (10 mg/mL), seguido da adição de 15µL de SDS a 20%, mistura suave e incubação a 37 °C por 1 hora. Após essa etapa, foram adicionados 500µL de clorofane (fenol, clorofórmio e álcool iso-amílico, na proporção 25:24:1), homogeneizados invertendo-se os tubos, e as amostras foram centrifugadas a 7000g por 3 minutos a 4 °C. O sobrenadante foi cuidadosamente transferido para novos microtubos de 1,5ml. Em seguida, foram adicionados 400µL de clorofil (clorofórmio e álcool iso-amílico, na proporção 24:1) ao sobrenadante, a solução resultante foi homogeneizada e centrifugada novamente a 7000g por 3 minutos a 4 °C, transferindo-se o sobrenadante para outro microtubo. Foi adicionado NaCl (para uma concentração final de 0,3 M) e 3 volumes de etanol 100% gelado, seguido de armazenamento a -20 °C por uma noite. No dia seguinte, as amostras foram centrifugadas a 12000 g, a 30 minutos a 4 °C, com descarte do sobrenadante e duas lavagens consecutivas com 500 µL de etanol 70% gelado, seguidas por centrifugação a 12000 g por 20 minutos a 4 °C. Após as lavagens, para secagem do pellet de DNA, os tubos foram colocados em um heatblock a 55°C por 5 minutos para remoção do etanol. Por fim, os pellets secos foram ressuspendidos em 50µL de água ultrapura.

Para as amostras de dentina cariada, após a incubação na solução de lise descrita acima (TE, Proteinase K (20 mg/mL), RNAse A (10 mg/mL) e SDS a 20%) foi realizada agitação mecânica como *microbeads* e o restante do protocolo de extração foi realizado conforme descrito acima. Todas as amostras foram mantidas em -80°C até o momento da extração, assim como o DNA extraído.

Os DNAs foram quantificados utilizando *NanoVue™ Plus Spectrophotometer* (GE Healthcare, UK). O espectrofotômetro foi calibrado utilizando água ultrapura como branco para garantir a precisão das leituras. Em seguida, um volume de 1 µL de cada

amostra de DNA foi pipetado diretamente na superfície de leitura do NanoVue™, conforme as instruções do fabricante, com a tampa fechada durante a leitura para evitar evaporação. Após cada leitura, a superfície do equipamento foi limpa com água destilada e papel sem fibras para evitar contaminação cruzada.

5.2.6 Análise da composição microbiana

5.2.6.1 PCR*:*

Foi realizada a amplificação a partir do DNA extraído de cada amostra, usando um par de iniciadores específicos para o gene *mcrA:* LuF (GGTGGTGTMGGATTCACACARTAYGCWACAGC) e LuR (TTCATTGCRTAGTTWGGRTAGTT) (26).

As reações de PCR foram realizadas em sistemas com volume final de 50µL, contendo 3µL de DNA total, em diferentes concentrações variando de 20ng a 100ng. As concentrações finais para os demais reagentes para as reações de PCR com os iniciadores LuF e LuR foram: 2X DreamTaq Green PCR Master Mix™ (Thermo Scientific™), 400ng/µL de soroalbumina bovina (BSA, Thermo Scientific™), 0,5µM de cada iniciador.

Os ensaios de PCR foram realizados em termociclador (Kasvi, k33-20TG, Paraná, Brasil) nas seguintes condições de ciclagem: desnaturação inicial em 95º C por 2 minutos, 30 ciclos incluindo desnaturação a 95º C por 30 segundos, anelamento a 56ºC por 30 segundos e a extensão a 72ºC por 1 minuto, com o passo de extensão final a 72ºC por 20 minutos.

Em todas as reações de PCR realizadas, dois controles negativos foram utilizados. O primeiro foi feito adicionando-se água ultrapura à reação em substituição ao DNA. Para o segundo, foi feito o crescimento do *Streptococcus mutans* (ATCC25175) em placa BHI, foi coletada uma colônia e diluída em 50 µl de água ultrapura. Para controle positivo utilizou-se o DNA de *Methanobrevibacter oralis* (*DMZ* 7256) na concentração de 10ng/µL.

Os produtos das reações de PCR foram analisados por eletroforese em gel de agarose 1,5%, corado com Sybr safe (Invitrogen, Califórnia, Estados Unidos), e o

tamanho das bandas foi estimado com o auxílio do marcador de massa molecular 1kb Plus DNA Ladder (Invitrogen, Califórnia, Estados Unidos).

5.2.6.2 qPCR*:*

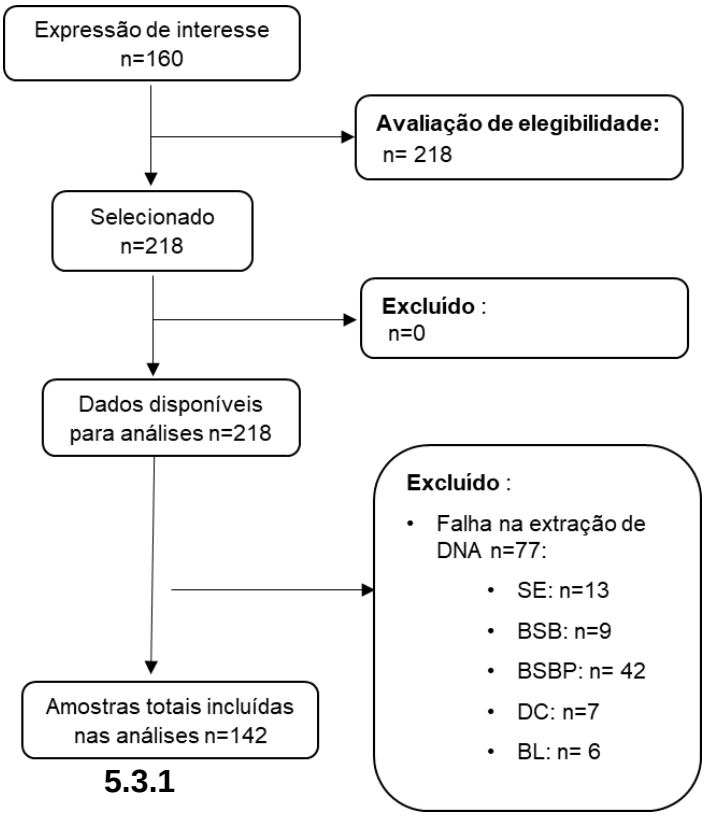
Os ensaios de qPCR foram realizados em um termociclador de PCR em tempo real (StepOnePlus™, Applied Biosystems™), utilizando-se os mesmos iniciadores específicos para arqueias metanogênicas - LuF e LuR (26). Cada reação continha um volume final de 20µL, composto por 2µL de DNA molde em concentrações 20ng/µL, 10µL de solução master mix (PowerTrack™ SYBR Green Master Mix, Applied Biosystems™), e concentrações finais dos iniciadores de 0,5µM cada. O protocolo de amplificação consistiu em uma etapa inicial de 95 °C por 2 minutos para ativação da DNA polimerase, seguida por 40 ciclos de desnaturação a 95 °C por 30 segundos, anelamento a 56 °C por 30 segundos, e extensão a 72 °C por 1 minuto.

Em todas as reações de qPCR, os controles negativos e positivos foram utilizados. Para o controle negativo foi adicionando água ultrapura à reação em substituição ao DNA e para o controle positivo foi usado o DNA de *Methanobrevibacter oralis* (7256) na concentração de 10ng/µL.

Para determinar a especificidade das amplificações, foi realizada uma curva de *melting* ao final dos ciclos de amplificação. Para comparação, Reações usando os iniciadores 519F (CAGCMGCCGCGGTAA) e 1041R (GGCCATGCACCWCCTCTC) (27), tendo como alvo o gene rRNA 16S também foram testadas, nas mesmas condições já citadas acima. Todos os experimentos foram realizados em duplicadas.

5.3 RESULTADOS

O estudo começou com a inclusão de 213 doadores, superando a meta inicial de 160 indivíduos (Figura 1, fluxograma STORMS). No entanto, devido a falhas no processo de extração de DNA, principalmente nas amostras de biofilme subgengival (BSBP), foram analisadas 142 amostras no total. Todas essas amostras foram submetidas à PCR e 110 foram analisadas por qPCR.



5.3.1

Figura 1: Fluxograma STORMS, descrevendo as amostras obtidas e analisadas no estudo.

5.3.2 Resultados clínicos

As principais características dos indivíduos doadores de amostras por grupo estão apresentadas na Tabela 1, incluindo a descrição de indivíduos com doenças crônicas como diabetes mellitus e hipertensão arterial sistêmica.

Os doadores de saliva apresentaram um fluxo salivar médio de 2,19 ± 1,38 ml/min, indicando uma variação significativa entre as amostras. O pH médio observado foi de 7,41 ± 0,576. A capacidade tampão da saliva teve uma média de 3,43 ± 1,48, sugerindo uma variabilidade na capacidade das amostras de neutralizar ácidos.

