UNIVERSIDADE DE BRASÍLIA
Faculdade de Ciências de Saúde
Programa de Pós-Graduação em Odontologia

Tese de Doutorado

Atividade de colagenases e seu envolvimento em lesões cariosas radiculares

Cecília de Brito Barbosa

Brasília, 9 de agosto de 2023

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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 Doutora em Odontologia.

Orientadora: Nailê Damé-Teixeira

Brasília, 2023

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Data da defesa: 9 de agosto de 2023

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Dedico este trabalho em primeiro lugar a Deus, por me guiar em direção aos meus sonhos. À minha família, em especial aos meus pais, Clédiston e Marliete, por não pouparem esforços para minha felicidade. Aos meus amados colegas de pós-graduação, por todo companheirismo. Aos meus amigos, que me incentivaram todos os dias e estiveram sempre ao meu lado. À minha tão querida orientadora, Profa. Dra. Nailê Damé Teixeira, por todo ensinamento compartilhado com tanta bondade e confiança.

AGRADECIMENTOS

Agradeço a Deus, pois devo a ele todas as oportunidades e sonhos alcançados em minha vida. A fé me fez conquistar muito mais do que mereço.

Aos meus pais, que foram o meu grande alicerce nestes anos de pós-graduação. Com muito amor apoiaram cada decisão tomada e, principalmente, abdicaram de muitos sonhos para que eu alcançasse os meus. Minha mãe, por todas as palavras de amor, conforto e colo insubstituível. Ao meu pai, professor há tantos anos e o mais sábio que pude conhecer, ensinando-me que a educação, ética e humildade são a chave do sucesso.

Ao meu namorado, Leonardo, por encorajar-me nos momentos de ansiedade, por todo amor, carinho e paciência nos períodos que precisei estar distante. Ao meu irmão gêmeo, Matheus, por todo bom humor e tranquilidade ao me acompanhar nesses anos de pós-graduação.

À Profa. Dra. Nailê Damé Teixeira, que acreditou em mim desde o primeiro contato no mestrado. Quando eu, tão nova e inexperiente, embarquei no sonho de tornar- me professora e pesquisadora. Graças à sua irretocável orientação, paciência, sabedoria e, principalmente, boa vontade em compartilhar conhecimento, completo esta etapa. Obrigada por confiar em mim e por mostrar-me todos os dias o quanto sou capaz. O seu amor pela profissão enche o coração dos alunos de incentivo e vontade de aprender. Que não seja o fim de um ciclo, mas o início de uma grande parceria.

À equipe do laboratório de Odontologia da FS e do laboratório de Biologia Molecular do IB, que acompanharam de perto minha jornada na pós-graduação. Sou grata a cada um que cedeu um pedaço do seu tempo para me ajudar, seja em experimentos ou até mesmo com uma palavra de conforto.

Aos incríveis professores que contribuíram com todo aprendizado ao longo dos anos. Jamais caberia todos os nomes em poucas linhas, mas deixo aqui meu muito obrigada especialmente aos professores, Profa. Dra. Cristine Stefani, Prof. Dr. Rodrigo Alex Arthur, Prof. Dr Fernando Araripe, Profa. Dra. Lídia de Moraes, Profa. Dra. Loise Salles, Profa. Dra. Janice Lisboa Del Marco por dedicarem seu tempo ao meu projeto e crescimento profissional.

À Universidade de Brasília e à Faculdade de Ciências da Saúde, que abriram portas para que este sonho se tornasse realidade.

Ao Programa de Pós-Graduação em Odontologia e à Secretaria do Curso de Pós-Graduação pela colaboração, auxiliando-me nos procedimentos acadêmicos formais.

À Profa. Dra. Soraya Coelho Leal e ao Prof. Dr. Leandro Hilgert e toda equipe da coordenação do Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília, pela atenção e disponibilidade.

Às Profas. Dra. Debora Heller, Profa. Dra. Bruna Genari, Profa. Dra.Paula Kruly e Profa. Dra. Fabricia Pereira, por aceitarem o convite para compor a banca examinadora da tese.

À Profa. Dra. Heliana Dantas Mestrinho pelos ensinamentos, desde o início da graduação, ao me apresentar o mundo da pesquisa científica. A partir de então, tenho traçado um caminho de dedicação e amor pela ciência. A senhora, sem dúvidas, tem uma grande colaboração na minha formação.

Aos meus amigos de UnB, especialmente, Patrícia, Fabio, Camila Coelho, Jessica Luiza, Rafaella Cristhina, Rayssa, Marta, Carlos Alexandre e Thiago pela parceria e todos os momentos de alegria compartilhados.

Ao querido team Root Caries, assim carinhosamente chamado nosso time de pesquisa. Que esse grupo forme grandes pesquisadores, empenhados e apaixonados evidência cientifica.

Aos amigos de laboratório que se tornaram amigos para vida, Luísa, Ana Laura, Myrna, Roberta, Igor, Pedro e Carmem.

Às minhas amigas e parceiras de pesquisa, Isabela Monici e Jéssica Cena, pela parceria tão agradável e frutífera. Nosso trabalho em equipe foi indispensável para que eu chegasse até aqui.

A todos os amigos e familiares que torceram por mim, por cada sorriso, palavra de conforto e incentivo destinado ao longo desta caminhada.

À CAPES, pelo financiamento ao Programa de Pós-Graduação em Odontologia e por me dar a oportunidade de ser bolsista.

A todos que colaboraram direta ou indiretamente para que eu pudesse vencer mais essa etapa. Meus sinceros agradecimentos.

RESUMO

O processo carioso ocorre de formas distintas nas superfícies dentárias coronária e radicular, diante de suas características bioquímicas específicas. A grande quantidade de material orgânico nos tecidos radiculares sugere que o desenvolvimento da lesão cariosa aconteça em um processo de dois estágios. O primeiro é caracterizado por dissolução mineral e o segundo pela degradação da matriz orgânica da superfície radicular por meio de proteases. Em revisão sistemática, foram identificadas tendências na direção do efeito para algumas metaloproteases em dentina cariada, ainda que com evidência muito baixa. Já em revisão de estado da arte da literatura, observou-se que a degradação da matriz de colágeno só poderia ser possível após a desmineralização, pois o substrato não fica acessível no tecido mineralizado para a ação das colagenases. O colágeno desmineralizado serviria, então, como nicho para microrganismos colonizadores, para os quais é atribuída a função de produção de ácidos que seguem a desmineralização da dentina. Destaca-se o *Streptococcus mutans*, um microrganismo reconhecidamente envolvido no desenvolvimento da doença cárie, a partir dos seus inúmeros fatores de virulência, principalmente, acidogenicidade e aciduricidade. Para cárie radicular, estudos já foram capazes de mostrar frequências de isolamento mais altas e/ou maiores proporções dessa bactéria do que em biofilmes de superfícies radiculares hígidas, além de uma superexpressão de genes codificadores de colagenases em biofilmes radiculares. Devido à necessidade de maior conhecimento sobre o estágio proteolítico da doença, o objetivo primário da presente tese de doutorado foi promover um estudo sobre o papel bacteriano na segunda fase da formação de lesões de cárie radicular, com ênfase no *Streptococcus mutans*. Foram realizados ensaios enzimáticos para determinação da capacidade colagenolítica do *S. mutans* em degradar colágeno sintético, colágeno tipo I e gelatina. Foram testadas diferentes condições de pH, tempo e concentrações celulares. Os resultados demonstraram atividade significativa apenas para colágeno sintético. Demonstramos aqui que há um potencial para degradação de colágeno a partir de bactérias bucais, inclusive do *S. mutans*. Acredita-se que isso sinaliza alguma influência bacteriana nesse processo e representa um alvo potencial para a modulação do biofilme. Mais estudos são necessários para avaliar a capacidade colagenolítica de *S. mutans* em condições laboratoriais mais próximas do que se encontra clinicamente.

PALAVRAS-CHAVE: Cárie dentária; cárie radicular; *Streptococcus mutans*; colagenase microbiana; colagenases humanas

ABSTRACT

The carious process occurs differently on the coronal and root dental surfaces, given their specific biochemical characteristics. The large amount of organic material in root tissues suggests that the development of the caries lesion occurs in a two-stage process. The first is characterized by mineral dissolution and the second by degradation of the organic matrix of the root surface trough proteases. In the systematic review, trends in the direction of the effect for some metalloproteinases in carious dentin were identified, although with very low evidence. In the state-of-the-art literature review, it was observed that the theory suggests the degradation of the collagen matrix could only occur after demineralization, as the substrate remains inaccessible in mineralized tissue for the action of collagenases. The demineralized collagen would then serve as a niche for colonizing microorganisms, which are attributed to the function of producing acids following dentin demineralization. *Streptococcus mutans* is highlighted as a microorganism known to be involved in the development of dental caries due to its numerous virulence factors, particularly acidogenicity and aciduricity. For root caries, studies have shown higher isolation frequencies and/or greater proportions of this bacteria compared to biofilms from healthy root surfaces. Additionally, there is an overexpression of genes encoding collagenases in root biofilms. Due to the need for a better understanding of the proteolytic stage of the disease, the primary objective of this thesis was to conduct a study on the bacterial role in the second phase of root caries lesion formation, with a particular emphasis on *Streptococcus mutans.* Enzymatic assays were conducted to determine the collagenolytic capacity of *Streptococcus mutans* in degrading synthetic collagen, type I collagen, and gelatin. Different conditions of pH, time, and cellular concentrations were tested during these assays. The results showed significant activity only for synthetic collagen. This study demonstrated the potential for collagen degradation by oral bacteria, including *Streptococcus mutans*. It is believed that this finding signals some bacterial influence in this process and represents a potential target for biofilm modulation. However, further studies are necessary to evaluate the collagenolytic capacity of *Streptococcus mutans* under conditions that closely resemble the clinical environment.

Keywords: Dental Caries, Root Caries, *Streptococcus mutans*, Microbial Collagenase, Human Collagenase

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LISTA DE ABREVIATURAS E SIGLAS (EM INGLÊS)

BSP	Bone sialoprotein
CTs	Cisteína catepsinas
CJE	Cranberry juice
FALGPA	Furylacryloyl-Leu-Gly-Pro-Ala
GE	Genipin
GA	Glutaraldehyde
GTE	Green tea extract
GSE	Grape seed extract
MMP	Matrix metalloproteinase
NGS	Next-generation sequencing
OD	Optical dentisty
OIS	Optimal information size
PBS	Phospate buffered saline
P. gingivalis	*Porphyromonas gingivalis*
PZ-PLGPA	PZ-Pro-Leu-Gly-Prop-Arg
PA	Proanthocyanidins
RC	Root caries
SDF	Silver diamine fluoride
S. mutans	*Streptococcus mutans*

SUMÁRIO

APRESENTAÇÃO

Dentre as doenças humanas, a cárie não tratada é considerada a campeã em prevalência mundial (Kassebaum, Bernabé et al. 2015). Além disso, junto às demais doenças bucais, impõe uma importante carga econômica à sociedade, diante do grande impacto gerado em saúde pública (Peres, Macpherson et al. 2019). Isso demonstra que a Odontologia ainda falha em controlar sua progressão, embora a Cariologia tenha mudado consideravalemente nos últimos anos, a partir da teoria da placa ecológica (Marsh 1994). Uma prova dessa falha é o aumento da incidência de cárie radicular na população idosa e a dificuldade em estabelecer tratamentos padrão – ouro. Isso, porque a cárie radicular apresenta algumas particularidades quando comparada à cárie coronária, como a degradação de matriz orgânica durante a formação das lesões (Damé-Teixeira, Parolo et al. 2017), mas os tratamentos atuais pouco consideram tais diferenças. Portanto, estudar e entender a etiopatogenia de cárie radicular é o caminho para que se desenvolva novos alvos para tratamentos em biofilmes disbióticos. Esta tese de doutorado foi delineada a partir de um gap da literatura, que é saber se microrganismos bucais são capazes de degradar colágeno da dentina durante o processo de formação de lesão radicular, e irá apresentar quatro capítulos, dos quais três correspondem a artigos científicos que, portanto, serão apresentados em língua inglesa.

O **capítulo 1** introduz o tema base a partir de uma revisão de literatura acerca da ação das proteases colagenolíticas na degradação da matriz dentinária em cárie radicular e possíveis estratégias para seu manejo. Tal revisão está em processo de revisão final para submissão para publicação em periódico científico internacional.

Já o **capítulo 2** traz uma revisão sistemática sobre a presença de proteases colagenolíticas derivadas do hospedeiro e de bactérias na dentina cariada. A revisão foi realizada em colaboração com outras colegas da pós-graduação e está ligada a disciplina de Revisão Sistemática em Odontologia do PPGODT. Essa revisão já foi submetida para publicação em periódico científico internacional.

O **capítulo 3** constitui um estudo *in vitro* para avaliar o potencial colagenolítico de *Streptococcus mutans* em diferentes níveis de pH e substratos a fim de avaliar a atividade enzimática dessa bactéria em condições distintas. O artigo será futuramente submetido à periódico internacional.

O **capítulo 4** aborda a discussão geral do trabalho e os principais achados da tese de maneira concisa e direta, trazendo ao leitor, uma visão geral do que foi discutido ao longo do trabalho.

Como parte da produção executada nesse período pela candidata, serão apresentados em anexo três estudos publicados no ano de 2022, que fazem parte da linha de pesquisa dessa tese de doutorado. Os artigos são denominados 1) "*Streptococcus mutans* e seu metabolismo a nível molecular no contexto ecológico da doença cárie", 2) "Manejo de cárie radicular: um guia para o dentista brasileiro baseado na tradução e adaptação cultural do consenso internacional/ORCA e EFCD" e 3) "Minimum intervention oral care: defining the future of caries management". Também está registrado nos anexos a participação como membro de banca avaliadora e coorientação em trabalhos de conclusão de curso da graduação em Odontologia, respectivamente "Impacto dos critérios de detecção nas estimativas de cárie coronária e radicular em adultos com e sem Diabetes Mellitus tipo 2" e coorientação de Trabalho de Conclusão de Curso de graduação.

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OBJETIVOS E JUSTIFICATIVA

Objetivo Geral

Estudar colagenases presentes em cárie radicular, caracterizando especialmente a capacidade colagenolítica do *Streptococcus mutans*.