A análise dos índices periodontais dos doadores de biofilme subgengival revelou que a profundidade de sondagem (P.S.) apresentou uma média de 5,89 ± 1,94, sangramento à sondagem (%SS) teve uma média de 37,1 ± 23,6 e o percentual de índice de placa visível (%IPV) apresentou uma média de 57,1 ± 27,5. Os doadores de biofilme subgengival tinham um número médio de dentes de 22,4 ± 4,90.

5.3.3 Resultados microbiológicos

Tabela 1*:* Característica dos doadores de amostras

Grupo	N	Média Idade	Desvio Padrão Idade	% Masculino	% Feminino	% Diabetes	% Hipertensão	% Doença Periodontal
SE	33	45,1	17,9	39,4%	60,6%	15,2%	30,3%	51,5%
BSP	27	46,7	14,3	40,7%	59,3%	33,3%	37%	66,7%
BSBP	22	53,2	12,9	27,3%	72,7%	54,5%	72,7%	100%
DC	28	40,6	15,3	51,9%	48,1%	17,9%	28,6%	28,6%
BL	32	32,9	19,2	18,8%	81,3%	25%	31,3%	18,8%

5.3.3.1 PCR

Houve detecção de amplificação de DNA em duas amostras de saliva estimulada e uma de biofilme subgengival, apresentando bandas no tamanho esperado, de aproximadamente 496 pb. Um exemplo dos géis de agarose gerados é apresentado na figura 2, na qual pode ser visualizada uma banda do tamanho esperado para o controle positivo e para a amostra SE23. As amostras que exibiram bandas no tamanho esperado foram consideradas positivas para o alvo em questão, sugerindo a presença de arqueias metanogênicas a ser confirmado após sequenciamento dos amplicons.

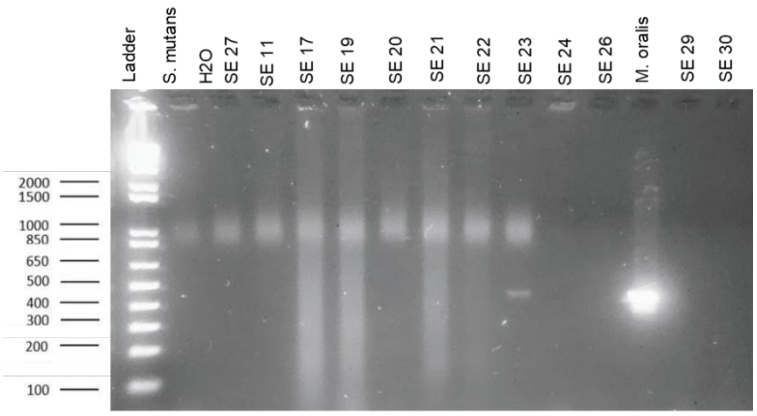


Figura 2: Eletroforese em gel de agarose 1,5% relacionado à amplificação por PCR do gene mcrA a partir de amostras de saliva estimulada. O perfil eletroforético revelou fragmento do tamanho esperado na amostra SE23 amplificado por PCR para os iniciadores escolhidos.

5.3.3.2 qPCR

A amplificação por qPCR foi realizada em 22 amostras de cada grupo que possuíam quantidade de DNA acima de 40ng/µL. Após a curva de melting, seis amostras de BSBP, três amostras de biofilme supragengival, quatro de dentina cariada e seis de saliva estimulada se mostraram com possíveis resultados positivos para a presença de arqueias metanogênicas.

Os resultados do experimento de qPCR indicaram variações na detecção do gene *mcrA* nos diferentes tipos de amostras. Nas amostras de biofilme subgengival, a abundância do gene *mcrA* foi relativamente estável em relação ao gene 16s.

Considerando que as análises de qPCR e PCR convencional de todos os sítios analisados, exceto a língua, apresentaram amplificação compatível com o gene alvo, os resultados sugerem a presença de *Archaea* nas amostras de saliva estimulada, biofilme supragengival, biofilme subgengival e dentina cariada investigadas, resultado a ser confirmado com sequenciamento dos *amplicons* (Figura 3).

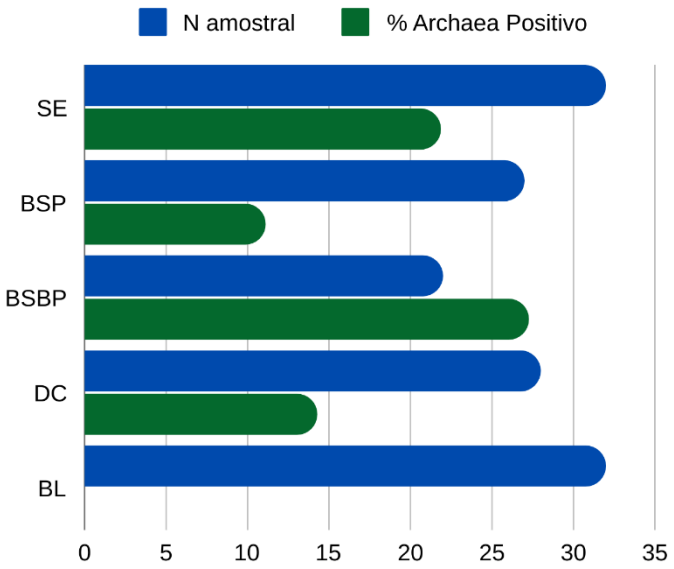


Figura 4: Prevalência de amostras putativas para presença de arqueias metanogênicas. Legenda: SE – saliva estimulada; BSP – biofilme supragengival; BSBP- biofilme subgengival, DC – dentina cariada, BL – pseudobiofilme lingual.

5.4 DISCUSSÃO

Nossos dados indicam que possivelmente há distribuição de arqueias metanogênicas nos principais sítios orais. Para a população avaliada, a prevalência variou de 11,1% a 27,2% de sítios orais positivos para arqueia, de certa forma corroborando com as revisões sistemáticas prévias onde metanalisamos médias da proporção de arqueia nos sítios periodontais em endodônticos (18, 22).

Foi observado a diferença de proporção de detecção entre a PCR convencional e a qPCR. Na literatura encontramos relatos que a qPCR favorece a detecção de espécies de baixa abundância (28), contudo é necessário considerar que houve variação do protocolo de ciclagem utilizado entre os ensaios de PCR e de qPCR, com um maior número de ciclos empregados nos ensaios de qPCR e padronizações dos protocolos devem ser realizadas para ambos os ensaios para a confirmação dessa diferença. Outra questão metodológica relevante foi a escolha do método de extração de DNA, visto que estudos prévios demonstraram que diferentes métodos de extração podem levar à identificação de conjuntos microbianos distintos, influenciando os resultados e interpretações (29, 30). Assim, é importante considerar essa variável em estudos futuros para garantir maior precisão nos dados obtidos.

Essas dificuldades inerentes as técnicas escolhidas podem justificar a ausência de amostras arqueias positivas para as amostras de língua, visto que, em estudo anterior (31) sequências arqueais foram identificadas em 73,0% das amostras de língua. Contudo, no estudo de Gohler (2018) (31) o gene alvo foi o gene 16s rRNA, que usamos como gene referência, e em análise *in silico* o par de iniciadores utilizados por Gohler teve uma baixa cobertura para arqueias (32). Como discutido no artigo do Capítulo II desta tese, a seleção de primers representa um desafio significativo na identificação precisa de arqueias em amostras humanas (32). Neste estudo, os primers LuR e LuF foram escolhidos para amplificação do gene mcrA, devido aos resultados positivos relatados na literatura para a detecção de metanogênicos em diferentes estudos sobre o arqueoma oral (26, 33-40). Contudo, diferenças de tamanho e *temperatura de melting* entre esses primers podem dificultar a otimização da técnica de amplificação. Assim, para futuros estudos, um novo par de primers mais seletivo, baseado nas mesmas regiões-alvo de LuR e LuF, mas com parâmetros

ajustados para melhorar a especificidade e eficiência da amplificação deve ser utilizado.

Além das questões metodológicas, outro fator que diferencia nosso estudo do de Gohler (31) é a diferença amostral já que nossos doadores do pseudobiofilme lingual eram em sua maioria adultos jovens, sem agravos de saúde oral ou sistémica e boa higiene oral. Os dados atuais indicam que arqueias tendem a estar presentes em biofilmes maduros onde é possível encontrar um microambiente anaeróbico o que pode ser difícil acontecer em doadores que realizam com frequência a escovação da área, a média de escovação diária foi de 2,625 ± 0,49 (41). Isso demonstra a importância de estudos englobando diferentes populações em diferentes condições de saúde como próximos passos dessa pesquisa.

As amostras de biofilme subgengival de pessoas com doença periodontal foram as que tiveram maior proporção de detecção positiva (6/22), assim como esperado. Nossa metanálise prévia demonstrou que quase metade de amostras de doença periodontal são positivas para arqueia (22). Os doadores dessas 6 amostras positivas eram indivíduos com profundidade de sondagem ≥5mm o que reforça a hipótese de que a presença de arqueais metanogênicas se correlaciona com o aumento da gravidade da doença periodontal (42, 43). Estudos futuros que analisem a atividade metabólica e expressão gênica nesse tipo de amostra podem auxiliar a elucidar o real papel das arqueias na doença periodontal.