Objetivos Específicos

1) Realizar uma revisão de estado da arte quanto ao papel de colagenases em lesões cariosas radiculares;
2) Revisar sistematicamente a literatura quanto a presença de colagenases do hospedeiro e microbianas em lesões cariosas dentinárias, bem como observar sua direção de efeito quando comparadas com dentinas hígidas;
3) Comparar a atividade colagenolítica de diferentes cepas de *S. mutans* utilizando diferentes substratos (colágeno sintético, colágeno tipo I, gelatina);
4) Comparar a atividade colagenolítica do *S. mutans* com outras bactérias bucais;
5) Comparar a atividade colagenolítica da cepa *S. mutans* UA159 em diferentes pHs (neutro e ácido).

CAPÍTULO 1 – DENTINAL MATRIX DEGRADATION IN ROOT CARIES: EXPLORING THE ROLE OF COLLAGENOLYTIC PROTEASES AND MANAGEMENT STRATEGIES - A COMPREHENSIVE STATE- OF- ART REVIEW

Autoria: Cecília de Brito Barbosa, Isabela Monici Silva, Nailê Damé-Teixeira

1.1 INTRODUCTION

Root caries (RC) is an incident condition (Christensen, Doblhammer et al. 2009, Hayes, Burke et al. 2017, Gao, Hu et al. 2018) associated with an increase in life expectancy worldwide and with the current reduction of edentulism due to better hygiene standards and fluoride access (Griffin, Griffin et al. 2004). Its incidence is also affected by biological and socioeconomic features, such as the presence of coronal caries lesions, educational level, income, and location of the county (Manji and Fejerskov 1990, Mamai-Homata, Topitsoglou et al. 2012). It is challenging to define the global prevalence and incidence of RC owing to the heterogeneity in the detection criteria and the high risk of bias in the related studies. Given the deficient data on its epidemiological characteristics (Hayes, Burke et al. 2017) and a lack of consensus on its development and progression (Takahashi and Nyvad 2011, Heasman, Ritchie et al. 2017), it can be stated that RC has been neglected in the scientific community.

Managing root caries also presents unique challenges, particularly in the clinical setting. The presence of dentin as the substrate for adhesion (Gostemeyer, da Mata et al. 2019) and the significant amount of organic material on the surface indicate the need for a different treatment approach compared to that used for coronal surfaces (Burrow and Stacey 2017, Damé-Teixeira, Parolo et al. 2017). The absence of a consensus regarding the optimal clinical and epidemiological approach to treat root carious lesions opens up opportunities for developing novel strategies in future treatments (Wierichs and Meyer-Lueckel 2015, Meyer-Lueckel, Machiulskiene et al. 2019). However, achieving this requires a thorough understanding of the specificities of RC ethiopatogeny. This comprehensive state-of-the-art review aimed to explore the characteristics

involved in the process of formation of caries root lesions, with emphasis on the degradation of the root collagenolytic matrix, clearly stating the knowledge gap on the role of bacterial collagenolytic proteases in this process. For that, we revisited the peculiarities on the composition of dental root surfaces and lesion development, and the action of proteases on collagenolytic matrix degradation. Finally, some future perspectives regarding the clinical aspects for prevention and management of root caries will be discussed.

1.2 LITERATURE REVIEW

1.2.1 Revisiting the peculiarities of the dental root surfaces composition

To understand the particularities of RC, it is best to describe its differences from coronal caries, which evidently starts with differences in the composition of the tissues involved. Dental enamel covering coronal surfaces is the most mineralized tissue in the body, consisting of 89% calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and small amounts of calcium carbonate (4%), calcium fluoride (2%), and magnesium phosphate (1.5%) (Gedalia, Azaz et al. 1969). On root surfaces, meanwhile, cement consists of 45%–50% inorganic content and 50% organic content, mostly type I collagen (Christner, Robinson et al. 1977). In root tissues, a higher amount of magnesium can confer greater solubility to hydroxyapatite crystals (Hoppenbrouwers, Driessens et al. 1986), as magnesium inhibit and regulate hydroxyapatite crystal growth by the replacement of calcium ions in the crystals (Teruel Jde, Alcolea et al. 2015, Klimuszko, Orywal et al. 2018). Collagen in the cementitious organic matrix forms cross-linked and induces biological mineralization, maintaining the structural integrity of the cement (Christner, Robinson et al. 1977). The main non-collagen proteins in the cementum are bone sialoproteins and osteopontin (Christner, Robinson et al. 1977, Bosshardt and Selvig 1997, Breschi, Maravic et al. 2018). Similarly to cement, the root dentin has a high organic content (approximately 18%). Other components include 70% inorganic materials and 12% water (Goldberg, Kulkarni et al. 2011, Damé-Teixeira, Parolo et al. 2017). Its inorganic content is mainly hydroxyapatite crystals within an organic matrix rich in collagen structural

components, with a bioactive molecular diversity (Okamoto, Takahashi et al. 2018).

It is now important to define the term “collagen”. It is used as a generic term for 28 types of proteins differing in size, function, and distribution in tissues. However, all types have a common property to form a supramolecular structure with a triple helix characteristic of three alpha polypeptide chains in an extracellular matrix (Gelse, Pöschl et al. 2003, Ricard-Blum and Ruggiero 2005). They are classified according to the complexity of their structure, splice variants, the presence of non-helical domains, and their assembly and function. They can be either homotrimers (when formed by three identical chains) or heterotrimers (when formed by two or more different chains) (Gelse, Pöschl et al. 2003). In both cases, the three chains supercoil around the central axis, forming an extended helix. Each chain is formed by groups of 18 amino acids (Hulmes and Miller 1979, Gelse, Pöschl et al. 2003). A structural prerequisite for mounting on a triple helix is a glycine residue (always positioned in the center, the smallest amino acid) in each third position of the polypeptide chains, resulting in a Gly-X-Y repeat structure that characterizes and identifies the “collagen” domains. The X and Y positions are often occupied by proline and hydroxyproline (Ricard-Blum and Ruggiero 2005). Type I collagen, found on root surfaces, is characterized by its ability to gather in oriented supramolecular aggregates with a characteristic heterotrimeric structure, which contributes to the molecular stabilization and mechanical properties of dentin. Its fibrils represent a structural pillar and are perpendicularly connected by non-collagen proteins (Hulmes and Miller 1979, Varma, Orgel et al. 2016). This molecular structure is quite complex, formed by a triple helix with approximately 300 nm, comprised of three parallel polypeptide chains coiled together to form fibrils (Figure 1). This arrangement is such that the N terminals of two adjacent triple helices along the axis are separated by a distance of D = 67 nm, and the N terminals of the two triple helices adjacent to the side are axially separated by 0.54 nm (Hulmes and Miller 1979). This staggered arrangement results in alternating regions of low and high protein density along the fibril axis with a repetitive unit of length D (67 nm) (Hulmes and Miller 1979, Varma, Orgel et al. 2016). Consequently, root caries lesions expose a more vulnerable area rich in this collagen component, which is susceptible to

enzymatic degradation. Understanding the molecular structure of collagen can provide insights into alternative management options for root caries.

Only a specific group of proteases, namely collagenases, can degrade native collagen. The triple helix is cut in its internal structure by digesting the amino group in a “Gly-Leu” bond in the aqueous phase before proteolytic degradation. This can cause intramolecular flexibility and allow specific proteolytic cleavage (Gelse, Pöschl et al. 2003, Takahashi and Nyvad 2016). In the presence of gingival recession, either due to periodontal diseases or inadequate control of local biofilms, a new ecological niche is formed on the root surface. This surface, which used to be an anaerobic microenvironment covered by gingival tissue, becomes an aerobic microenvironment with varying availability of nutrients (Gross, Beall et al. 2012). The exposed root region then constitutes a favorable site for microbial infiltration by converting the collagen fiber system into channels which bacteria can penetrate through (Takahashi and Nyvad 2016). In addition, improper brushing of teeth or periodontal treatment itself can often damage or remove cement, exposing the dentin to the oral environment quickly. When demineralization of dental surfaces begins with acidification of the oral microbiota due to carious process, it leads to the formation of root carious lesions (Takahashi and Nyvad 2016).

1.2.2 Do root caries lesions develop in two phases - demineralization and degradation of the collagenolytic matrix?

The first ecological concept of caries was proposed by Marsh (1994) and later extended by Takahashi and Nyvad (Marsh 2010, Takahashi and Nyvad 2016). This hypothesis emphasizes that the enrichment of some species in the oral microbiota, previously considered odontopathogens, occurs in response to a change in the environment caused by the high consumption of fermentable carbohydrates, i.e., dental caries is not caused by a pre-established set of microorganisms but by changes in the composition of the community due to external factors that modify the balance of the microbiota toward demineralization (Takahashi and Nyvad 2011, Takahashi and Nyvad 2016). Nonetheless, emphases on the processes that affect the enamel surface have been placed,

and little has been discussed about specificities of caries on root surfaces. Irrespective of the differences in the substrate between the crown and root of teeth, dental caries develops on both surfaces due to an imbalance of the microbiota, influenced by the availability of fermentable carbohydrates in the individual's diet. Consequently, the development of both enamel caries and root caries is driven by the same factors, even though the lesions may progress at different rates during the processes.

Dental biofilms are naturally constituted of a diverse and multifunctional microbiota. In some diseases, an essential role played by bacteria is the ability to form a multidimensional set known as biofilm, which consists of highly complex communities firmly attached to teeth or other solid oral structures (Aas, Griffen et al. 2008). The main components of the biofilm formed on the surface of the teeth include glucan, fructan, and proteins, and they differ from the surrounding saliva in terms of levels of lipids, calcium, magnesium, fluorine, and phosphorus. *In situ*, 80% of dental biofilm consists of water (Bowen and Koo 2011). Therefore, the formation of an amorphous membrane with ideal conditions for bacterial survival determines the virulence of the biofilm structure. On the root surface, however, the biofilm is comprised by a variety of saccharolytic, aciduric, and acidogenic organisms, as well as proteolytic bacteria, which can produce acid or ammonia from the catabolism of nitrogenous substrates, available exogenously or from the organic matrix of dentin (Syed, Loesche et al. 1975, Dame-Teixeira, Parolo et al. 2016, Do, Damé-Teixeira et al. 2017).

Furthermore, the recent integrated ecological hypothesis for caries and periodontitis (Nyvad and Takahashi 2020) points to a common risk factor for both diseases, which are originated in the dynamic stability stage and emerges in response to nutritional imbalances in the microbiota. Although they are diseases of different pathophysiology, there is an integrated hypothesis of dental caries and periodontal diseases, in which both originate in the stage of dynamic stability and arise in response to nutritional imbalances in the microbiota (Nyvad and Takahashi 2020). In this context, teeth, gingival tissue and the surfaces of the oral mucosa remain clinically healthy when the functional balance of the oral microbial ecosystem is maintained, even in the presence of dental biofilm. However, if the pH balance of the microbial community is disrupted by external

environmental pressures, such as frequent periods of carbohydrate consumption, normal homeostatic reactions will collapse, triggering a loss of homeostatic balance. Didactically, these periods can be divided into dynamic stability stage, aciduric stage and acidogenic stage. The dynamic stability stage is associated with health, in which pH fluctuations are balanced over time due to infrequent exposures to diets high in fermentable carbohydrates. When inflammatory nutrients are intense and prolonged, the pH of the microbiota can move from the dynamic stability stage to stages of proteolytic degradation, whereas the more intense and prolonged the episodes of exposure to dietary carbohydrates, the greater the pH shift to acidogenic levels and acidurics associated with caries (Nyvad and Takahashi 2020).

Takahashi and Nyvad proposed two stages of RC development. A large amount of organic material in the hard root tissues suggests that a proteolytic stage occurs during the formation of a caries lesion, which occurs after a demineralization stage (Figure 2) (Takahashi and Nyvad 2011, Takahashi and Nyvad 2016). Ultrastructural studies confirmed that demineralization and degradation of the organic matrix occur in two successive stages (Nyvad and Fejerskov 1990). In the early stages of cement and dentin caries, minerals are dissolved by a pH gradient from the outer surface, maintaining the original cross-links between collagen fibers (Nyvad and Fejerskov 1990, Deyhle, Bunk et al. 2011). Demineralized collagen serves as a support for colonizing bacteria. In more advanced stages, the exposed collagen is degraded by proteolytic enzymes, and the collagen fibers lose their structural characteristics (Takahashi and Nyvad 2016). Indeed, the collagen covered by the mineral became accessible to enzymatic degradation only if the concentration of mineral ions in solution was below one critical value dependent on pH (Klont and ten Cate 1991). However, a recent study suggested that cross-collagen bands may be degraded during demineralization (Tjaderhane, Buzalaf et al. 2015), in which an exposed region of the collagen molecule is degraded by the activity of host-derived collagenolytic proteases.

Matrix metalloproteinases (MMPs) are an important group of enzymes responsible for the degradation of the extracellular matrix and basement membranes and are strongly related to physiological and pathological oral

processes. Studies on metalloproteinases were introduced in 1962, by Gross and Lapièrre, by observing an enzyme active in the fragments culture of the skin of rats, which degraded the triple helix of mature type I collagen. Since then, many studies have investigated metalloproteinases in the oral microenvironment to better understand the role of these enzymes in different pathological processes (Gross and Lapiere 1962). They are present, for example, in periodontal tissue destruction, RC lesions, metastases in some types of tumors, and disorders of the temporomandibular joint (Birkedal-Hansen 1993). An *in-situ* study by Van Strijp et al. identified the presence of metalloproteinases (MMP-2) in saliva and completely demineralized dentin samples. Their results suggest a potential role in the degradation of the extracellular matrix during the carious process (van Strijp, Jansen et al. 2003).

Regarding the mechanism of action, according to Birkedal-Hansen et al., the activity of MMPs on extracellular matrix substrates is regulated in four ways: 1) transcriptional regulation of MMP genes; 2) activation of precursors; 3) differences in substrate specificity; and 4) MMP inhibitors. The release and production of these metalloproteinases occur through cells, such as keratinocytes, polymorphonuclear leukocytes, macrophages, monocytes, fibroblasts, and mesenchymal cells, which in the presence of growth factors and cytokines (interleukin-1, TNF-α, and TGF-α) release MMPs into the extracellular environment (Birkedal-Hansen 1993).

MMPs are present in dental biofilms, gingival crevicular fluid, and saliva (Van Strijp, Klont et al. 1992). When the root is exposed in the oral cavity, rapid demineralization occurs exposing collagen fibers. These are accessed by bacteria and their acidic metabolites, activating MMPs. Bacterial enzymes may be responsible for the positive regulation of interleukin-1 present in gingival crevicular fluid and saliva. This interleukin is responsible for stimulating polymorphonuclear leukocytes and macrophages to release MMPs. These MMPs can be activated by bacterial enzymes or host-derived proteases (Simon-Soro, Belda-Ferre et al. 2013). After they are activated, MMPs are usually involved in proteins removal from the dentin, cement, and enamel matrix during their maturation, resulting in a highly mineralized tissue for the latter.

For classification, more than 30 different types of human MMPs were identified and divided into five large groups according to the specificity of the substrate and its internal homology: matrilisins, collagenase, gelatinases, stromelysins, and metastases; and MT-MMP (1, 2, 3, and 4). All MMPs have catalytic zinc and calcium-binding domain of approximately 165 residues that allow substrate hydrolysis, as well as autolytic cleavage. They are secreted primarily in the form of zymogenes, that is, inactive proenzymes, activated by the segmentation of a part called propeptide (Birkedal-Hansen 1993, Bartlett, Simmer et al. 1996, Murphy and Knauper 1997). One of the ways to control MMP activity is the presence of specific inhibitors, known as tissue MMP inhibitors (TIMPs). TIMPs are small, multifunctional proteins widely targeted by the scientific community, as they can regulate the level of MMP activation and their ability to hydrolyze a particular substrate. It means that creating a synthetic inhibitor of metalloproteinases would be a great advance in the control of several pathologies that involve these enzymes (Visse and Nagase 2003). However, proteases related to dentin matrix degradation cannot reach protein substrates or be activated in mineralized tissues. Also, intact collagen molecules are resistant to several proteases, except for collagenases, due to their conformational triple helix structure. As explained before, their internal structure must be solubilized and denatured in the aqueous phase before proteolytic degradation (Takahashi and Nyvad 2016). There is evidence that this role is performed by host metalloproteinases, capable of digesting proteins from the extracellular matrix and that have important functions in several biological processes. Tjaderhane et al. showed that once the collagen molecules are exposed, the telopeptide activity of the host-derived collagenase (present even in saliva) begins to partially break the telopeptide region of the molecules and can further degrade the molecules and turn them into more water-soluble components (Tjaderhane, Buzalaf et al. 2015). Other proteases, such as matrix metalloproteinases (MMP-2, 3, 8, 9, and 20) and cysteine cathepsins (B and K), are present in the organic dentinal matrix and are automatically activated under acidic conditions (around pH 4.5) at the moment when the dentin is demineralized or exposed to the aqueous phase, initiating surface degradation (Tjaderhane, Buzalaf et al. 2015, Takahashi and Nyvad 2016).

1.2.3 Does bacteria play a role in collagen degradation in root caries?

In addition to endogenous (host) enzymes, microbial proteases may also participate in the process of collagen fibrils degradation (Figure 2). The first sign of this can be the isolation of many bacteria that possess their own collagenases from root lesions, such as *Prevotella* and *Propionibacterium.* Although this is not directly linked to their role in dentinal collagen degradation, it suggests some proteolytic activity in RC biofilms (Aas, Griffen et al. 2008, Preza, Olsen et al. 2008). Studies have shown that the oral microbiota is modified during changes in the dental surface with the development of caries lesions, from the initial dominance of non-mutans *Streptococci* and *Actinomyces* to the dominance of *Streptococcus spp*. and other bacteria, including *Lactobacillus* and *Bifidobacterium* in the later stages of dental caries (Takahashi and Nyvad 2011, Simon-Soro, Belda-Ferre et al. 2013).

In the begging of demineralization stage, high proportions of *S. mutans* and other aciduric bacteria are in high abundance, as they can be benefited by dietary sucrose. In root tissues, in addition to *S. mutans, Lactobacillus spp.,* and *Actinomyces*, other species, such as *Atopobium, Olsenella*, *Pseudoramibacter*, *Propionibacterium*, and *Selemonas*, have also been observed (Becker, Paster et al. 2002, Preza, Olsen et al. 2008, Takahashi and Nyvad 2008). More recently, a study by Takenaka et al. also suggested this diversity from the identification of periodontal pathogens in root caries lesions. In this scenario, depending on the location of the lesion, the gingival crevicular fluid could modulate the local microbial composition and present a microbial diversity along the gingival margin. The microbial profile of the carious lesion extending beyond the gingival margin would be more diverse and complex than at the supragingival site. However, it is still unclear how these bacteria contribute to collagen matrix degradation during caries progression (Takenaka, Edanami et al. 2021).

Indeed, some well-known oral bacteria involved in oral diseases produce their own collagenases that can be capable of breaking down the dentinal collagen. It was reported the presence of bacteria with the ability to degrade collagen or synthetic analogues, such as FALGPA, in clinical samples of root

caries (Harrington 1996). Interestingly, a recent study showed that the bacterial composition of root caries lesions located under the gingival margin is likely to have periodontal pathobionts: *Porphyromonas, Selenomonas, Filifactor, Peptococcus* and *Tannerela* inhabit root caries lesions that extend beyond the gingival margin (Takenaka, Edanami et al. 2021). This suggests that the microbiome in root caries lesions expanding across the gingival margin would show an increase in proteolytic bacterial diversity. The expression of *P. gingivalis* collagenases-related genes in root carious lesions has also been demonstrated (Damé-Teixeira, Parolo et al. 2018).

Another example of this is the study by Hashimoto et al. (Hashimoto, Sato et al. 2011), which identified a high prevalence of *Prevotella* and *Bifidobacterium* sp. in root biofilms, which are known to possess collagenolytic activity (Keudell and Conte 1976, Steffen and Hentges 1981, Robertson, Lantz et al. 1982, Uitto, Haapasalo et al. 1988). On the same way, the expression of genes related to collagenases from *P. gingivalis* in root caries lesions has also been demonstrated (Damé-Teixeira, Parolo et al. 2018) and suggests once again that these proteases are in action at the time of the disease. There are many indications that these microbial enzymes are in motion and should not be neglected in the study of caries lesions or periodontal diseases.

The importance of microorganisms and the effects of the oral environment on the collagen of the dentin matrix were also revealed by van Strijp in a series of *in situ* studies that evaluated the amount of denatured collagen after an experimental period under different cariogenic conditions (Van Strijp, Klont et al. 1992, van Strijp, van Steenbergen et al. 1994, van Strijp, Jansen et al. 2003, van Strijp, Takatsuka et al. 2015). For this, completely demineralized dentin specimens were placed on the buccal surfaces of the partial dentures used by the participants. After an intraoral period of seven weeks, dentin specimens were analyzed for denatured collagen using light microscopy and transmission electron microscopy. Intra-individual and inter-individual differences in collagen loss were found, probably due to variations in the composition of the microbiota that colonized the demineralized specimens. In addition, differences in the ability of microorganisms to degrade the collagen matrix may be responsible for the deviations in collagen loss (Van Strijp, Klont et al. 1992).

In 1994, the same research group, after identifying the colonizing microbiota of the decalcified dentinal matrix, evaluated the gelatinolytic activity of the isolated strains related to the degradation of the dentinal matrix. The predominant species found were *Streptococcus mitis, Peptostreptococcus productus*, *Lactobacillus casei*, *Propionibacterium* species, and *Veillonella parvula*. Although no correlation was found with the severity of dentin matrix degradation, the microflora showed gelatinolytic activity (van Strijp, van Steenbergen et al. 1994). In addition to the diversity of the bacterial community, the presence and activity of metalloproteinases 1, 2, and 9 (MMP-1, MMP-2, MMP-9) in the saliva and completely demineralized dentin specimens were investigated by van Strijp. The intention was to analyze the correlation between these enzymes and the level of collagen degradation. Samples of demineralized dentin were attached to the partial dentures of 17 volunteers, and saliva samples were collected at 0, 2, and 4 weeks. After 4 weeks, the enzymes were extracted from the specimen dentin, and collagen loss was assessed. Despite revealing gelatinolytic enzyme activity in both saliva and dentin collagen, no correlation was observed between the levels of enzyme activity and the loss of collagen in the dentin specimens (van Strijp, Jansen et al. 2003).

Since the studies conducted by van Strijp and the research group, there has been limited published research on the role of oral bacteria in root collagen degradation. Only a handful of molecular studies have contributed to our understanding of the actions of microorganisms and their proteases in caries progression. Notably, Simon-Sóro et al. proposed a tissue-dependent hypothesis of caries, which involved comparing the metagenomics of carious biofilms from enamel and dentin caries (Simon-Soro, Belda-Ferre et al. 2013). The researchers demonstrated distinct metabolic events taking place in each carious tissue. In enamel caries biofilms, genes associated with acid stress tolerance and dietary sugar fermentation were significantly overexpressed, whereas in coronal carious dentin, collagenases and proteases were among the groups of genes showing significant overexpression (Simon-Soro, Belda-Ferre et al. 2013). Notably, dentin lesions displayed low levels of genes involved in sugar fermentation from the diet and pH stress, but showed an abundance of genes related to monosaccharide and disaccharide metabolism. In addition, deep dentin samples showed an

overrepresentation of genes associated with the host immune response. According to Simon-Sóro, dental caries can be categorized into two stages: enamel and dentin lesions.

In another metatranscriptomics study, other microorganisms overexpressed genes related to collagenases in RC, demonstrating their potential involvement in protein degradation, such as *Veillonella parvula DSM 2008 (VPAR_RS05935* and *VPAR_RS05390), Veillonella dispar ATCC 17748 (VEIDISOL_RS04770),* and *Leptotrichia buccalis (LEBU_RS05040*). Furthermore*, S. mutans* also presented two genes highly expressed in root caries biofilms. Those genes encode the collagenase-type protease family PrtC of the peptidase family U32 (Ajdic, McShan et al. 2002). SMU_761 encodes a 428 aa protein, whereas SMU_759 encodes a 308 aa protein. The U32 family of peptidases is a broad family of enzymes with little known structure and catalytic mechanism. The presence of this family has also been described in other pathogenic bacteria such as *Porphyromonas gingivalis*, *Proteus mirabilis*, *Helicobacter pylori*, and *Aeromonas veronii.* In all cases, these bacteria are putative collagenases, which are generally related to bacterial infections (Navais, Méndez et al. 2014, Damé-Teixeira, Parolo et al. 2018). For example, in *Porphyromonas gingivalis*, studies have reported the potential role of U32 collagenase in bacterial virulence. It is a microorganism capable of degrading soluble fibrillar collagen type I and reconstituted at or below body temperature (Zhang, Ran et al. 2015). These analyzes were possible from the characterization of a purified protease expressed from the bacterium's *prtC* gene (Kato, Takahashi et al. 1992).

Overexpression of genes in *S. mutans* (SMU_761 and SMU_759 - *S. mutans* UA159) were related to proteolytic activity and collagenases in RC lesions (Damé-Teixeira, Parolo et al. 2018). These findings are relevant due to the higher abundance of this organism in carious lesions. There is few evidence on *S. mutans* collagenolytic activity, such as its capacity to degrade collagen from rodent tendons (Rosengren and Winblad 1976). Still, another study showed that the same strain of *S. mutans* GS-5 collected from human carious lesions, was able not only to induce caries in rodents, but also to promote bone tissue

degradation, reinforcing the microbial role hypothesis (Rosengren and Winblad 1976).

A recent study showed, through functional analysis of *S. mutans* in the metatranscriptome of sound root surfaces (SRS) and carious root surfaces (RC) biofilms, a similar pattern of gene expression, and only a few genes were differentially expressed between SRS biofilms and those of radicular carious lesions. However, *S. mutans* showed greater functional abundance in samples of carious lesions. Although studies have claimed that *S. mutans* constitutes only a small proportion of the microbiota (Bowden 1990), this microorganism demonstrates a strong relationship with the disease and is present at higher frequencies on decayed root surfaces than on biofilms on root surfaces (Ellen, Banting et al. 1985, Nyvad and Kilian 1990) and can play an important role in the progression of RC (Ellen, Banting et al. 1985, Do, Damé-Teixeira et al. 2017, Santos, Do et al. 2022).

1.2.4 Clinical aspects and future perspectives for prevention and management of root caries

As discussed before, the management for root caries is still a challenge to restorative dentistry. Invasive treatments (restorations) of root caries lesions present several operational adversities, including difficulties in moisture control and adhesion to dentin (Schwendicke and Gostemeyer 2017). Therapies with fluoride in high concentrations (toothpaste 5000ppm/F) can control root caries lesions (Wierichs and Meyer-Lueckel 2015), and other agents such as silver diamine fluoride (SDF) also efficiently prevent and arrest initial lesions. However, cavities that extend under gingival margin and with a extensive loss of structure are complex to treat and have a fast evolution, frequently causing tooth loss.

Distinctive characteristics, such as the considerable amount of organic material in dentin and cementum, indicate that the management of these lesions often does not follow the same protocol applied to coronal surfaces (Burrow and Stacey 2017, Do, Damé-Teixeira et al. 2017). This fact demonstrates the need to seek treatment alternatives that aim to control the development and decrease the

speed of progression of lesions, particularly regarding the dentin (Wierichs and Meyer-Lueckel 2015, Meyer-Lueckel and Paris 2016, Meyer-Lueckel, Machiulskiene et al. 2019). Promoting the remineralization of demineralized dentin is one of the important strategies for the control of root caries (Mellberg and Sanchez 1986, Baysan, Lynch et al. 2001, Clarkson and Rafter 2001). Natural non-invasive therapies may be the key to mineral gain and have shown promising initial results. These are sources of new therapeutic agents and have been the focus of research in the prevention of oral diseases (Duarte, Koo et al. 2003, van Strijp, Takatsuka et al. 2015).

In root and coronal caries, most of the available therapeutic agents act as antimicrobials and little is discussed about substances with phytochemical effects on tissue demineralization and remineralization processes (Cai and Wu 1996, Li, Cai et al. 1997, Chu, Li et al. 2007). The biomodification of the dental surface is still little studied. It corresponds to using substances that promotes cross-links in collagen, significantly reducing the rates of biodegradation of the dentin matrix and increasing the biomechanical properties of healthy tissue (Walter, Miguez et al. 2008). Several reagents have been recognized as cross-linkers throughout the literature, including glutaraldehyde (GA), formaldehyde, carbodiimide and epoxy compounds (Walter, Miguez et al. 2008, Wang, Green et al. 2021). However, adverse effects such as toxicity and/or instability limited the use of these substances in *in vivo* studies. Recently, natural agents, available in fruits, bark, leaves, and seeds, have been considered biocompatible and stable for a long period of time in animals. Examples include proanthocyanidins (PA) genipin (GE), and cranberry (Wang, Green et al. 2021).