Proporcionalmente ao tamanho amostral, nosso estudo reforça os achados de Bouzid (2024) (44) e Grine (2018) (45) em de que há arqueias metanogênicas na saliva. A hipótese das arqueias serem achados de uma “contaminação temporária” perde força devido as diferenças culturais, de hábitos e idade dos indivíduos doadores dos estudos. Apesar da saliva parecer ser um sítio somente aeróbico, estudos tem mostrado que há formação de complexos microbianos em que determinas espécies “protegem” a outras permitindo a sobrevivência de espécies extremamente anaeróbicas (46, 47), como as arqueias. A saliva humana contém uma diversidade de espécies microbianas colonizadoras precoces e tardias, que interagem entre si e aderem a diversas superfícies da cavidade oral, resultando na formação de biofilmes (46). Sendo assim, a presença de arqueias na saliva expande a distribuição arqueal

na cavidade oral, não se limitando a bolsas periodontais como pensado anteriormente (48).

Os biofilmes supragengivais estão entre as comunidades microbianas mais diversificadas e têm sido essenciais para o avanço do conhecimento sobre a biologia e o desenvolvimento biofilmes (49). Embora os microrganismos que o compõe possam contribuir para a saúde oral e geral, eles também desempenham um papel central nas disbioses bucais (46). Em estudo prévio, observamos a superexpressão de adesinas arqueais em amostras de biofilmes supra gengival de individuos sem cárie, o que indica que as arqueias podem ter um papel secundário na formação do biofilme supragengival em homeostase (Damé-Teixeira, $^{in\ prelo}$). Ainda não entendemos se eles diminuem em situação de doença por não suportarem o ambiente ácido, ou se tem papel protetor contra doença cárie.

Devido as condições de nutrição e metabolismo a possibilidade de identificar arqueias em biofilmes maduros é maior o que justificaria a baixa frequência de amostras positivas em biofilmes supragengivais (3/27). Outro fator é a metodologia que utilizamos, voltada para identificação apenas de arqueias metanogênicas e em estudo piloto encontramos níveis detectáveis de outros tipos de arqueias em amostras de biofilme supragengival sem cárie (17). Nesse estudo piloto também realizamos o primeiro relato de presença de arqueias em dentina cariada ao obtermos um OTU afiliado ao clado *Methanocella*, e outro afiliado ao grupo I.1b do filo *Thaumarchaeota*. No presente estudo ampliamos o tamanho amostral e tivemos identificação positiva de arqueia metanogênica em 14% das amostras de dentina cariada.

Não podemos afirmar que as arqueias estão diretamente envolvidas com a saúde oral ou com processos de disbiose. Contudo, nossos resultados são indícios da presença de membros desse domínio em diversas condições da cavidade oral. Os dados de detecção obtidos pela qPCR, sugere que as arqueias estão presentes em baixa abundância. No entanto, a confirmação da amplificação dos genes pretentidos (mcrA e rRNA 16S de arqueia) e, por sua vez, da presença de arqueias metanogênicas nas amostras avaliadas, dependerá do sequenciamento dessas amostras. Nossos próximos passos incluem a realização de *next-generation sequencing.*

Um ponto limitador do nosso estudo foi a utilização de uma amostragem por conveniência. Esse método não permitiu o controle de fatores confundidores, como a variabilidade interindividual na composição do microbioma, no estilo de vida, na dieta, no consumo de álcool e na qualidade da higiene oral. Estudos futuros que investiguem essas variáveis de forma mais abrangente são necessários para aprofundar o entendimento sobre a biogeografia do arqueoma oral. Além disso, apesar dos resultados obtidos sugerirem a presença de arqueias nos sítios analisados, os protocolos utilizados podem ser aprimorados para garantir maior confiabilidade e precisão nos ensaios. A ocorrência de bandas inespecíficas, bem como a intensidade reduzida das bandas específicas, indica a necessidade de ajustes nos parâmetros da reação de PCR, como a otimização da concentração de primers, condições de anelamento e ciclos de amplificação, para melhorar a especificidade das reações. Além disso, a padronização das quantidades de DNA inicial, bem como a inclusão de curvas padrão nos ensaios de qPCR, é fundamental para obter informações quantitativas robustas e reprodutíveis. Outro aspecto essencial é a realização do sequenciamento para confirmar a amplificação dos alvos pretendidos, validando os resultados obtidos

5.5 CONCLUSÃO

Concluímos, nesse estudo preliminar, que arqueias metanogênicas podem estar amplamente distribuídas nos principais sítios orais, embora em baixa prevalência na população avaliada. A maior detecção de arqueias em biofilmes subgengivais de pacientes com bolsas profundas, reforça a hipótese de que arqueias estão associadas a condições periodontais severas, enquanto sua presença na saliva e em biofilmes supragengivais expande a compreensão sobre sua distribuição ubíqua na cavidade oral. Diferenças metodológicas, como o tipo de PCR utilizado, e fatores amostrais, incluindo variabilidade individual e hábitos de higiene oral, podem ter influenciado os resultados. Estudos futuros devem incluir análises metagenômicas, para esclarecer o papel das arqueias na saúde oral e nas condições de disbiose.

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6 CAPÍTULO VI - DISCUSSÃO GERAL E CONCLUSÕES DA TESE

6.1 DISCUSSÃO GERAL

> "*Para a imprensa e o público, a descoberta das arqueias foi um evento altamente significativo; tocou naquela antiga preocupação humana básica sobre de onde viemos - que interessava mais ao leigo do que a promessa de um futuro mais brilhante por meio da tecnologia biomédica. A comunidade biológica, por outro lado (embora não os cientistas em geral), teve uma reação decididamente diferente... no nível mais alto expressando os caracteres abrangentes da organização celular procariótica e eucariótica. Afirmar que um terceiro grupo primário existia era patentemente absurdo!" (Carl Woese, 1993(1)*)

Originalmente descritas como habitantes de ambientes extremos, as arqueias revelaram sua presença difundida e foram reconhecidas em abundância como parte integrante do microbioma humano nos últimos anos (2). Embora os avanços metodológicos tenham melhorado a detecção dessas arqueias, elas frequentemente ainda são negligenciadas em muitos estudos que investigam as interações entre os membros da microbiota humana (3).

Os estudos relativamente recentes consideram que os representantes *Archaea* na cavidade oral são limitados (4), o foco principal em organismos abundantes com potenciais patogênicos faz com que o entendimento dos microrganismos de baixa abundância, que poderiam gerar um efeito borboleta na homeostasia do microbioma seja insuficiente (5). A importância dos domínios microbianos minoritários, como as arqueias, na disbiose oral é cada vez mais destacada.

A diversidade bacteriana nos nichos orais e suas interações metabólicas são bem documentadas. Contudo, a presença de arqueias nesses ambientes trouxe um novo fator a ser considerado. Evidências sugerem que arqueias possam desempenhar um papel ativo em infecções polimicrobianas (6), interagir sintroficamente com bactérias (7) ou ser antagonista a outros micro-organismos que competem pelo hidrogênio, como bactérias redutoras de sulfato (7, 8).

As arqueias são componentes do microbioma oral humano, presentes em diferentes nichos e com potenciais implicações na saúde e nas doenças orais (9). Atualmente, não há evidências substanciais que sustentem as propriedades patogênicas das arqueias, possivelmente devido às dificuldades associadas à detecção, identificação, isolamento desses microrganismos.

Pesquisas futuras, com abordagens metodológicas diversificadas e padronizadas, são essenciais para aprofundar a compreensão do arqueoma oral humano e esclarecer os papéis específicos das arqueias nos diferentes sítios orais, que ainda permanecem pouco compreendidos.

6.1.1 Limitações

Durante o meu percurso de doutorado, enfrentei desafios que ilustram bem as dificuldades enfrentadas por pesquisadores no Brasil. Logo no início do processo, deparamo-nos com uma pandemia que atrasou o início e dificultou o processo de coleta das amostras. Apesar de a pandemia ser uma circunstância fora do nosso controle, decidimos focar em revisões sistemáticas para manter o andamento da pesquisa.

Além disso, enfrentei de perto os desafios que o pesquisador brasileiro encara, indo além das constantes ameaças de cortes de verbas que afetam diretamente a continuidade e a qualidade da pesquisa científica. Após a conquista das amostras, deparei-me com uma dificuldade específica: a obtenção de controles positivos de arqueias, essenciais para validar as metodologias que eu buscava implementar. Descobri que, no Brasil, não havia fornecedores disponíveis dessas espécies, o que nos obrigou a recorrer à importação. Esse processo de importação revelou-se longo e desafiador, levando cerca de dois anos para ser concluído.