Promising results have shown that PA could efficiently stabilize collagen increasing its resistance against caries *in vitro* under artificial lesion formation (Walter, Miguez et al. 2008). Similarly, other study used an in vitro pH cycling model to evaluate the effect of GSE (grape seed extract that contains proanthocyanidins) on the remineralization of artificial root caries, showing a positive effect in the demineralization and/or remineralization processes (Xie, Bedran-Russo et al. 2008). Favorable results were also seen with the use of hesperidin (a citrus flavonoid antioxidant) on dentine collagen and remineralization in dentine lesion. In this study, the flavonoid preserved collagen

and inhibited demineralization, and enhanced remineralization even under the fluoride-free condition (Hiraishi, Sono et al. 2011). Extracts rich in polyphenols – such as grape seed (GSE), green tea (GTE) and cranberry juice (CJE) have also been studied in the literature with the aim of evaluating the effects of these agents on the interaction of cross-linking, digestion resistance and activities. of endogenous matrix metalloproteinase (MMP) from dentinal collagen. CJE-treated dentin collagen rapidly increased its resistance to digestion and MMP inhibition and, according to the authors, an application of CJE as short as 30 seconds may be a clinically viable approach to improve the longevity of dentin bonding in composite resin restorations (Wang, Green et al. 2021).

It is important to point out that the good results of cross-links are still based on *in vitro* experiments and simulate demineralization conditions with cyclic pH models. These conditions make immediate clinical applicability difficult, but within the limits of the studies, they show that cross-linkers agents may have the potential to promote the remineralization. More studies are needed to elucidate the action mode of these agents in human tissues. If the effectiveness of these treatments are confirmed, they might represent low-cost and easy-to-apply adjunctive non-invasive therapies for reducing the size of root caries lesions.

In conclusion, it is very likely that root caries is a 2-stage process, where the collagen breaking down is subsequent to the mineral loss, as collagenases need to physically access the collagen matrix. Presence of MMPs is indisputable and must be linked to collagen denaturation, while proteolytic bacteria and of microbial collagenase genes are prevalent in root caries may also make some contribution. If this function is confirmed, biofilm modulation may include collagenases as targets for root caries treatments and prevention, such as the use of substances capable to cross-link the root dentin or bacterial collagenase inhibitors.

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FIGURES

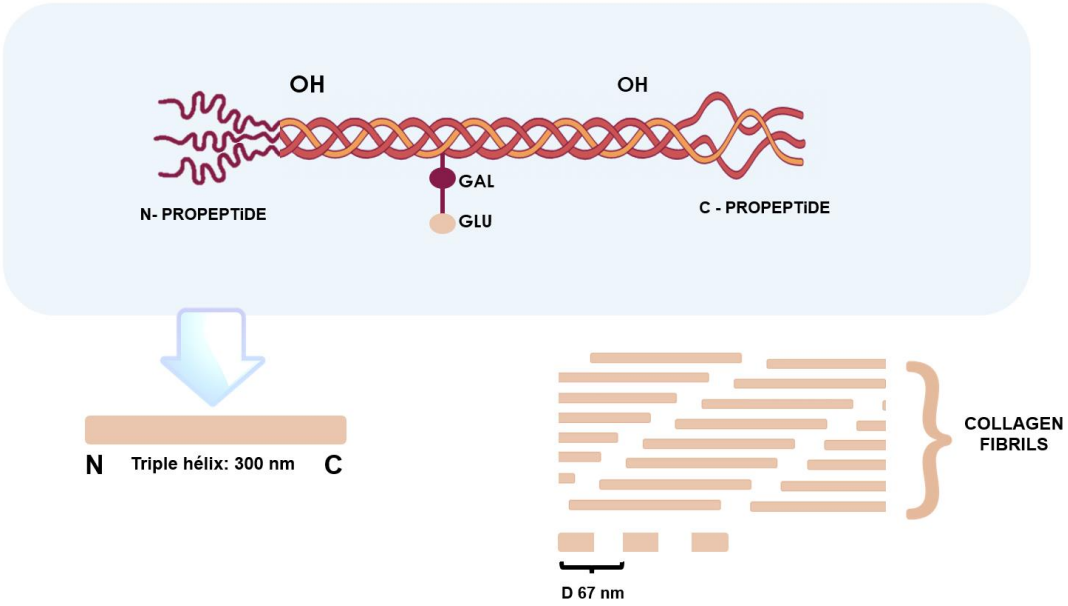

Figure 1: Representation of type I collagen adapted from Gelse et al. (2003) and Varma et al. (2016). N = N-terminal telopeptide region with 16 aa residues. C = C-terminal telopeptide region with 26 aa residues D = repeating unit of collagen fibril of length 67mm.

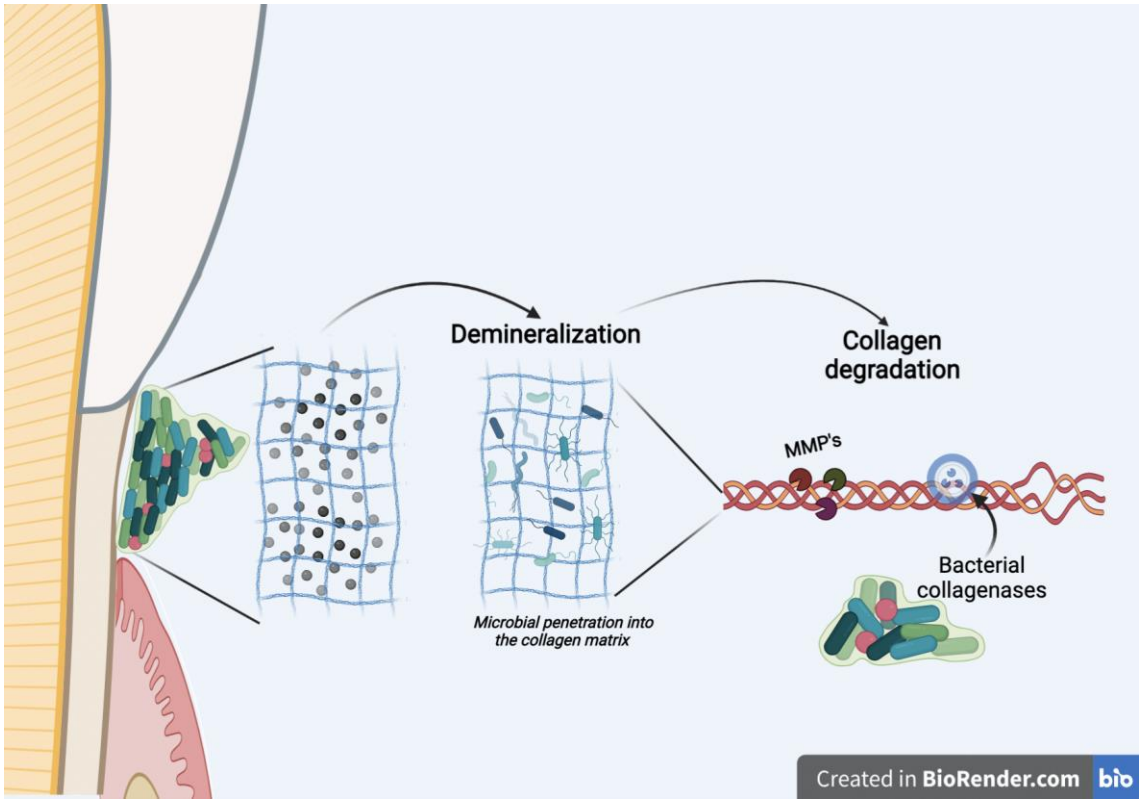

Figure 2: Representation of the action of collagenases on the root surface. When there is a disruption of the microenvironment's homeostasis, an imbalance of the microbiota caused by the low pH can cause caries lesions toward demineralization. Upon exposure of the root surface, rapid demineralization is expected due to the critical pH of the cementum and dentin, which can be as higher as 6. This critical pH which is reached in the presence of weak acids coming from the microbial biofilm saccharolytic metabolism. The demineralization exposes the root collagen fibers, which become accessible to bacteria and their acidic metabolites, thereby activating MMPs (matrix metalloproteinases). In addition to endogenous proteases, microbial collagenases may play a role in this process.

CAPÍTULO 2 – PRESENCE OF HOST AND BACTERIAL-DERIVED COLLAGENOLYTIC PROTEASES IN CARIOUS DENTIN: A SYSTEMATIC REVIEW OF EX VIVO STUDIES

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ABSTRACT

Objectives: The aim was to assess the host and bacterial-derived collagenolytic proteases profile in root and coronal dentin carious lesions. **Design:** The search was performed in eight databases and the grey literature. Studies evaluating *ex vivo* dentin, extracted teeth, or biofilms from natural caries lesions were included. The methodological quality of studies was assessed using the Joanna Briggs Institute tool. Synthesis of the results and the certainty of evidence were performed following the Synthesis without Meta-analysis (SWiM) checklist and GRADE approach for narrative synthesis, respectively. **Results**: From 935 recovered articles, 18 were included. Although the evidence was very uncertain, it was possible to assume that 1) MMP-2, MMP-9, MMP-13, and CT-B may be increased in carious dentin when compared to sound dentin; 2) there is no difference in MMP-2 presence, while MMP-13 may be increased in root when compared to coronal carious dentin; 3) there is no difference of MMP-2 and MMP-9 expression/activity before and after cavity sealing; 4) MMP-8 may be increased in the dentin before cavity sealing compared to dentin after cavity sealing; 5) there is no difference of MMP-20 in irradiated vs. non-irradiated carious dentin. MMP-20 probably reduces in carious outer dentin when compared to carious inner dentin (moderate certainty). Genes encoding bacterial collagenolytic proteases and protein-degrading bacteria were detected in coronal and root carious lesions (Protocol register-PROSPERO: CRD42020213141). **Conclusion**: Trends in the direction of the effect were observed for some collagenolytic proteases in carious dentin, that may represent a potential target for development of new treatments.

Keywords: Dentin Caries, Root Caries, Collagenase, Microbial Collagenase, Host Collagenase

2.1 INTRODUCTION

Untreated carious lesions at the cavity level are frequent in the worldwide population (Kassebaum et al., 2015), significantly affecting the individual's quality of life (Severo Alves et al., 2013). Besides, a growing incidence of root caries is being observed (Griffin et al., 2004; Kassab & Cohen, 2003; Severo Alves et al., 2013). Both coronal cavitated and root caries lesions share several key features, particularly the involvement of dentin. To enable the development of future strategies of prevention and control, expanding the molecular targets for interventions, it is important to better explore the etiology of the carious process in both, coronal and root dentin caries. When compared to enamel, root and dentin lesions have received considerably less attention, particularly concerning dentinal collagen breakdown.

The issue has grown in importance in light of the ecological hypothesis of dentin and root caries (Takahashi & Nyvad, 2016). It was suggested that the demineralization in the dentin caries is followed by a proteolytic stage, in which the exposed organic matrix is degraded (Takahashi & Nyvad, 2016). The collagen in the dentin matrix is subjected to denaturation by enzymatic degradation after exposure to collagen fibrils due to the low pH triggered by the bacterial acidogenesis (Tjaderhane et al., 2015; Tjäderhane et al., 1998). During demineralization, it is also expected some breakdown of the dentin matrix cross-links (Dayan et al., 1983). Then, the type I collagen is subjected to proteolysis by collagenolytic enzymes that, when activated, can site-specifically cleave its molecule (Tjaderhane et al., 2015).

Traditionally, it has subscribed to the belief that host proteases are present in carious dentin lesions and are associated with the proteolytic stage of caries (Nascimento et al., 2011; Tjäderhane et al., 1998; Vidal et al., 2014). Some studies have shown that matrix metalloproteinases are present in the dentinal organic matrix and might be activated under acidic conditions (around pH 4.5). Subsequently, the collagen would be denatured during the remineralization phase of the pH cycle, once the pH is neutralized by salivary buffers (Chaussain-Miller et al., 2006; Kawasaki & Featherstone, 1997; Takahashi & Nyvad, 2016;

Tjaderhane et al., 2015). However, most of these studies also showed the presence of the same proteases in the sound dentin matrix. Despite the debate on the presence of endogenous and salivary proteases in dentin organic matrix degradation in critical reviews (Chaussain-Miller et al., 2006; Tjaderhane et al., 2015), there is still no robust evidence on their presence in dentin/root caries. It seems that the current evidence is essentially based on *in vitro* models, roughly reflecting the natural caries lesions development (Tjaderhane et al., 2015).

When the bacterial collagenases are under debate, the real situation is far less clear. There is a relatively small body of literature that is concerned with a microbial role in this process, mostly *in vitro* or *in situ* study designs. Studies have shown that bacterial collagenases would not resist the acidic pH drop (pH 4.3) during the demineralization phase of a pH cycling model (Kawasaki & Featherstone, 1997), a fact that support the hypothesis that they do not play an important role in the carious process. Such approaches, however, have failed to address bacterial collagenolytic proteases in their natural system could be activated in the remineralization phase during the pH cycles, similarly to host collagenases. Furthermore, complex biofilms and interconnected metabolisms between different members of dental biofilms could be activating collagenases, which might not be detectable *in vitro*. Additionally, reports suggesting that the predominant microorganisms are not capable to degrade collagen matrix in cavitated caries lesions are previous to the application of NGS technologies in the oral microbiology field, mostly testing culturable bacteria(Kawasaki & Featherstone, 1997; Tjäderhane et al., 1998).

Studies evaluating *ex vivo* natural caries lesions could help in identifying factors such as variations in pH, the complex microbiota role, and substrates possibly influencing the pattern of activation of different proteases. Considering the progress of the knowledge in this area in the past decades, a systematic review could establish the current evidence, opening a new avenue for caries preventive strategies using collagenases as a target for new drugs (Chaussain-Miller et al., 2006). Therefore, the purpose of this study was to systematically review the host and bacterial-derived collagenolytic proteases profile in root and coronal dentin carious lesions.

2.2 MATERIALS AND METHODS

2.2.1 Eligibility criteria

The acronym PECO (Aromataris & Munn, 2020; Munn et al., 2018) (Population; Exposure; Comparator; Outcomes) was used to design the research question:

Participants/population: human dental tissue or biofilms collected from human dental tissue (*ex vivo* samples*);*

Exposure: caries in dentin and/or root surface;

Comparator/control: sound dentin / no control / sealed dentin;

Outcome: collagenolytic (or gelatinolytic / collagenolytic protease) presence / activity / gene expression.

Inclusion criteria: Studies eligible for this review were the ones analyzing human dentin or biofilms with natural caries lesions. These comprised *ex vivo* extracted teeth or clinical carious/biofilm samples from observational (cross-sectional, case-control, or cohort studies), and experimental studies (randomized or non-randomized clinical trials), with no limitation of publication year.