Tivemos duas tentativas frustradas em que as encomendas foram devolvidas. A burocracia envolvida nas exigências da ANVISA foi um obstáculo significativo, resultando em procedimentos que nos faziam andar em círculos sem solução prática a tempo. A solução veio apenas na terceira tentativa, graças à colaboração da Prof. Dra. Thuy Do, que providenciou o envio, e de um despachante que, mediante uma taxa, nos auxiliou com o preenchimento de formulários não divulgados no site da ANVISA.

Finalmente, após quase dois meses de tramitação e muita expectativa, conseguimos receber os controles em Brasília. Somente a partir desse momento, já faltando cinco meses para a data de defesa, pude iniciar os experimentos necessários para o andamento e conclusão da pesquisa. Essa experiência evidenciou o quanto a

burocracia e a falta de infraestrutura científica interna podem impactar a eficiência e o sucesso de projetos de pesquisa no país.

Apesar de todos esses desafios, com criatividade e soluções inovadoras, conseguimos obter muitos sucessos ao longo desses quatro anos. Fomos convidados a desenvolver dois capítulos de livro e publicamos cerca de dez artigos, fruto de parcerias com diferentes grupos de pesquisa, inclusive internacionais. Os trabalhos condensados nesta tese chamaram a atenção da comunidade científica mundial, e nosso time foi convidado a palestrar em eventos de destaque da odontologia, como o AADR e o IADR.

Essas são apenas algumas das conquistas de um projeto que começou como um PIBIC, iniciado lá em 2018 quando ainda não conhecia quem eram as arqueias. Esse mesmo projeto permitiu, ainda na graduação, a publicação da revisão de escopo sobre o arqueoma oral que embasou os objetivos desta tese no maior periódico de odontologia, o *Journal of Dental Research* (JDR).

No entanto, ainda há um longo caminho a percorrer para a plena compreensão das arqueias na cavidade oral. Esse percurso inclui a necessidade urgente de disseminar o conhecimento sobre esses microrganismos como um domínio próprio e único, especialmente entre os acadêmicos e profissionais da odontologia, onde sua relevância ainda é subestimada. É igualmente essencial reforçar a defesa do papel crucial das pesquisas básicas, que são a base para a ampliação do conhecimento aplicado. Sem essa fundamentação, muitas oportunidades para avanços na prática clínica, como novas abordagens diagnósticas e terapêuticas, podem ser perdidas. O estudo das arqueias, além de fornecer *insights* sobre seu impacto no microbioma oral e na saúde geral, abre portas para colaborações interdisciplinares, que são indispensáveis para a evolução da ciência odontológica. Para alcançar esses objetivos, é necessário integrar esforços para fomentar interesse e investimentos no campo, criar plataformas de discussão científica e promover a conscientização sobre a relevância desse domínio, tanto no Brasil quanto no cenário internacional.

6.1.2 Conclusões

As principais conclusões dessa tese são:

- Arqueias fazem parte da microbiota oral;
- Arqueias são prevalentes em casos de periodontite, mas seu papel no desenvolvimento e progressão da doença ainda é pouco compreendido;
- A terapia adjuvante com antibióticos sistêmicos pode não aumentar significativamente a redução de células arqueanas já observadas após a terapia periodontal convencional;
- Arqueias compõe a microbiota endodôntica e participar de interações sintotróficas com táxons bacterianos, contribuindo para processos infecciosos.
- Cerca de um quinto dos canais radiculares infectados contem arqueias;
- A metanogênese é superexpressa em biofilmes e saliva de pessoas livres de cárie quando comparada a pessoas com cárie;
- Estudos adicionais, com metodologias que permitam uma detecção mais abrangente da diversidade arqueana, principalmente a não metanogênica, são necessários para esclarecer o papel dessas células no arqueoma periodontal.

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7 CAPÍTULO VII - PRESS RELEASE

Arqueias são microrganismos únicos que, apesar de já terem sido estudados no início do século XX, eram inicialmente associados apenas ao trato gastrointestinal humano. Mais tarde, descobriu-se que elas também habitam a cavidade oral, ampliando nossa compreensão sobre a microbiota bucal.

Os dados dessa tese sugerem que arqueias podem desempenhar um papel como "patógenos secundários" em ambientes onde ocorre disbiose – um desequilíbrio na microbiota. Elas parecem ser favorecidas por condições inflamatórias, como a periodontite, já que nossos estudos mostram que sua prevalência é maior em indivíduos com doenças bucais do que em pessoas saudáveis. Essa presença mais comum em condições patológicas sugere que elas podem interagir com bactérias, potencialmente contribuindo para o avanço de doenças.

A detecção de arqueias na boca, incluindo em biofilmes dentários e canais radiculares infectados, foi possível graças a tecnologias como o sequenciamento de DNA. No entanto, as metodologias atuais ainda limitam nossa capacidade de identificar toda a diversidade de arqueias e compreender seu papel na saúde e nas doenças bucais.

Estudos futuros, com técnicas mais sensíveis e diversificadas, são fundamentais para confirmar se esses microrganismos contribuem diretamente para doenças bucais ou se apenas coexistem com outros microrganismos. Entender o "arqueoma" da cavidade oral pode levar a novas estratégias de tratamento para doenças como a periodontite e a cárie.

8 ANEXO 1: THE CARIES AND CARIES-FREE ARCHAEOME

Submetido para publicação, em revisão por pares

8.1 ABSTRACT

Aim: The difficulty of establishing a relationship between archaea and oral diseases such as dental caries stems from the challenges of detecting, identifying, and isolating these microorganisms. This study aimed to detect archaea in publicly available datasets comprising caries and caries-free saliva and supragingival biofilms by using a tailored bioinformatic pipeline for shotgun sequencing analysis.

Methods: A systematic search was performed to identify studies using shotgun metagenomics or metatranscriptomics on samples obtained from individuals with dental caries. Two reviewers selected studies based on eligibility criteria. Sequencing and metadata from each study were retrieved from their SRA Bioproject. A count table was generated for each database by mapping reads against an archaea genome database, specifically tailored for this study, using stringent filtering parameters of above 95% similarity and 90% query coverage. Archaeal prevalence was determined using an arbitrary cut-off point (>500 reads). An effect size meta-analysis was performed to determine the overall prevalence. Phyloseq and DESeq2 packages were used to determine alpha and beta diversities, differential abundance in different taxonomic levels, and differential expression comparing caries and caries-free samples. Spearman correlation was performed with the bacteriome.

Results: The search yielded 154 titles, from which a collection of 7 datasets from 8 studies was obtained. N=63 samples out of 397 samples were positive for archaea using a post-filtering, comprising a putative prevalence of 20% (CI = 0-40%), and identifying Euryarchaeota, Thermoplasmatota, and Nitrosphaeria. Methanogens were present in both caries and caries-free groups (Methanobrevibacter spp., Methanosarcina and Methanosphaera), and positively correlated with Strephotrophomonas, Streptococcus, Actinomyces, Abiotrophia, Gemella, and Corynebacterium. Several methanogenesis genes, including Methyl-coenzyme M reductase, which catalyzes the final step of methane production in methanogens, were overexpressed in caries-free samples compared to caries-active samples.

Conclusion: Saliva and supragingival biofilms emerged as sites of low-abundance archaea, with methanogenesis genes overexpressed in caries-free samples.

Keywords: Archaea domain, Oral Archaeome, Methanogens, Oral microbiome, Dental Caries, bioinformatic analysis

8.2 INTRODUCTION

Woese *et al*(1990) revolutionized our understanding of life on Earth by proposing a 3-domain system, classifying living organisms into Bacteria, Archaea and Eucarya (1). Prior to this, owing to their morphological resemblance and prokaryote classification, archaea were taxonomically grouped with bacteria. However, the discovery of important phenotypic distinctions, such as the absence of peptidoglycan cell walls and unique enzymes involved in their methanogenic metabolism, prompted the recognition of the need for this reclassification. There was an initial belief that all archaea were exclusively extremophiles. However, the later detection of archaea in several environments, including the isolation of archaea in humans (2), supported the hypothesis that archaea are not exclusive extremophiles and in fact they are ubiquitous and recyclers of organic and inorganic matter.

It is already known that members of the *Archaea* domain constitute a minor portion of the oral microbiome (3). Indeed, small proportions of DNA sequences from methanogenic archaea have been identified in ancient dental calculus; for example, a nearly complete genome of *Methanobrevibacter oralis* was discovered in a Neanderthal with signs of dental abscess (4), indicating that the archaea have been part of the human/humanoid oral microbiome for a long time. Evidence suggests that their prevalence has diminished significantly due to changes in the diet and lifestyle of *Homo sapiens* (5), although studies have demonstrated that archaea are present in at least 40% of subgingival biofilms associated with periodontitis in modern samples (6).

There is very limited evidence of archaea presence or their potential functions in dental caries, potentially representing fundamental gaps in understanding cross-domain interactions in either homeostasis or dysbiosis status (7). In a pilot study, we

have shown the presence of DNA sequences belonging to the *Archaea* domain in dentinal caries lesions, including the low abundance of *Thaumarchaeota* and methanogenic archaea (8). Archaea likely have implications in countless biogeochemical processes, including the nitrogen and carbon cycles, as well as increasing the efficiency of bacterial metabolism by contributing to energy flux using cross-feeding strategies(9). While *Thaumarchaeota* (recently reclassified as *Nitrososphaerota*, with the majority comprised by ammonia-oxidizers) could be disturbing a natural buffer system, potentially influencing the supragingival biofilm balance, methanogenic archaea have an energy metabolism related to methane production. These archaea play a crucial role in the carbon cycle within biofilms, with some species capable of thriving under microoxic conditions (10).

The difficulty of establishing a relationship between archaea and oral diseases such as dental caries stems from the challenges due to several factors including their low abundance (underreported low-abundant microbiota less than 1% of relative abundance in next generation sequencing-NGS studies)(11), primers mismatches (12), unsuitable DNA extraction methods, incomplete reference databases often missing archaeal sequences, and the lack of clinical interest on archaea as no archaeal pathogens are yet identified (13). Also, metagenomic bioinformatic pipelines aims at defining the core microbiome often overlook low-abundance microorganisms (11), such as those belonging to the *Archaea* domain. Nevertheless, due to the challenges associated with accurately identifying true richness and diversity within sequencing data, particularly concerning low-abundance sequences, there is a risk of underestimating the significance of rare organisms within the oral microbiome(11). Therefore, this study aims to develop a rigorous bioinformatic pipeline to identify archaea in shotgun sequencing datasets related to dental caries. To investigate the presence of *Archaea* domain members in dental caries samples, we systematically selected datasets and reanalyzed their sequences using our tailored pipeline.

8.3 METHODS

8.3.1 Eligibility criteria, information source and search strategy

Included studies comprised the ones analyzing DNA shotgun metagenomics and cDNA shotgun metatranscriptomics in samples of individuals with dental caries,

with or without a control group. More details including the question design and exclusion criteria are in Supplementary Table 1. The complete search strategy can be found in Supplementary table 2. A flowchart detailing the process of identification, inclusion, and exclusion of studies is shown in Figure 1A.

8.3.2 Datasets selection process

Two independent reviewers (ND-T and JAC) selected studies based on title and abstract assessment while applying the eligibility criteria. In a second stage, both reviewers selected the eligible studies based on full-text reading. The availability of the sequencing and metadata from each study were retrieved from their SRA Bioproject. Studies without data availability were then excluded.

8.3.3 Bioinformatics (tailored pipeline) and metanalysis

A full non-redundant archaea database was specifically tailored for this study, extracted from the National Center for Biotechnology Information (NCBI). A total of 519 different genomes were included. After downloading each dataset and corresponding metadata from NCBI, a count table was generated for each dataset by mapping reads against this curated archaea genome database, using stringent filtering parameters of above 97% sequencing similarity and 90% query coverage using the Diamond sequence alignment tool (14).

Archaeal prevalence was determined using an arbitrary post-filtering cut-off point, considering the proportion of samples with over 500 archaeal reads in total. This filter was used to avoid false-positive data. For metagenomic analysis, after excluding 10 samples not derived from caries in one dataset (PRJNA396840), samples with more than 500 overall archaeal reads from four studies were used for further analysis. The overall prevalence of archaea was meta-analyzed using the restricted likelihood model for crude proportions with 95% confidence interval (CI) (Jamovi software version 1.6 obtained from https://www.jamovi.org/). For metatranscriptomic analysis, three studies with available mRNA sequences were included. Count tables were imported into R for visualization with Phyloseq (15) and DESeq2 R packages (16). For alpha diversity, ANOVA and post hoc Tukey HSD test were used for normally distributed data, while Kruskal-Wallis and post hoc Dunn’s test were used when data were not normally

distributed. For beta diversity, permutational multivariate analysis of variance tests were used.

A Spearman correlation analysis was conducted using MetaPhlAn 4 to identify the top 20 bacterial genera in the same samples, along with *Tannerella* (previously correlated with methanogens) (17). These genera were correlated with the top 20 most abundant archaeal genera in post-filtered DNA samples. P-values were adjusted using the Benjamini-Hochberg method. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to map the methanogenesis within the carbon metabolism pathway (map01200) using KEGG mapper to identify the methanogenesis overexpressed genes (https://www.genome.jp/kegg-bin/color_pathway_object).

8.4 RESULTS

8.4.1 Datasets selection process

In the initial search, 185 articles were identified. After removing duplicates, 184 articles were screened by title and abstract, and 33 were selected for full-text review. Following a thorough assessment and retrieval of sequencing data and metadata, 26 articles were excluded, leaving 7 studies for inclusion. These studies provided a total of 211 caries-active samples and 168 caries-free samples for DNAseq, and 88 caries-active samples and 98 caries-free samples for RNAseq. For clarity in communication, "caries-active" refers to samples collected from donors with caries, regardless of the specific lesion activity status or index used. A flowchart detailing the process of identification, inclusion, and exclusion of studies is shown in Figure 1A and the number of samples in total and according to presence of archaea for DNA is presented in Figure 1B.

Most datasets had only DNAseq data, but three had both RNAseq and DNAseq (18-20). The studies employed various methods for diagnosing caries, resulting in a highly heterogeneous disease group analyzed here. ICDAS system was the most commonly used for detailed visual classification of caries stages (18, 19, 21). ICCMS and definition disease group as early caries defined as having at least one tooth with

early, non-cavitated carious lesion focusing on lesion activity and severity was also used (22). Another study identified untreated caries affecting three or more surfaces, highlighting severe cases (20), and others used the DMFT index for an epidemiological measure of caries experience (Baker et al. 2021), d1mft index for early caries stages in children (23), or assessed caries prevalence (23). The main characteristics of each included study are shown in Table 1.

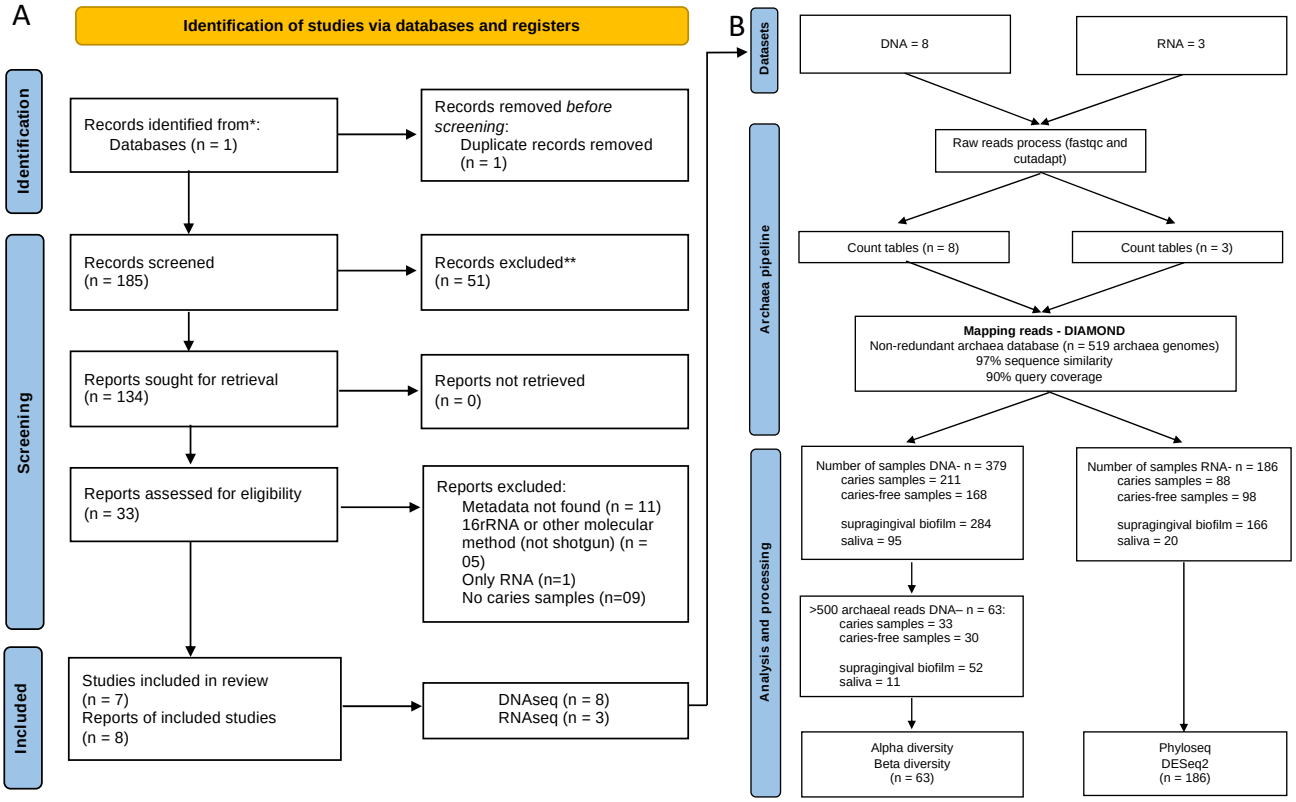

Figure 1. Flowchart (adapted from PRISMA): A) Selection process of the studies and their databases. B) Tailored archaea pipeline. For the metagenomic analysis, 10 non-caries related samples from one dataset under the BioProject PRJNA396840 were excluded (subgingival samples). For the metatranscriptomic analysis, three studies with available mRNA sequences were included and analyzed for differential gene expression.