Exclusion criteria: Exclusion criteria were the following: (1) reviews, letters, conference abstracts, personal opinions, book chapters, and protocols; (2) *in vitro* studies, including artificial caries models; (3) studies performed in dental enamel caries/erosion lesion; (4) studies evaluating only sound dentin; (5) studies evaluating other than enzymatic activity of collagenases / gelatinases / collagenolytic proteases; (6) studies written in the non-Latin alphabet; and (7) animal studies. Although not foreseen in protocol phase, studies on matrix metalloproteinases (MMPs) inhibitors were not included after the full-text reading due to the particularities of their research questions.

2.2.2 Data sources and search strategy

The search was performed in March 2022. Supplementary Table 1 shows the complete search strategy. “Dental caries” and “Collagenases”, their

synonyms and variations were used as main search terms for PubMed search strategy elaboration, which was adapted for each electronic database: MEDLINE via PubMed, LILACS, Web of Science, Scopus, Cochrane, EMBASE, and Livivo. Gray literature search was also performed in Google Scholar, ProQuest (Dissertations and Thesis), and OpenGrey. Reference lists from included studies were assessed to identify other potentially eligible studies. No language or interval time restrictions were applied. Duplicates were identified through EndNoteWeb (Clarivate Analytics, Mumbai) and then manually identified on Rayyan QCRI® (Qatar Computer Research Institute, Qatar).

2.2.3 Study selection and data extraction

Two independent reviewers (IMS and CBB) selected the included articles. First, both reviewers independently read titles and abstracts, applying the eligibility criteria within a web application tool designed for systematic reviews (Rayyan QCRI®, Qatar Computing Research Institute). In a second stage, the same reviewers performed a full-text reading while applying for the eligibility criteria. In both stages, all the retrieved information was crosschecked by a third reviewer (JAC). Once a study was selected for the second stage and it was not available in any way through online sources, the corresponding author was contacted to provide the full-text.

2.2.4 Methodological quality assessment

The methodological quality assessment of the included studies was evaluated by two independent reviewers (IMS and CBB) using the JBI Critical Appraisal Checklist for Analytical Cross-Sectional Studies (https://jbi.global/critical-appraisal-tools) (Aromataris & Munn, 2020). Despite all questions of the adopted appraisal tool are considered important, four of them were considered critical items to this systematic review due to the design of included articles. These items included: “Were the criteria for inclusion in the sample clearly defined?”; “Were the study subjects and the setting described in detail?”; “Was the exposure measured in a valid and reliable way?” and “Were objective, standard criteria used for measurement of the condition?”.

Criteria adopted for considering a low methodologic quality were: two or more “no” answers in those critical items; or one “no” and two or more “unclear” answers in those critical items; or one “no” answer in a critical and two or more “no” answers in non-critical items. High methodological quality was considered when a study gets a maximum one “no” answer or two “unclear” answers in non-critical items. Studies were considered with a moderate methodological quality when they did not fit the criteria, as described before (Dame-Teixeira et al., 2021).

2.2.5 Data analysis

Data analysis was reported according to the synthesis without meta-analysis (SWiM) reporting guideline (Campbell et al., 2020), in addition to chapter 12 of the Cochrane Handbook (McKenzie et al., 2022). Due to the high heterogeneity of the included studies, turning out a meta-analysis was unfeasible. The studies and their results were grouped and analyzed according to the origin of collagenase (host or bacterial-derived), the method used to detect collagenases presence or activity (collagenases assays, including westernblots and commercial kits, gene expression, immunohistochemistry), and paired comparisons (sound versus carious dentin; root versus coronal dentin; outer versus inner dentin; irradiated versus non-irradiated dentin). The metrics to measure the outcome were the statistically significant differences related to the primary studies in collagenase presence/activity at the paired comparisons (effect direction).

The certainty of the evidence was addressed through the GRADE approach for narrative synthesis (in the absence of a single effect estimate) (Murad et al., 2017), considering the main five domains for downgrading (risk of bias, inconsistency, indirectness, imprecision, and likelihood of publication bias) for the main collagenases found for each paired comparison. When average differences between groups and standard deviations were available in the literature, the optimal information size (OIS) was calculated to determine the imprecision. Results were then standardly reported according to GRADE guidelines, based on the size of the effect and the certainty of the evidence (Santesso et al., 2020).

2.3 RESULTS

A total of 935 records were found by searching the databases. After removing duplicates, 568 remained for titles and abstracts reading. From 50 records selected for full-text reading, 18 studies were included for qualitative synthesis as they met the eligibility criteria: Boushell *et al*., 2011 from USA; Ballal *et al*., 2017 from Switzerland, Lee *et al*., 2013 from South Korea; Loreto *et al.,* 2014 from Brazil; Nascimento *et al*., 2011 from Brazil; Gomes-Silva *et al*., 2017; Gomes-Silva *et al*., 2017b from Brazil; Chibinski *et al*., 2014 from Brazil; Charadram et al., 2012 from Australia; Kuhn *et al.*, 2016 from Brazil; Vidal *et al.,* 2014 from Brazil, Damé-Teixeira *et al*., 2018 from Brazil and United Kingdon; Shimada *et al.,* 2009 from Japan; Hashimoto *et al*., 2011 from Japan; Toledano *et al.,* 2010; Simon-Soro *et al*. 2015 from Spain; Bello Arroyo and Simón-Soro, 2015 from Spain; Tjaderhane et al, 1998 from Canada/Finland/England (Fig. 1; Supplementary Appendix 2). Only four studies evaluated bacterial collagenases in dental caries samples, and all others were devoted to the study of host collagenases. While studies on bacterial collagenases evaluated mainly *ex vivo* biofilms using microbial nucleic acids analysis, studies on host collagenases mostly used immunohistochemistry direct to the dentin tissue, so that the host and bacterial-derived collagenolytic proteases could be differentiated. No studies reported both, host and bacterial collagenases.

Due to the characteristic of the studies, low sample sizes were observed, ranging from 3 to 42 samples (on average 15 samples). As for the methodological quality, 4 studies were classified as “high” methodological quality (Bello Arroyo, 2016; Damé-Teixeira et al., 2018; Simon-Soro et al., 2013), 11 as “moderate”(Ballal et al., 2017; Chibinski et al., 2014; Gomes-Silva et al., 2017; Gomes-Silva et al., 2017; Hashimoto et al., 2011; Kuhn et al., 2016; Loreto et al., 2014; Nascimento et al., 2011; Shimada et al., 2009; Tjäderhane et al., 1998; Yeon Lee et al., 2013), and 3 as “low” methodological quality (Boushell et al., 2011; Toledano et al., 2010; Vidal et al., 2014) according to the JBI tool (Table 1, Table 3, Supplementary Appendix 3).

The heterogeneity in the methods and protocols made comparisons across studies particularly difficult. The qualitative synthesis of selected articles will be presented by dividing them into host collagenases and bacterial collagenases.

2.3.1. Host-derived Collagenolytic Proteases

Data on host-derived proteases were generally reported according to the location within the lesions and the enzymatic activity abundance in the different sites. Two studies applied enzymatic assays, and nine applied immunohistochemistry methods. No studies considered the age of the individuals or other clinical characteristics as confounding factors, except the ones from Gomes-Silva et al. (Gomes-Silva et al., 2017; Gomes-Silva et al., 2017), Nascimento et al. (Nascimento et al., 2011), and Charadram et al. (Charadram et al., 2012) (Table 1, Supplementary appendix 4).

Ten out of 18 included studies assessed the prevalence of the gelatinase MMP-2, comprising a total of 210 carious samples and 163 sound dentin samples evaluated across all the literature. From those, five evaluated more than one sample per teeth considering the lesion depth (Chibinski et al., 2014; Shimada et al., 2009; Toledano et al., 2010; Vidal et al., 2014) or location (Kuhn et al., 2016; Vidal et al., 2014). This represented the most studied MMP. Table 2 summarizes of the qualitative, quantitative and certainty of the evidence for MMP-2, MMP-8, MMP-9, MMP-13, MMP-20, cysteine cathepsins (CTs) B and K for different paired comparisons (sound versus carious dentin; root versus coronal caries; lesion depth and location - outer versus inner dentin; irradiated versus non-irradiated), as detailed below.

2.3.1.1 Sound vs. Carious

MMP-2 may be increased in carious dentin when compared to sound dentin, but the evidence is very uncertain. Two out of seven studies found no significant differences between sound and carious tissues (Boushell et al., 2011; Shimada et al., 2009) or regarding the level of caries severity (Boushell et al., 2011). However, other five studies showed significant higher presence/activity of MMP-2 in carious dentin when compared to sound ones, suggesting an effect

direction (Table 2). The low methodological quality of 3 out of 7 studies was the main reason for serious risk of bias, while the imprecision was serious as the total sample size (N=210 carious and 163 sound) did not reach the Optimal Information Size (OIS) (Supplementary Appendix 6.1).

Also, MMP-9, MMP-13 and CT-B may be increased in carious dentin when compared to sound dentin, but the evidence is very uncertain. Due to the conflicting results of the included studies, no effect direction was found so the evidence is very uncertain regarding the MMP-8 presence in carious vs. sound dentin (Supplementary Appendix 6). Higher intensity of immunodetection of CTs B and K, and MMPs in general was registered in carious dentin than in sound dentin, though only five samples were analyzed per group (Vidal et al., 2014). The same pattern of increased immunoexpression was also observed regarding CT-B (Nascimento et al., 2011) and MMP-13 (Loreto et al., 2014). The opposite direction was observed by Shimada et al. (Shimada et al., 2009); they found a significant decrease of MMP-8 and MMP-9 in the inner carious dentin compared to sound dentin, and, in their study, the MMP-20 was the highest prevalent in sound dentin (Shimada et al., 2009).

2.3.1.2 Coronal vs root dentin caries

For this paired comparison, two host-derived collagenases were studied across the literature. There is no difference in MMP-2 activity in root versus coronal carious dentin. Immunofluorescence results from one study (Toledano et al., 2010) showed that MMP-2 was present in both coronal and root dentin in all specimens of extracted teeth (sound and carious). MMP-13 may be increased in root caries when compared to coronal, but the evidence is very uncertain (Supplementary Appendix 6.1 and 6.4). It has to be noted that no MMP-13 activity was observed in the crown region by western blot methods, suggesting its exclusive presence in root caries lesions (Yeon Lee et al., 2013). However, another study showed an increase in coronal caries when compared to sound dentin (Loreto et al., 2014).

2.3.1.3 Lesion depth and location

Figure 2 illustrates a summary of the location of host collagenases MMPs according to the included studies. Some MMPs activity and/or presence were

more significant the closer the lesion was to the pulp and in root dentin (when compared to coronal dentin) (Ballal et al., 2017; Toledano et al., 2010; Vidal et al., 2014; Yeon Lee et al., 2013). Eight studies used immunohistochemistry assays to evaluate the presence and location of host collagenases in carious samples (Ballal et al., 2017; Boushell et al., 2011; Kuhn et al., 2016; Nascimento et al., 2011; Shimada et al., 2009; Vidal et al., 2014). Although some of them described an increase in the collagenolytic proteases expression and activity according to the closer proximity to the pulp tissue, they evaluated different types of collagenases making the cross-study comparison intricate (Ballal et al., 2017; Nascimento et al., 2011; Toledano et al., 2010; Vidal et al., 2014; Yeon Lee et al., 2013).

While a study showed statistically significant increase in the level of MMP-2 immunoreactivity in tubules affected by caries across all samples (Boushell et al., 2011), other studies showed that MMP-2 was ubiquitous (present in sound and carious, root and coronal dentin) and varied considerably in different regions of the dentin (Kuhn et al., 2016; Toledano et al., 2010). Consequently, the evidence is very uncertain about the MMP-2 location in dentin, as no effect direction was found (Supplementary appendix 6.1). The same pattern was observed regarding MMP-8 and 9, i.e. generalized distribution in the intertubular dentin, with no statistical differences when considering their location (Kuhn et al., 2016). The evidence is very uncertain about the MMP-9 expression in different locations and lesion depth, as studies showed opposite effect directions. However, MMP-20 probably reduces in carious outer dentin when compared to carious inner dentin (moderate evidence). The MMP-20 expression was intense along the dentin-enamel junction (Gomes-Silva et al., 2017).

2.3.1.4 Before and after cavity sealing

Although the evidence is very uncertain, there is no difference of MMP-2 and MMP-9 expression/activity before and after cavity sealing, however, MMP-8 may be increased in the dentin before when compared to dentin after cavity sealing (Supplementary appendix 6). The imprecision was the reason for downgrading, as the OIS was not reached. The intensity of immunostaining of MMP-2 and MMP-9 was similar between dentin samples collected before and after restoration with glass ionomer cement (after 60 days), being the MMP-9

distributed in the intertubular dentin for both periods in all samples (Kuhn et al., 2016). The same study showed that MMP-8 was observed in all samples at baseline, but significantly reduced after 60 days of sealing (Kuhn et al., 2016). Chibinski et al., that evaluated the expression of the same proteases, in addition to type I collagen and bone sialoprotein (BSP) in infected dentin after sealing with glass ionomer, revealed a significant decrease of MMP-8 in carious dentin after cavity sealing (Chibinski et al., 2014). MMP-2 and 9 were more concentrated around the dentinal tubules.

2.3.1.5 Irradiated vs. non-irradiated dentin

There is no difference about the MMP-20 expression in irradiated vs. non-irradiated carious dentin, but the evidence is very uncertain (Supplementary appendix 6.5). Gomes-Silva et al. (Gomes-Silva et al., 2017; Gomes-Silva et al., 2017) evaluated the expression and activity of MMP-2 e MMP-20 in dentin-enamel junction and in the dentin-pulp complex of irradiated subjects, with no significant difference in either activity or gelatinolytic expression between the irradiated and non-irradiated groups.

2.3.2. Bacterial-derived Collagenolytic Proteases

Even though the number of studies is limited, a possible activity of bacterial-derived collagenases in dentinal/root caries has been shown (Table 3, Supplementary appendix 5). The synthesis of the results will be separated by the type of methodology applied. Because of the limited number and diversity of studies, the GRADE system was not applied, highlighting the urgent need for further research.

2.3.2.1 Collagenase enzymatic assays

Two studies assayed samples from nine subjects for microbial collagenase using ELISA and SDS-PAGE methods (Bello Arroyo, 2016; Hashimoto et al., 2011). The results are inconclusive: while one identified bacterial isolates capable of degrading protein in root lesions (Hashimoto et al., 2011), another has failed to demonstrate collagenolytic activity in the biofilm of dentinal lesions (Bello Arroyo, 2016). This fact, however, can be explained by the small amount of

collagen present in the culture medium used or the small sample size (only 3 subjects) (Bello Arroyo, 2016).

Some protein-degrading bacteria were detected in the biofilm of root carious lesions, such as *Actinobaculum*, *Prevotella* and *Propionibacterium*. For this study, the clinical characteristics of six individuals were evaluated, such as age, gender, plaque and counts of colony unity forming. However, it is important to emphasize that the sample number was small to be able to infer the importance of this result (Hashimoto et al., 2011). These bacteria were capable of degrading collagen. The proportions of protein-degrading and protein-clotting bacteria (acid-production capable of protein denaturation) in root caries lesions, supragingival biofilm from sound sites and periodontitis subgingival biofilm were 7 and 33%, 0 and 26% and 17 and 40% of the microbiota, respectively (Hashimoto et al., 2011).

2.3.2.2 Omics

Two studies found the gene expression of bacterial collagenases using omics data, both showing significant expression of genes coding for collagenolytic proteases in coronal (Simon-Soro et al., 2013) and root caries (N. Damé-Teixeira et al., 2018). A few bacterial collagenolytic proteases had high gene expression values in root surfaces biofilms (SMU_761 and SMU_759 from *S. mutans* and RS05935 from *V. parvula*), while others were overexpressed on root caries (Log2 fold change > 8) when compared to sound root surfaces biofilms comprised *P. alactolyticus* [HMPREF0721_RS02020], *S. inopinata* JCM 12537 [SCIP_RS02440], *P. alactolyticus* [HMPREF0721_RS04640] and *O. uli* DSM7084 [OLSU_RS02990] [Damé-Teixeira et al., 2018].

2.4 DISCUSSION/CONCLUSION

The role of host and bacterial collagenolytic proteases in distinct mechanisms involved in the development of carious lesions has not been fully elucidated yet. Although some research has been conducted linking dentinal collagen degradation and caries progression with host proteases activation, most studies corresponded to *in vitro* and *in situ* designs. In this systematic review, we found 18 studies evaluating *ex vivo* samples from clinical studies. A high heterogeneity precluded quantitative comparisons between studies, however,

trends in the direction of the effect were observed for some host-derived collagenolytic proteases in carious dentin. Genes coding for bacterial collagenolytic proteases and protein-degrading bacteria were detected in coronal and root dentin carious lesions.

The GRADE approach confirmed a very low certainty of the body of evidence for almost all analysed comparisons. The reasons for each downgrade and documentation of all assessments of the certainty of the body of evidence are available in Supplementary Appendix 6 (Supplementary file 2). However, it is important to emphasize that the GRADE approach, when used for systematic reviews of association, always consider observational studies as low certainty of evidence, which means that any downgrading will result in a very low certainty of evidence. Main reasons for other downgrading were methodological issues (risk of bias or methodological quality) of included studies, imprecision and inconsistency. The GRADE rule of thumb for imprecision considers an Optimal Information Size (OIS) of 800 samples (considering tests and controls) for continuous variables. For this reason and considering the specificities of this kind of study (*ex vivo*), we preferred to calculate the OIS whenever it was possible. However, for most comparisons it was not possible, then a conservative attitude was accomplished by following the GRADE recommendation.

Inconsistency was the reason for few downgrades, as studies had conflicting results and no clear effect direction was found for the presence of MMP-2 (lesion depth and location) and MMP-9 (sound vs. carious dentin; lesion depth and location). These conflicting results are potentially associated with differences in the sensitivity of the assays measuring the collagenolytic activity. Other potential explanation for discrepancies may rely on the absence of information about the clinical characteristics of the samples' donors, such as sex, age, diet, salivary flow, caries activity, etc. as studies were mostly descriptive in nature and not always explore the potential role of proteases in the carious process according to other clinical variables.

It worth noting that achieving certainty regarding the absence of publication bias is a challenging task, and determining the appropriate threshold and rate for its likely presence is equally complex. Given the comprehensive nature of our search in several databases and grey literature, without restriction

on language or time, a reduced chance of publication bias we classified it as undetected (Page et al. 2022). For this reason, there was no downgrade due to publication bias in the GRADE approach (Guyatt et al., 2011). Also, indirectness resulted in no downgrading, since all included studies answered one or more review questions, and fulfilled all the eligibility criteria. Whether there is no downgrading due to the main GRADE domain, it is possible to consider upgrading the evidence certainty for some comparison, in the presence of large magnitude of effect, dose-response gradient or if plausible confounding can increase confidence in estimated effects (Guyatt et al., 2011). In this review, the referred large magnitude was only observed for MMP-20 comparison in carious outer dentin vs. carious inner dentin, which resulted in moderate certainty of evidence for higher expression in carious outer dentin.

It has been suggested that two stages are present in the development of carious lesions on dentin (Nyvad & Fejerskov, 1990). First, a demineralization stage occurs, maintaining the characteristic cross-banding of collagen fibers (Deyhle et al., 2011; Nyvad & Fejerskov, 1990). In a second moment, the dentinal collagen would be degraded by proteolytic enzymes, damaging the structural characteristics of collagen fibers (Takahashi & Nyvad, 2016). Tjaderhane et al. (Tjaderhane et al., 2015) suggested that collagen bands could also be degraded during the demineralization phase by host-derived collagenolytic proteases activation. Although the physiological roles of those enzymes in dentin are not well understood, they might participate in the formation of peritubular and tertiary dentin and in the release of dentinal growth factors which, in turn, would regulate defensive reactions in the pulp (Charadram et al., 2013; Hannas et al., 2007; Mazzoni et al., 2012; Muromachi et al., 2012; Tjäderhane et al., 2015; Tjäderhane et al., 2013; Wahlgren et al., 2002) capable and sufficient to degrade demineralized dentin *in vitro* (Tezvergil-Mutluay et al., 2010; Tjäderhane et al., 1998). In this context, the gelatinase MMP-2 can be involved in the spread of caries. More attention should be given to MMP-9, MMP-13, and CT-B in further studies as they may also be increased in carious dentin.

The controversy about scientific evidence for bacterial collagenases activity in dentin has raged unabated. There is a relatively small body of literature that is concerned with a microbial role in this process, mostly *in vitro* or *in situ*

study designs. It has been reported that the predominant microorganisms are not capable to degrade collagen matrix in cavitated caries lesions (Kawasaki & Featherstone, 1997; Tjäderhane et al., 1998), and that cariogenic bacteria could not completely degrade the organic matrix of dentin after demineralization (Lynch & Ten Cate, 2006). A potential role was previously discarded by investigating 32 streptococci and lactobacilli isolates, from which only one colony showed faint gelatinolytic activity in enzymography *ex vivo* (Tjäderhane et al., 1998). However, these studies analyzed isolated microorganisms, disregarding the biofilm as a whole, the presence of other microorganisms and their complex metabolic interaction. On the other hand, recent advances in molecular methods (including NGS technologies) suggest a potential role of these collagenases in caries, showing a massive presence of proteolytic bacteria and the overexpression of genes encoding collagenases and other proteases (Damé-Teixeira et al., 2018; Hashimoto et al., 2011; Simon-Soro et al., 2013), although it is important to take into account that gene expression does not mean enzymatic activity. However, some well-known oral bacteria involved in oral diseases produce their own collagenases that can be capable of breaking down the dentinal collagen (Harrington, 1996).

Interestingly, a recent study showed that the bacterial composition of root caries lesions located under the gingival margin is likely to have periodontal pathobionts: *Porphyromonas, Selenomonas, Filifactor, Peptococcus* and *Tannerela* inhabit root caries lesions that extend beyond the gingival margin (Takenaka et al., 2021). This suggests that the microbiome in root caries lesions expanding across the gingival margin would show an increase in proteolytic bacterial diversity. Furthermore, the recent integrated ecological hypothesis for caries and periodontitis (Nyvad & Takahashi, 2020) points to a common risk factor for both diseases, which are originated in the dynamic stability stage and emerged in response to nutritional unbalance in the microbiota.

The difficulty of comparing the findings across all included studies due to high heterogeneity is a limitation of this study. In addition, few included studies were classified as “low” methodological quality, which reduces the strength of the scientific evidence. These findings reinforce the need for further research aiming to identify and characterize both host and bacterial collagenolytic proteases. In

the long term, unravelling the role of proteolytic bacteria in the degradation of the dentin matrix could open the way for new therapeutic measures in the treatment of dental caries.

In conclusion, although the evidence was very uncertain, it was possible to assume that 1) MMP-2, MMP-9, MMP-13, and CT-B may be increased in carious dentin when compared to sound dentin; 2) there is no difference in MMP-2 presence, while MMP-13 may be increased in root when compared to coronal carious dentin; 3) there is no difference of MMP-2 and MMP-9 expression/activity before and after cavity sealing; 4) MMP-8 may be increased in the dentin before cavity sealing when compared to dentin after cavity sealing; 5) there is no difference about the MMP-20 expression in irradiated vs. non-irradiated carious dentin. MMP-2 was present in almost all samples studied across the literature, and no effect direction was found in its presence regarding lesion depth and location. MMP-20 probably reduces in carious outer dentin when compared to carious inner dentin (moderate certainty of the evidence). For bacterial proteases, there is controversy over the scientific evidence of their activity in carious lesions, in addition to a significantly smaller number of studies focused on microbial proteolysis. However, genes encoding bacterial collagenolytic proteases and protein-degrading bacteria have already been seen with considerable prevalence in carious lesions and can represent a potential target for biofilm modulation.

Protocol and registration

This systematic review was reported according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist (Page et al., 2021), and designed according to the Joanna Briggs Institute (JBI) Manual for Evidence Synthesis - systematic reviews of etiology and risk (Moola et al. 2020). The Cochrane Handbook was used as a support paper for our systematic review (Higgins et al., 2022). A study protocol was registered at the International Prospective Register of Systematic Review (PROSPERO) database, under the identification number CRD42020213141.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) [grant numbers 88887.703237/2022-00; 88887.613390/2021-00; and 88887.687833/2022-00].

Data Statement

The data that support the findings of this study are openly available in Supplementary files 1 (Supplementary appendix 1 to 5) and 2 (Supplementary appendix 6).

Acknowledgments

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq). CBB, IMS and JAC gratefully acknowledge the scholarships from CAPES. ND-T acknowledges the UK's Academy of Medical Sciences Newton International Fellowship (NIF\R5\242) and the CNPq/EBSERH (no. 408020/2021-0) for their support. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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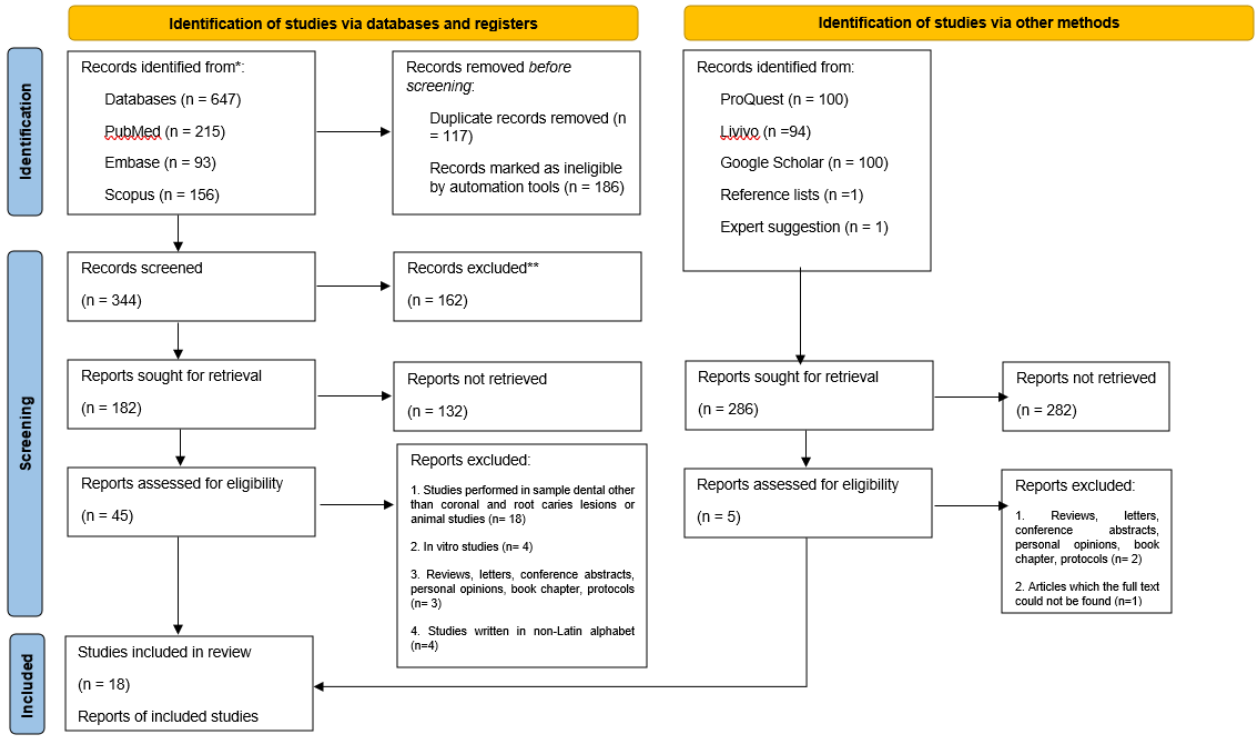
FIGURES



From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/

Figure 1. Flowchart of the study. PRISMA 2020 flow diagram for new systematic reviews which included searches of databases, registers and other sources