Table 1. Characteristics of the selected studies, datasets and metadata.

Cod.	Authors	Year	Country	Nucleic acid	Sample	N caries*	N caries free*	Age	Caries diagnosis	obs.
PRJNA766357	Pang et al.	2021	China	DNA	Biofilm	20	20	12–13 years old	ICDAS-II	
20180420	Al-Hebshi et al.	2019	USA	DNA	Biofilm	20	10	6–10 years old	ICCMS	Deciduous teeth

PRJNA712952	Carda-Diéguez et al.	2022	Spain	DNA, RNA	Biofilm	26	27	≥ 16 years old	ICDAS	
PRJNA383868	Espinoza et al.	2018	Australia	DNA, RNA	Biofilm	50	38	5–11 years old	ICDAS II	Deciduous teeth
PRJNA396840	Belstrøm et al.	2017	Denmark	DNA, RNA	S.saliva	10	10	37.6 years old (22–70)	Untreated caries ≥ 3 surfaces	
PRJNA478018	Baker et al.	2021	USA	DNA	S.saliva	23	24	≥3 years old	dmft	Deciduous teeth
PRJNA752888	Blostein et al.	2022	USA	DNA	Biofilm and saliva	99	90	2 months to 5 years old	d1mft	Deciduous teeth
	Blostein et al.	2023	USA	DNA	Saliva	87	75	2 months to 5 years old	Prevalence	Deciduous teeth

S. saliva = stimulated saliva; *Number of samples described in the study

8.4.2 Prevalence and diversity of archaea signs across all samples

DNAseq samples were used to calculate putative prevalence of archaea, independently of their taxonomy across datasets. N=63 samples out of 379 samples were positive for archaea using our post-filter strategy. Using our post-filtering strategy, archaeal reads were not detected in one dataset, and most had very few samples that passed the filter. Most studies had only 1-3 samples from each dataset positive for archaea (18, 19, 21) and one had no signs of archaea (20). Meanwhile, two studies concentrated the highest prevalence of archaea-positive samples (22, 23). While Blostein et al.(23) had the highest number of samples in general, Al-Hebshi et al (22). had a few samples but represented the highest prevalence. It appears that both saliva biofilms can harbor archaea. The relative prevalence for archaea was calculated using a meta-analysis, resulting in 20% (CI = 0 – 40%), independently of their caries status and type of sample (Figure 2A).

Alpha diversity of the archaeome was calculated for the overall samples (Supplementary Figure 2), as well as for the n=63 samples filtered according to the prevalence of archaea (Figure 2B). No differences in diversity between groups, caries and caries-free in both analyses using observed, Chao1 and Shannon indices. Figure 2C shows the beta diversity in general, and for phyla and genus levels. The compositional homogeneity of multivariate dispersions among archaea-positive samples was calculated for 999 permutations. For the pairwise comparison of caries and caries-free samples, a significant variability was observed (p=0.038).

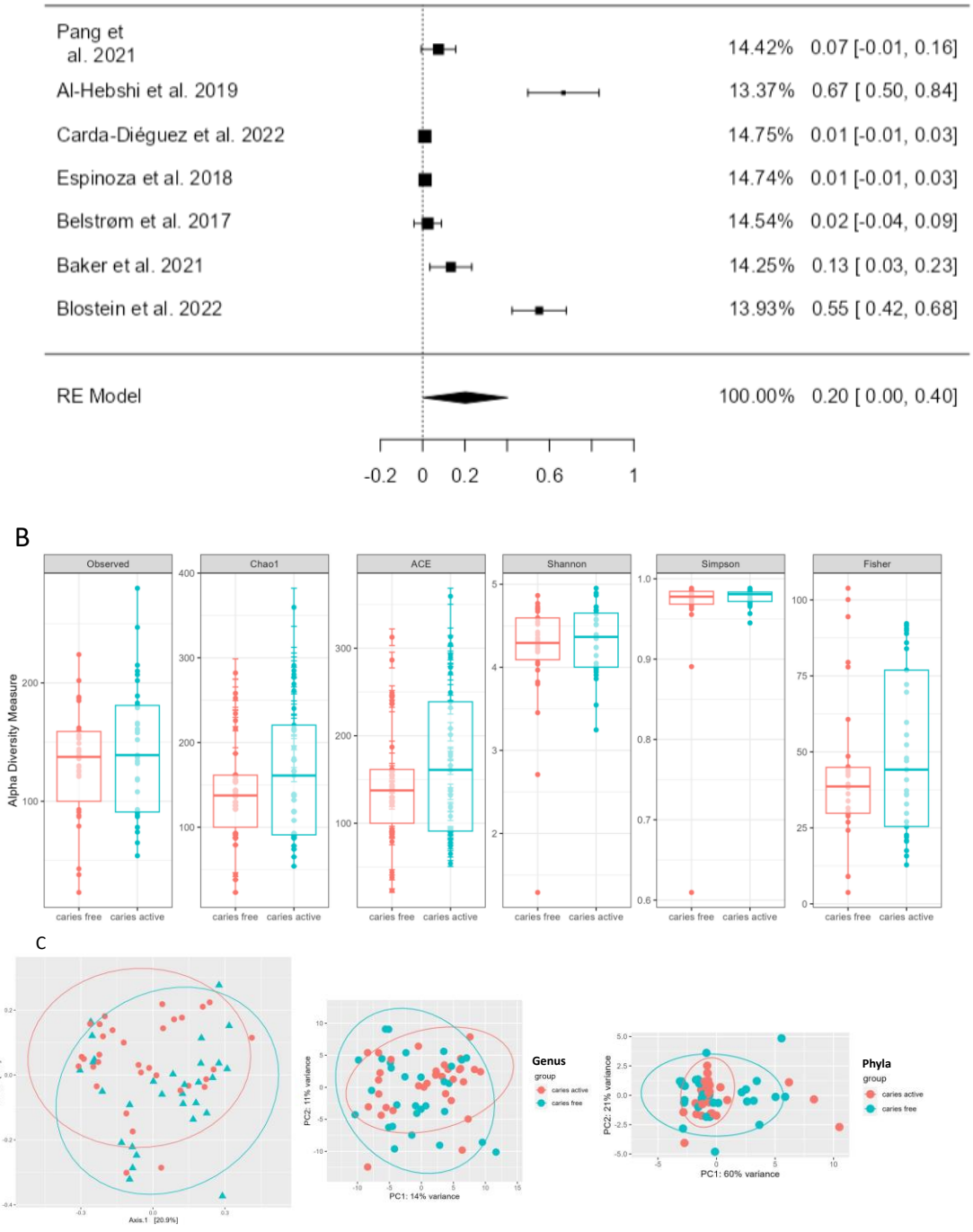

Figure 2. Prevalence and diversity of the archaeome in caries and caries-free samples. A) Overall prevalence of the Archaea domain in caries and caries-free biofilm and saliva samples in 7 metagenomic (DNAseq) studies. The box in the middle of each horizontal line (CI [confidence interval]) represents the point estimate of the effect for a single study, while the diamond represents the overall effect estimate of the meta-analysis. RE (random-effects) Model. B) Alpha and C) Beta Diversity of the archaeome in caries and caries-free samples in general, and in genus and phyla levels. The compositional

homogeneity of multivariate dispersions among archaea-positive samples was calculated for 999 permutations.

8.4.3 Taxonomy of caries vs. caries-free archaeome and their correlation with the bacteriome

Taxonomy averages at the phylum level for post-filtered samples using the >500 reads cut-off is shown in Figure 3A. The taxonomy appears to be more accurate following post-filtering, as it only includes taxa previously identified in oral cavity: *Euryarchaeota* as the most prevalent, followed by *Thermoplasmatota*, *Nitrososphaerota*, and *Thermoproteota*. No significant taxonomy differences between groups with and without caries were observed at the phylum level (p-adjusted ranging from 0.7 to 0.9). Among 150 non-redundant taxa at the genus level, *Methanobrevibacter* had the highest number of reads across all samples, which is expected considering its common occurrence in the oral cavity. It was succeeded by *Methanosarcina, Thermococcus, Methanothermobacter, Methanobacterium, Pyrococcus,* and *Methanococcus.*

Figure 3B shows the differential abundance of archaea in caries versus caries-free samples. While *Nitrososphaeraceae* was enriched in caries samples, the *Euryarchaeota* families *Halococcaceae* and *Thermococcaceae* were depleted (Figure 3B). At the genus level (Figure 3C), *Nitrososphaera* (*Thaumarchaeota* phylum) and *Ferroplasma* (*Euryarchaeota* from *Thermoplasmata* class) were enriched in caries.