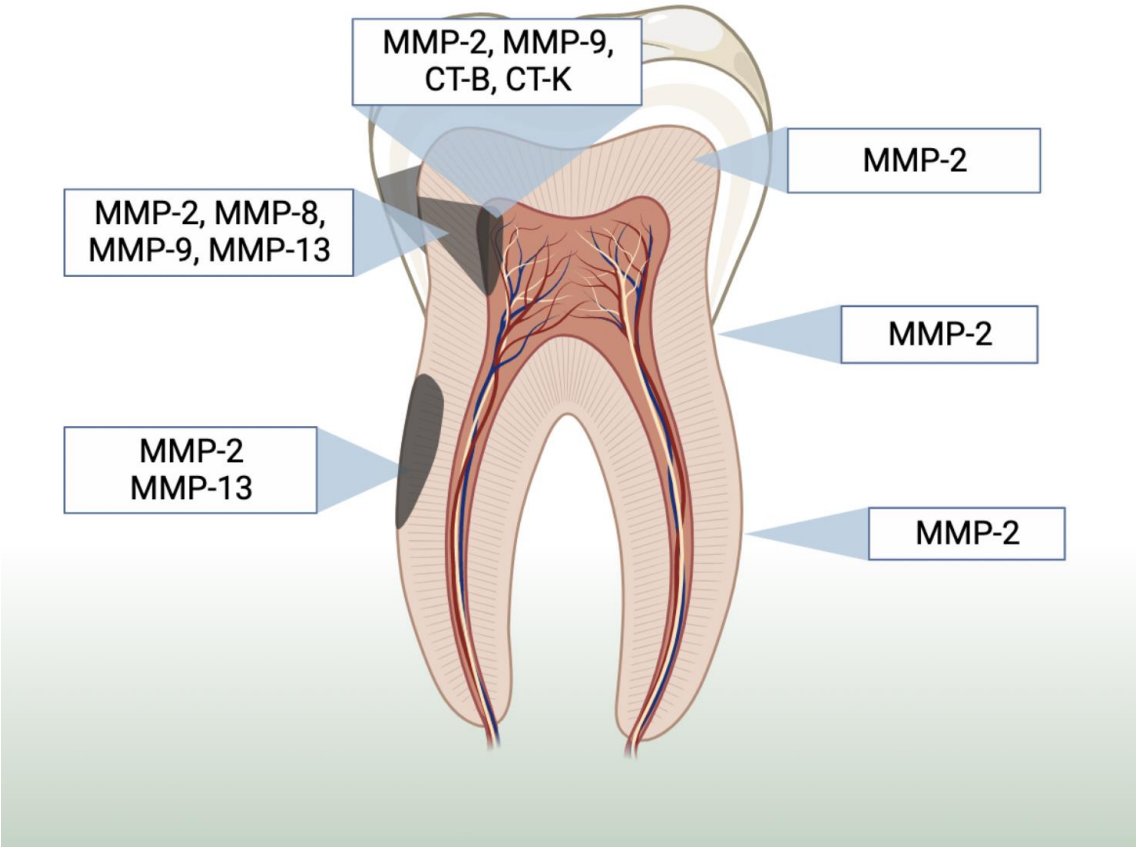


Figure 2. Summary of the location of host collagenases MMPs according to included studies. MMP-2 was observed in both sound and carious, radicular and coronal dentin (Boushell et al., 2011; Chibinsky et al., 2014; Vidal et al., 2014; Gomes-Silva et al., 2017; Toledano et al., 2010; Kuhn et al., 2016; Shimada et al., 2009). MMPs- 8 and 9 were found in coronal samples, suggesting a predilection for carious sites for the MMP-9 (Shimada et al., 2009; Gomes-Silva et al., 2017; Chibinski et al., 2014; Kuhn et al., 2016; Vidal et al., 2014). The presence of MMP-13 was observed more frequently in root tissues (Lee et al., 2013) and in the carious sites (Lee et al., 2013; Loreto et al., 2014). Cysteine cathepsins B and K (CT-B and CT-K) can be more expressed in carious tissues of coronal samples, with intensity increasing with proximity to the pulp chamber (Nascimento et al., 2001; Vidal et al., 2014).

TABLES

Table 1. Characteristics and methodological quality of individual studies evaluating host collagenases according to the comparative analysis (N= 14; some studies are duplicated as presented results for more than one comparative analysis).

Author, year, country	Sample location and size	Type of collagenase	Method used to identify/quantify collagenases	Main results	Methodological quality
Sound vs. Carious dentin					
Boushell *et al.*, 2011; USA	Coronal; Caries (n=6) vs. sound (n=10)	MMP-2 and BSP	Immunohistochemistry	No differences between carious vs. sound or caries severity.	+
Charadram et al., 2012 Australia	Coronal; Caries (n=30) vs. sound (n=15)	MMP- 2	Immunohistochemistry Collagenase assay	MMP-2 activity in the reactionary dentin was significantly higher than in the sound dentin.	+++
Loreto *et al.,* 2014; Brazil	Coronal; Caries (n=10) vs. sound (n=3)	MMP-13	Immunohistochemistry	**MMP-13 increased in caries**; very weak in sound dentin.	++
Nascimento *et al*., 2011; Brazil	Coronal; Caries (n=8) vs. sound (n=4)	CT-B MMPs and Cysteine proteinases	Immunohistochemistry Collagenase assay	CT-B less intense in sound than carious dentin; MMPs and cysteine proteinase activity increase with the age; Cysteine proteinase increase with the lesion depth, higher in active than inactive lesions.	++
Shimada *et al.,* 2009; Japan	Coronal (n=5)	MMP-2, MMP-8, MMP-9 and MMP-20	Immunohistochemistry	MMP-2 present in all conditions, either sound or caries; **MMP-8 and MMP-9 increased at the outer carious dentin when compared to sound.**	++
Toledano *et al.,* 2010; Spain	Coronal and root (n=10)	MMP-2	Immunohistochemistry	MMP-2 present in all conditions, either sound or caries, increased closer to the wider dentinal tubules; Demineralised (“affected”) dentin exhibited a low intensity of MMP-2 (but higher than sound).	+

Tjaderhane et al, 1998 Canada; Finland; England	Coronal (n = 37)	MMP-2, MMP-8 and MMP-9	Western blot enzymography	MMP-2, MMP-8 and MMP-9 were identified in carious dentin lesions; Overall, MMP-9 appeared to be the predominant gelatinolytic enzyme in dentin caries lesions.	++
Vidal *et al.,* 2014; Brazil	Coronal; Caries (n=5) vs. sound (n=5)	CT-B, CT-K and MMP-2	Immunohistochemistry	**CT-B, CT-K, MMP-2, and MMP-9 significantly higher in in caries than sound dentin.**	+
Root vs. Coronal					
Lee *et al*., 2013; USA	Coronal and root; (n=7)	MMP-13	Collagenase assay	**MMP-13 increased in root caries**, and absent in coronal caries.	++
Toledano *et al.,* 2010; Spain	Coronal and root (n=10)	MMP-2	Immunohistochemistry	No differences root and coronal dentin.	+
Lesion depth and location					
Ballal *et al*., 2017; Switzerland	Coronal (n=33)	MMP-2 and MMP-9	ELISA	**Significantly more MMP-9 in deep carious lesions than shallow;** No difference for MMP-2	++
Boushell *et al*., 2011; USA	Coronal; Caries (n=6) vs. sound (n=10)	MMP-2 and BSP	Immunohistochemistry	MMP-2 and BSP in carious and sound dentin (**higher activity closer to odontoblastic processes**). Significantly more immunostaining for MMP-2 and BSP in caries-affected tubules**.**	+
Shimada *et al.,* 2009; Japan	Coronal (n=5)	MMP-2, MMP-8, MMP-9 and MMP-20	Immunohistochemistry	MMP-2 present in all conditions; **MMP-8 and MMP-9 increased at the outer carious dentin when compared to inner. MMP-20 reduced toward the outer caries.**	++
Gomes-Silva *et al*., 2017; Brazil	Coronal; Irradiated (n=19) vs. non-irradiated (n=17) carious dentin	MMP-2 and MMP-9	Immunohistochemistry	**MMP-9 predominantly positive in the non-irradiated**; MMP-2 and MMP-9 more expressed along the dentin-enamel junction, and highly positive in carious dentin.	++
Gomes-Silva *et al*., 2017b; Brazil	Coronal; Irradiated (n=19) vs. non-irradiated (n=17) carious dentin	MMP-20	Collagenase assay	No differences, but more expressed along the dentin-enamel junction.	++

Vidal et al, 2014;	Coronal; Caries (n=5) vs. sound (n=5)	CT-B, CT-K and MMP-2	Immunohistochemistry	MMP-2 is more expressed in carious dentin	+
Before vs. after cavity sealing					
Chibinski *et al*., 2014; Brazil	Coronal (n=25)	MMP-2, MMP-8 and MMP-9	Immunohistochemistry	**Significant increase of MMP-8 before cavity sealing**	++
Kuhn *et al.*, 2016; Brazil	Coronal; (n=23)	MMP-2, MMP-8 and MMP-9	Immunohistochemistry	**Reduction of MMP-8 after 60 days of cavity sealing**.	++
Irradiated vs. Non – irradiated					
Gomes-Silva et al. 2017; Brazil	Coronal; Irradiated (n=19) vs. non-irradiated (n=17) carious dentin	MMP-20	Collagenase assay	**No significance difference between irradiated and non-irradiated.**	++

MMP = Metaloproteinase
CT-B = Cathepsin B
CT-K = Cathepsin K

Table 2. Summary of the findings of the qualitative, quantitative, and certainty of the evidence produced analyses for MMPs in the included studies.

MMP	Studies included / Methodological quality	Caries Group (n)	Sound Group (n)	Studies/Findings	Certainty of evidence GRADE (see Appendix 6 for more details, supplementary file 2)
MMP-2	[Tjäderhaneet al., 1998] ++ [Vidalet al., 2014] + [Chibinskiet al., 2014] ++ [Ballalet al., 2017]++ [Gomes-Silvaet al., 2017a] ++ [Shimadaet al., 2009] ++ [Kuhnet al., 2016]++ [Boushellet al., 2011] + [Toledanoet al., 2010] + [Charadram et al.], 2012 Australia +++	210	163	***Sound vs. Carious dentin*** [Tjäderhaneet al., 1998]: Present in carious dentin – NA [Vidalet al., 2014]: More in carious dentin – NA [Boushellet al., 2011]: More in caries-affected tubules ($p < 0.05$) [Shimadaet al., 2009;Gomes-Silvaet al., 2017a]; NS carious vs. sound ($p > 0.05$) [Toledanoet al., 2010]: More in caries-affected – NA [Charadram et al.], 2012 : More in carious dentin - carious vs. sound ($p < 0.05$) ***Lesion depth and location*** [Ballalet al., 2017] NS Location ($p > 0.05$) [Boushellet al., 2011] More in outer sound dentin ($p < 0.05$) (Shimada, Ichinose et al. 2009): NS Location ($p > 0.05$) [Vidalet al., 2014]: More in inner dentin – NA [Shimadaet al., 2009;Gomes-Silvaet al., 2017a]: More in dentin-enamel junction – NA **Root vs. Coronal** [Toledanoet al., 2010] No diferences – NA ***Before vs. after cavity sealing*** [Chibinskiet al., 2014];[Kuhnet al., 2016] NS before vs. after sealing ($p > 0.05$)	⊕◯◯◯ Very low
MMP-8	[Tjäderhaneet al., 1998] ++ [Chibinskiet al., 2014] ++ [Shimadaet al., 2009]++ [Kuhnet al., 2016] ++	90	90	***Sound vs. Carious dentin / Lesion depth and location*** [Tjäderhaneet al., 1998]: Present in carious dentin – NA [Shimadaet al., 2009]: More in sound dentin, but also increased in outer carious dentin ($p < 0.05$) ***Before vs. after cavity sealing*** [Chibinskiet al., 2014]);(Kuhn, Reis et al., 2016): More in baseline (carious dentin) ($p < 0.05$)	⊕◯◯◯ Very low
MMP-9	[Tjäderhaneet al., 1998] ++ [Vidalet al., 2014] + [Chibinskiet al., 2014] ++ [Ballalet al., 2017]++ [Gomes-Silvaet al., 2017a] ++	164	128	***Sound vs. Carious dentin / Lesion depth and location*** [Tjäderhaneet al., 1998]: Present in carious dentin – NA [Vidalet al., 2014]: More in carious dentin – NA [Shimadaet al., 2009]: More in sound dentin, but also increased in outer carious dentin ($p < 0.05$) [Ballalet al., 2017]: More in deep than shallow carious tissue ($p < 0.05$) [Gomes-Silvaet al., 2017a]: Positive in carious dentin – NA	⊕◯◯◯ Very low

	[Shimadaet al., 2009] ++ [Kuhnet al., 2016]++			***Before vs. after cavity sealing*** [Chibinskiet al., 2014];[Kuhnet al., 2016]: NS before vs. after sealing (p > 0.05)	
MMP-13	[Loretoet al., 2014] ++ [Yeon Leeet al., 2013] ++	17	10	***Sound vs. Carious dentin*** [Loretoet al., 2014]: More in coronal caries group (p < 0.05) ***Root vs. Coronal*** [Yeon Leeet al., 2013]: More in root lesions – NA	⊕◯◯◯ Very low
MMP-20	[Gomes-Silvaet al., 2017b] ++ [Shimadaet al., 2009] ++	41	41	**Irradiated vs. non irradiated** NS **Lesion depth and location** [Shimadaet al., 2009]: Significantly lower in outer dentin when compared to inner.	⊕◯◯◯ Very low ⊕⊕⊕◯ Modarate
CT-B	[Vidalet al., 2014] + [Nascimentoet al., 2011] ++	13	9	**Sound vs. Carious dentin** [Vidalet al., 2014]; [Nascimentoet al., 2011]: Significantly higher in in caries than sound dentin (p < 0.05)	⊕◯◯◯ Very low

MMP = Metaloproteinase
CT-B = Cathepsin B
NA = statistics not available
NS = non-significant
+ = low methodological quality; **++** moderate methodological quality; **+++** high methodological quality

Table 3. Characteristics and methodological quality of individual studies evaluating bacterial collagenases according to collagenase activity or gene expression (N=4).