Figure 3D shows the Spearman correlation of the archaeome and the core bacteriome. *Strephotrophomonas* was significantly correlated with the highest number of archaea taxa. Interestingly, *Streptococcus* has shown correlations with methanogens, while *Actinomyces* is associated specifically with *Methanobrevibacter*. Other bacteria like *Abiotrophia* and *Gemella* have positive correlations with methanogenic archaea such as *Methanococcus*, *Pyrococcus*, and *Thermococcus*, with *Corynebacterium* being linked to the latter two. In contrast, *Saccharibacteria* is negatively correlated with *Halorussus* and *Methanosphaera*. *Porphyromonas* and *Tannerella* did not show significant correlations with any archaea, which was unexpected given their presence in similar environments.

8.4.4 Archaea functions in caries vs. caries-free

Genes frequently identified across all DNAseq samples included ATP synthase subunit A, PFL family protein, ribulose-bisphosphate carboxylase, exonuclease ABC subunit UvrB, sodium-translocating pyrophosphatase, and ATP-dependent chaperone ClpB (Supplementary Figure 3). No archaeal gene was significantly overrepresented in caries-free samples using the DNAseq data using our post-filtering strategy. Meanwhile, several genes were significantly overrepresented in caries samples (Supplementary Table 3): cation-transporting P-type ATPase, ATP synthase subunit A, replication-associated recombination protein A, V-type ATP synthase subunit A, pyruvate phosphate dikinase, NAD-dependent protein deacylase, cell division protein FtsZ, DNA topoisomerase subunit B, anthranilate synthase component I, and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase.

Additionally, several genes showed overexpression in caries-free samples when only RNAseq were analyzed (Supplementary Table 3). Genes with potentially significant roles in oral biofilms, such as adhesins, glycosyltransferases, growth factors, and agmatinase, were found to be overexpressed in caries-free RNAseq samples (Table 2). What stands out from this analysis is the overexpression of several genes related to methanogenesis in caries-free samples, such as CoB--CoM heterodisulfide reductase iron-sulfur, tetrahydromethanopterin S-methyltransferase, coenzyme-B, and especially coenzyme-M (Table 2). Figure 4 shows the KEGG carbon metabolism pathway confirms that methanogenesis is overexpressed in caries-free samples, including overexpression genes from the beginning to the end of the pathway.

Table 2. Methanogenesis genes overexpressed in caries-free samples compared to caries-active samples (n=186 samples), and their potential functions described for archaea.

Protein Name	Function Description	Methanogenesis phase	Log2 Fold Change	p-adjusted
Formate dehydrogenase subunit alpha	Catalyzes the oxidation of formate to carbon dioxide.	Activation and Conversion of Substrates	1.39	1.83E-05

Formylmethanofuran dehydrogenase subunit A	Catalyzes the reversible reduction of CO2 and methanofuran to N-formylmethanofuran.	Activation and Conversion of Substrates	1.15	8.51E-05
Formylmethanofuran dehydrogenase subunit B	Catalyzes the reversible reduction of CO2 and methanofuran to N-formylmethanofuran.	Activation and Conversion of Substrates	0.97	0.0008
Methanol--corrinoid protein MtaC	Involved in methanogenesis from methanol, transferring methyl groups from methanol to a specific corrinoid protein.	Activation and Conversion of Substrates	0.89	0.0011
Methanol--corrinoid protein co-methyltransferase MtaB	Methyltransferase involved in the methanol pathway, catalyzing the methylation of the MtaC-bound cob(I)amide.	Activation and Conversion of Substrates	0.85	0.0044
Coenzyme F420 hydrogenase subunit beta	Participates in the formatehydrogenylase system in methanogenic archaea.	Activation and Conversion of Substrates	1.34	9.08E-06
Coenzyme F420 hydrogenase/dehydrogenase, beta subunit C-terminal domain	Functions as a bidirectional enzyme in the formatehydrogenylase system.	Activation and Conversion of Substrates	1.31	9.31E-06
Tetrahydromethanopterin S-methyltransferase subunit C	Coenzyme M methyltransferase that drives a sodium ion pump.	Formation of Methyl-Coenzyme M	1.65	1.14E-07
Tetrahydromethanopterin S-methyltransferase subunit E	Coenzyme M methyltransferase that drives a sodium ion pump.	Formation of Methyl-Coenzyme M	1.54	5.83E-07
Tetrahydromethanopterin S-methyltransferase subunit H	Part of a complex catalyzing the formation of methyl-coenzyme M from coenzyme M and methyl-tetrahydromethanopterin.	Formation of Methyl-Coenzyme M	0.79	0.0062
F420-dependent methylenetetrahydromethanopterin dehydrogenase	Catalyzes the fourth reaction step of CO2 reduction to methane.	Formation of Methyl-Coenzyme M	0.67	0.0144
CoB--CoM heterodisulfide reductase iron-sulfur subunit A family protein	Catalyzes the reversible reduction of heterodisulfide of methanogenic thiol-coenzymes.	Reduction to Methane	1.98	6.58E-09
CoB--CoM heterodisulfide reductase subunit B	Catalyzes the reversible reduction of CoM-S-S-CoB to thiol-coenzymes.	Reduction to Methane	0.71	0.0114

Methyl-coenzyme M reductase operon protein D	Catalyzes the last step of methane reduction in methanogens.	Reduction to Methane	0.69	0.0111
Methyl-coenzyme M reductase I operon protein C	Catalyzes the last step of methane reduction in methanogens.	Reduction to Methane	0.57	0.0289
Coenzyme-B sulfoethylthiotransferase subunit gamma	Catalyzes the last step in methanogenesis.	Reduction to Methane	1.35	9.08E-06
Coenzyme-B sulfoethylthiotransferase subunit alpha	Catalyzes the final step in methane formation.	Reduction to Methane	0.75	0.0178
5,10-methenyltetrahydromethanopterin hydrogenase	Catalyzes the reversible reduction of methenyl-H4MPT+ to methylene-H4MPT.	Reduction to Methane	0.89	0.0012

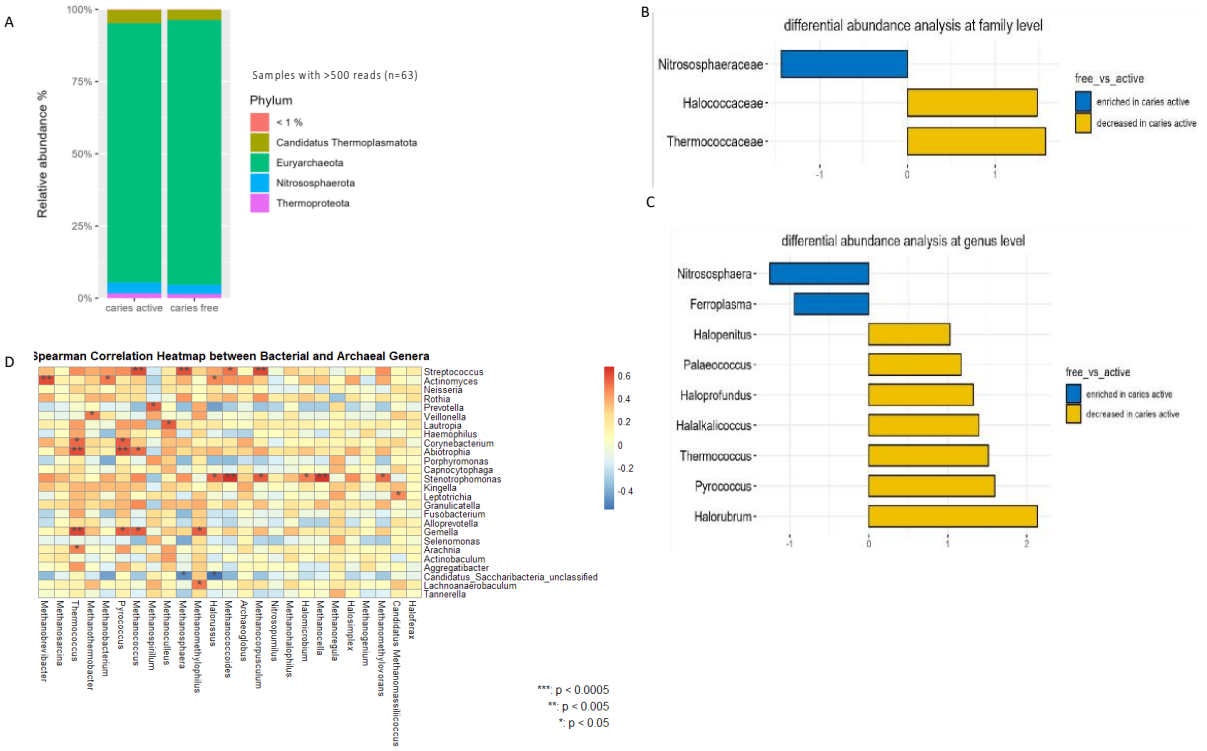

Figure 3. Taxonomy of the caries and caries-free archaeome. Averages at the phylum level (A) (n=63 post-filtered samples using the >500 reads cut-off); Differential abundance of archaea in caries vs. caries free samples, calculated by DESeq at family (B) and genus (C) levels; D) Spearman correlation of the archaeome and bacteriome.

8.5 DISCUSSION

Archaea are often overlooked in oral microbiome studies, largely due to methodological constraints including primer sensitivity. Our recent *in silico* analysis of primers used for detecting archaea in human samples revealed significant gaps in coverage, highlighting the need for improved methodologies (13). To overcome this primer issue, omics studies can expand our understanding of the oral archaeome. To address limitations on the bioinformatics noise in mapping the shotgun sequencing reads, we advocate for a shift towards considering not just the abundance but also the prevalence of low-abundance taxa, thereby encompassing a broader spectrum of microbial diversity. We re-examined metagenomics data from 379 samples, including 63 samples that tested positive for archaea. The domain was considered as "detected" only if it met our defined threshold; otherwise, it was reported as "not detected". Even if each detected taxon is present in low abundance, archaea could still be classified as positive if their signal surpasses the threshold of 500 reads in total. The relative prevalence found here aligns with findings from endodontic samples analyzed through various molecular methods, where archaea were similarly present in 20% of individuals in a meta-analysis(24). This suggests that the actual prevalence of archaea in most oral samples can be ~10-20%. Therefore, it seems that our post-filtering could be applied in metagenomics to detect archaea ubiquity.

There has been no exploration of archaea potential commensal or protective role within the oral microbiome, despite extensive discussions on the role of archaea in oral pathogenesis. Some propose that archaeal pathogenesis does not occur due to the absence of essential virulence factor genes or because their cell walls do not allow interdomain DNA changes with the host, however, another hypothesis is that archaea has not yet been fully explored (“not-yet-discovered hypothesis”)(25). To better explore the oral archaeome, we advocate for the development of multifaceted approaches that encompass both abundance and prevalence metrics, alongside advancements in bioinformatic pipelines and primer design. For instance, we did not detect archaea in the same samples when using the MetaPhlAn 4 pipeline for correlation analysis. This outcome was expected, especially for low-abundance organisms, as prior studies analyzing the human microbiome with different pipelines have consistently yielded varying results (Sun et al. 2021). By using our curated database and a tailored pipeline designed for archaea, we were able to detect them and identify several of their potentially relevant pathways within the oral microbiome. As next steps, we plan to run the tailored pipeline on environmental microbiomes known for high archaea abundance, using other conventional pipelines to verify the accuracy of our pipeline in these samples. Also, we are planning to check if our pathway is capable of identifying low abundance archaea in species

level using longer de novo assembled contigs, as this will provide higher confidence levels in the genes aligned though with the loss of some diversity. Another strategy will be using metagenome assembled genomes (MAGs) tools to find novel oral archaea.

Euryarchaeota is the most abundant phylum in the dataset, while *Thermoplasmatota* and *Nitrososphaerota (Thaumarchaeota*) are less abundant. They were all previously found in the oral cavity (13). Some *Euryarchaeota* representatives are methanogenic and can act in periodontal and endodontic biofilms in syntropy with disease-associate bacteria, however, here they seem to be acting in favor to the host in caries-free individuals. On the other hand, *Nitrososphaeraceae*, a member *Thaumarchaeota* family, was enriched in caries samples. Our previous pilot study identified representatives of the *Thaumarchaeota* phylum in samples of coronal caries in low abundance(8). These archaea were characterized by their ability to nitrify ammonia, contributing to the disruption the natural buffering system that prevents demineralization processes, which can lead to the formation of caries lesions. Other archaeal groups were depleted in caries when compared to caries-fee samples: *Halococcaceae* includes halophilic archaea that thrive in high-salt environments, and *Thermococcaceae* are hyperthermophilic archaea, typically found in environments with high temperatures. It is rather surprising that archaea commonly associated with extreme environments may not tolerate low pH caries environment, given their low-porosity cell walls, which would seemingly offer protection against acidic conditions. Therefore, it is likely that another factor contributes to their depletion in caries environments, beyond their inability to withstand low pH conditions.

To observe cross-kingdom interactions, we made correlation between archaeome and core bacteriome. Archaea might have the ability to team up with bacteria typically found in mature supragingival biofilms forming microbial networks, probably due to their metabolic capacity at the end of the food chain and the presence of microoxic niches within mature biofilms. Despite the aerobic nature of the mouth, early bacterial colonizers rapidly consume oxygen, creating conditions suitable for obligate anaerobes such as methanogens, particularly in dense dental biofilms. Furthermore, an interesting correlation with *Strephotrophomonas* and several methanogen genera was found. In environmental microbiomes, *Strephotrophomonas* plays a role in nitrogen fixation and the oxidation of sulfur, highlighting its contribution to nutrient cycling (26).

The overexpression of adhesins in caries-free samples suggests that archaea may also play a minor role in supragingival biofilm formation (Supplementary table 3). This make sense considering that studies have demonstrated that certain strains can form biofilms on various substrates, including mucous membranes, by producing relatively low amounts of extracellular polysaccharides composed of glucose, mannose, and galactose (27). It is known that these

organisms possess cell surface structures, such as type IV pili and the archaellum, that allow them to move, adhere to surfaces, and interact with other microbial cells (28, 29).

The formation of biofilms by archaea is an emerging field of study, with recent evidence highlighting this ability in various species and could be another function of the most abundant archaea in supragingival biofilms. For instance, we found methanogens RNA reads showing its activity in laboratory-engineered biofilms(30). Other *in vitro* studies showed that *Methanobrevibacter* and *Methanosarcina* form monospecies biofilms with low amounts of extracellular polysaccharides(31). Also, it has been reported that *Methanothermobacter thermautotrophicus* develops monospecies biofilms on nickel grids, using Mth60 fimbriae for robust adhesion(32). Other archaea has been shown to be involved in biofilms using archaella for adhesion or a sulfated extracellular polymeric substance, ensuring stability in harsh environments(33). All of these genera were present and with gene expression here.

Our pipeline was efficient to indicate for the first time that methanogenesis can be an active function in supragingival biofilms and saliva in homeostasis. Methanogenesis is an exceptional metabolic process in which archaea use carbon dioxide(CO_2) and hydrogen(H_2), produced by anaerobic bacterial fermentation, as an electron donor to generate methane(CH_4). This process involves several stages, including the formation of intermediates like formic acid, acetic acid, and methyl coenzyme M (34). The proteins involved in methanogenesis are categorized into three essential phases: substrate activation and conversion, methyl-coenzyme M formation, and methane reduction. Key enzymes such as formate dehydrogenase and formylmethanofuran dehydrogenase activates substrates converting them into intermediates. Enzymes like tetrahydromethanopterin S-methyltransferase facilitate the formation of methyl-coenzyme M, while proteins such as CoB--CoM heterodisulfide reductase and methyl-coenzyme M reductase operon proteins drive the final steps in methane production(35).

Methanobrevibacter are not commonly prevalent in the oral microbiome of modern industrialized societies, but highly prevalent in ancient dental calculus suggesting its relation to lifestyle (oral hygiene, diet- refined carbohydrate, less protein, etc)(5). The overexpression of function of carbohydrate metabolism in the caries archaeome confirms the biological plausibility of our analysis and advocates for the presence of archaea in supragingival samples, probably in very low abundance. In vitro studies have demonstrated that methane production can be increased in the presence of sucrose, especially under conditions of elevated pH(36). While there is limited data on the characteristics of the caries archaeome, methanogenic archaea may play a role in maintaining the environment in caries by regulating

pH levels, which could be essential for the survival of the main microorganisms involved in the disease.

8.5.1 Limitations

The datasets exhibit significant heterogeneity due to the differences in caries classification. We have previously discussed this issue of inconsistent definitions and descriptions of caries cases, even for the same condition, when using NGS data for meta-analysis(37). The criteria for determining caries cases are crucial in studying caries-associated microbiota and should be standardized for further NGS studies in caries. As this study is based on secondary data, we had no control on metadata quality, and more information on oral health of participants would have add value to the understanding of the findings. Our next steps involve wet lab experiments, combining relevant metadata.

Datasets containing RNA are not included among the post-filtered samples of DNA in the three studies where both nucleic acids were analyzed. While this indicates a significant risk of false negatives resulting from the arbitrary nature of our post-filtering strategy, at this stage of the oral archaeome evidence, prioritizing the avoidance of false positives is more crucial than minimizing false negatives. We applied a stringent filter, which may seem contrary to our concept that we need to find low abundance microorganisms. However, this step was crucial to minimize false positives and accurately identify where archaea are truly present.

8.6 CONCLUSION

Saliva and supragingival biofilms emerged as sites of low archaeal abundance, and methanogenesis genes were overexpressed in caries-free samples. This analysis, tailored to minimize false-positive outcomes, uncovered compelling evidence of archaea in dental caries-associated samples. Given that the putative prevalence of archaea was similar to that observed in other oral sites, our pipeline seems suitable for integration with other methods to screen for archaea in shotgun analysis of oral samples.

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