Author, year, country	Sample location	Type of collagenase	Method used to identify/quantify collagenases	Main results	Methodological quality
Collagenase activity					
Arroyo and Simón Soro, 2015; Spain	Coronal (n=3 dentin samples)	Non-characterized bacterial collagenases	Collagenase assay testing isolates	No collagenolytic activity (low concentration of collagen in the culture media used).	+++
Hashimoto *et al*., 2011; Japan	Root (n=6 dentin samples)	Non-characterized bacterial collagenases	SDS-PAGE testing isolates for collagen degradation	Protein-degrading bacteria (the ones forming clear zones around their colonies on the FAA-skim milk plates) **isolated from biofilm on root caries lesions were capable of degrading collagen** (*Prevotella, Actinobaculum* and *Propionibacterium* were predominant within this group); Protein-coagulating bacteria did not degrade collagen (the ones forming whitish-coagulating zones around their colonies on the FAA-skim milk plates), but produced enough organic acids to denature proteins; The proportion of protein-degrading in root caries was 7%.	++
Collagenases gene expression					
Damé-Teixeira *et al*., 2018; Brazil	Root; Caries (n=9) vs. sound (n=10)	Most peptidase U32 Collagenase-like protease, PrtC family	Omics	42 bacterial collagenolytic proteases with significant differential expression, from which **18 were superexpressed in root caries**	+++
Simon-Soro *et al*. 2015; Spain	Coronal; Dentin (n=3) vs. enamel (n=3)	Non-characterized bacterial collagenases or proteases	Omics	Genes coding for **collagenases and other proteases overrepresented in dentin caries when compared to enamel caries.**	+++

Supplementary Appendix 1. Search strategy

Database	Search strategy	Results
Cochrane Library	(“Dental Caries“ OR “Root Caries“) in Title Abstract Keyword AND (“Collagenases“ OR “Microbial Collagenase“ OR “metalloproteinase collagen“) in Title Abstract Keyword - (Word variations have been searched)	4
LILACS	(tw:(Dental Caries OR Dental Decay Caries OR Dental Decay OR Carious Dentin OR Carious Dentins OR Coronal Dentin OR coronal dentin OR Root Caries OR Cervical Caries OR Cervical Cary OR Caries)) AND (tw:(Collagenases OR Collagen-Degrading Enzyme OR Collagen Degrading Enzyme OR Collagenase OR Collagen Peptidase OR Peptidase, Collagen OR Microbial Collagenase OR Collagenase-Like Peptidase OR Collagenase Like Peptidase OR metalloproteinase collagen))	2
Google Scholar	(“Dental Caries“ OR “Carious Dentin“ OR “Root Caries“ OR “Caries“) AND (“Collagenases“ OR “Collagen Degrading Enzyme“ OR “Microbial Collagenase“)	101
PubMed	(Dental Caries [MeSH Terms] OR Dental Decay Caries OR Dental Decay OR Carious Dentin OR Carious Dentins OR Coronal Dentin OR coronal dentin OR Root Caries[MeSH Terms] OR Cervical Caries OR Cervical Cary OR Caries) AND (Collagenases [MeSH Terms] OR Collagen-Degrading Enzyme OR Collagen Degrading Enzyme OR Collagenase OR Collagen Peptidase OR Peptidase, Collagen OR Microbial Collagenase [MeSH Terms] OR Collagenase-Like Peptidase OR Collagenase Like Peptidase OR metalloproteinase collagen)	215
Web of Science	(Dental Caries OR Dental Decay Caries OR Dental Decay OR Carious Dentin OR Carious Dentins OR Coronal Dentin OR coronal dentin OR Root Caries OR Cervical Caries OR Cervical Cary OR Caries) AND (Collagenases OR Collagen-Degrading Enzyme OR Collagen Degrading Enzyme OR Collagenase OR Collagen Peptidase OR Peptidase, Collagen OR Microbial Collagenase OR Collagenase-Like Peptidase OR Collagenase Like Peptidase OR metalloproteinase collagen)	176
Embase	(Dental Caries OR Dental Decay Caries OR Dental Decay OR Carious Dentin OR Carious Dentins OR Coronal Dentin OR coronal dentin OR Root Caries OR Cervical Caries OR Cervical Cary OR Caries) AND (Collagenases OR Collagen-Degrading Enzyme OR Collagen Degrading Enzyme OR Collagenase OR Collagen Peptidase OR Peptidase, Collagen OR Microbial Collagenase OR Collagenase-Like Peptidase OR Collagenase Like Peptidase OR metalloproteinase collagen)	93

Scopus	("Collagenases" OR "Collagen Degrading Enzyme" OR "collagenase" OR "Microbial Collagenase") AND ("Dental Caries" OR "Dental Decay Caries" OR "Dental Decay" OR "Carious Dentin" OR "Coronal Dentin" OR "Root Caries" OR "Cervical Caries" OR "Caries")	156
Livivo	(“Dental Caries“ OR “Dental Decay Caries“ OR “Dental Decayv OR “Carious Dentin“ OR “Carious Dentins“ OR “Coronal Dentin“ OR “coronal dentin“ OR “Root Caries“ OR “Cervical Caries“ OR “Cervical Cary“ OR “Caries“) AND (“Collagenases“ OR “Collagen-Degrading Enzyme“ OR “Collagen Degrading Enzyme“ OR “Collagenase“ OR “Collagen Peptidase“ OR “Peptidase, Collagen“ OR “Microbial Collagenase“ OR “Collagenase-Like Peptidase“ OR“ Collagenase Like Peptidase“ OR “metalloproteinase collagen“)	94
Proquest	(Dental Caries OR Dental Decay Caries OR Dental Decay OR Carious Dentin OR Carious Dentins OR Coronal Dentin OR coronal dentin OR Root Caries OR Cervical Caries OR Cervical Cary OR Caries) AND (Collagenases OR Collagen-Degrading Enzyme OR Collagen Degrading Enzyme OR Collagenase OR Collagen Peptidase OR Peptidase, Collagen OR Microbial Collagenase OR Collagenase-Like Peptidase OR Collagenase Like Peptidase OR metalloproteinase collagen)	100
OpenGrey	Caries AND colagenases	0

Supplementary Appendix 2. Excluded studies and reasons for exclusion (n= 33).

Author/Year	Reasons for exclusion*
Armstrong, 1958	2
Ashwini, 2020	1
Beltz, 1999	2
Damé-Teixeira, 2017	3
Dayan, 1983	2
Gonçalves, 2021	1
Guirado, 2021	3
Harrington, 1994	1
He, 2005	5
Hedenbjork-Lager, 2014	3
Hedenbjork-Lager, 2015	2
Hedenbjork-Lager, 2016	3
Hu, 2019	1
Huang, 2021	1
Khan, 2021	6
Kawasaki, 1997	3
Leonardi, 2010	6
Niu, 2011	1
Schmidt, 2018	1
Sulkala, 2004	1
Tananure, 2011	1

Tannure, 2012	1
Van-Strijp, 1992	1
Van-Strijp, 1994	1
Van-Strijp, 2003	1
Vasconcelos, 2019	1
Wang, 2018	5
Xu, 2011	1
Yan, 2014	5
Yang, 2006	5
Zheng, 2011	1
Zheng, 2012	1
Zhu, 2012	4

* 1. Studies performed in sample dental other than coronal and root caries lesions or animal studies (n= 18)

2. In vitro studies (n= 4)

3. Reviews, letters, conference abstracts, personal opinions, book chapter, protocols (n= 5)

4. Articles which the full text could not be found (n=1)

5. Studies written in non-Latin alphabet (n=4)

6. Outcome including other bacterial enzymatic activity that does not collagenases

References of the excluded articles:

1. Armstrong, W. G. "Further studies on the action of collagenase on sound and carious human dentin." Journal of Dental Research 37.6 (1958): 1001-1015.
2. Ashwini, Ajay, et al. "Dentin degradonomics–The potential role of salivary MMP-8 in dentin caries." Journal of Clinical and Experimental Dentistry 12.2 (2020): e108.
3. Beltz, R. E., E. C. Herrmann, and H. Nordbö. "Pronase digestion of carious dentin." Caries research 33.6 (1999): 468-472.
4. Damé-Teixeira, Nailê, Clarissa Cavalcanti Fatturi Parolo, and Marisa Maltz. "Specificities of caries on root surface." Root caries: From prevalence to therapy. Vol. 26. Karger Publishers, 2017. 15-25
5. Dayan, D., I. Binderman, and G. L. Mechanic. "A preliminary study of activation of collagenase in carious human dentine matrix." Archives of Oral Biology 28.2 (1983): 185-187.
6. Gonçalves, Rafael Simões, et al. "Two-year randomized clinical trial of different restorative techniques in non-carious cervical lesions and MMP activity in gingival crevicular fluid." Clinical Oral Investigations 26.2 (2022): 1889-1902.
7. Guirado, E., and A. George. "Dentine matrix metalloproteinases as potential mediators of dentine regeneration." Eur. Cells Mater 42 (2021): 392-400.

8. Harrington, Dean J., and Roy RB Russell. "Identification and characterisation of two extracellular proteases of Streptococcus mutans." FEMS microbiology letters 121.2 (1994): 237-241.
9. He, Chang-Li, et al. "The effect of matrix metalloproteinase-1 on root surface dentin matrix: a scanning electron microscope observation." Hua xi kou Qiang yi xue za zhi= Huaxi Kouqiang Yixue Zazhi= West China Journal of Stomatology 23.2 (2005): 113-115.
10. He, Chang-Li, et al. "The effect of matrix metalloproteinase-1 on root surface dentin matrix: a scanning electron microscope observation." Hua xi kou Qiang yi xue za zhi= Huaxi Kouqiang Yixue Zazhi= West China Journal of Stomatology 23.2 (2005): 113-115.
11. Hedenbjörk-Lager, Anders, et al. "Caries correlates strongly with salivary levels of matrix metalloproteinase-8." Caries Research 49.1 (2015): 1-8.
12. Hedenbjörk-Lager, Anders, et al. "Collagen degradation and preservation of MMP-8 activity in human dentine matrix after demineralization." Archives of oral biology 68 (2016): 66-72.
13. Hedenbjörk-Lager, Anders. Dentine caries: acid tolerant microorganisms and aspects on collagen degradation. Diss. Malmö University, Faculty of Odontology, 2014.
14. Hu, Xiao-Pan, et al. "Association of ENAM, TUFT1, MMP13, IL1B, IL10 and IL1RN gene polymorphism and dental caries susceptibility in Chinese children." Journal of International Medical Research 47.4 (2019): 1696-1704.
15. Huang, Bo. Identification and Characterization of Proteolytic Activities from S. mutans that Hydrolyzes Dentinal Collagen Matrix. Diss. University of Toronto (Canada), 2021.
16. Khan, Zaid Majeed, et al. "Differentially expressed salivary proteins in dental caries patients." BioMed Research International 2021 (2021).
17. Kawasaki, K., and J. D. B. Featherstone. "Effects of collagenase on root demineralization." Journal of dental research 76.1 (1997): 588-595.
18. Leonardi, Rosalia, and Carla Loreto. "Immunohistochemical localization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in human carious dentine." Acta histochemica 112.3 (2010): 298-302.
19. Niu, LN1, et al. "Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine." Journal of dentistry 39.8 (2011): 536-542.
20. Schmidt, Jana, et al. "aMMP-8 in correlation to caries and periodontal condition in adolescents—results of the epidemiologic LIFE child study." Clinical Oral Investigations 22.1 (2018): 449-460.
21. Sulkala, Merja, et al. "Matrix metalloproteinase-13 (MMP-13, collagenase-3) is highly expressed in human tooth pulp." Connective tissue research 45.4-5 (2004): 231-237.
22. Tannure, P. N., et al. "MMP13 polymorphism decreases risk for dental caries." Caries research 46.4 (2012): 401-407.
23. Tannure, Patricia Nivoloni, and Erika Calvano Küchler. "Patients with manifest caries lesions have higher levels of salivary matrix metalloproteinase-8 than patients with no caries lesions." Journal of Evidence Based Dental Practice 16.1 (2016): 77-78.
24. Van Strijp, A. J. P., B. Klont, and J. M. Ten Cate. "Solubilization of dentin matrix collagen in situ." Journal of dental research 71.8 (1992): 1498-1502.
25. Van Strijp, A. J. P., et al. "Bacterial colonization and degradation of demineralized dentin matrix in situ." Caries research 28.1 (1994): 21-27.
26. Van-Strijp, A. J. P., et al. "Host-derived proteinases and degradation of dentine collagen in situ." Caries Research 37.1 (2003): 58-65.
27. Vasconcelos, Katia Regina, et al. "MMP13 contributes to dental caries associated with developmental defects of enamel." Caries Research 53.4 (2019): 441-446.
28. Wang, Xiao, and M. Qin. "A preliminary study of saliva matrix metalloproteinases (MMP-2 and MMP-9) in children with caries." Beijing da xue xue bao. Yi xue ban= Journal of Peking University. Health Sciences 50.3 (2018): 527-531.
29. Yan, G. et al. "Relationship between dental caries and salivary proteome by electrospray ionization ion-trap tandem mass spectrometry in children aged 6 to 8 years." West China Journal of Stomatology 32.3 (2014).

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31. Zheng, X., et al. "Real-time enzymatic degradation of human dentin collagen fibrils exposed to exogenous collagenase: an AFM study in situ." Journal of microscopy 241.2 (2011): 162-170.
32. Zheng, Xinyu, et al. "AFM study of the effects of collagenase and its inhibitors on dentine collagen fibrils." Journal of Dentistry 40.2 (2012): 163-171.
33. Zhu, Zi-yuan, Tian Zhou, and Bao-wei Zhang. "Characterization and analysis of matrix metalloproteinases 8 and 20 in the human crown and root dentin." Chinese Journal of Tissue Engineering Research 16.24 (2012): 4526.

Supplementary Appendix 3. Quality assessment of the individual included studies (n = 17) using The Joanna Briggs Institute Critical Appraisal Checklist for Cross-sectional Studies. Methodological quality was categorized as high, low or moderate according to critical domain of each question. Criteria adopted to this systematic review for considering a low methodological quality was two “no” or one “no” and one “unclear” in critical domains. High methodological quality was considered when an article got a maximum one “no” answer or two “unclear” answers in non-critical domains. The other ones were classified as moderate.

Questions	Author, Year																
	Arroyo and Simón Soro,	Boushell et al,	Charadram et	Damé-Teixeira	Hashimoto et	Simon-Soro, A.	Ballal et al,	Chibinski et al,	Gomes-Silva et	Gomes-Silva et al,	Kuhn eta l,	Lee et al, 2013	Loreto et al,	Nascimento, F.	Shimada, Y, et.	Toledano, M	Vidal et al,	Tjäderhane
Q1*	UN	N	Y	N	Y	Y	Y	Y	Y	Y	Y	U N	U N	Y	U N	N	U N	N
Q2*	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	U N	Y
Q3*	Y	U N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U N	Y
Q4*	Y	U N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U N	N	U N	Y
Q5	N	N A	N A	N A	N	N A	N	N	N	N	N	N	N	N	N A	N	N	U N
Q6	N	N A	N A	N A	N	N A	N	N	N	N	N	N	N	N	N A	N	N	N
Q7	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q8	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N A	Y	Y	Y	N A	Y	N A
MQ	**H**	**L**	**H**	**H**	**M**	**H**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**L**	**L**	**M**

Critical domain (*); methodological quality (MQ); yes (Y), no (N), unclear (U); not applicable (NA); low (L); high (H). Q1 – CRITICAL: Were the criteria for inclusion in the sample clearly defined? Q2 – CRITICAL: Were the study subjects and the setting described in detail? Q3 – CRITICAL: Was the exposure measured in a valid and reliable way? Q4 – CRITICAL: Were objective, standard criteria used for measurement of the condition? Q5 – NON-CRITICAL: Were confounding factors identified? Q6 – NON-CRITICAL: Were strategies to deal with confounding factors stated? Q7– NON-CRITICAL: Were the outcomes measured in a valid and reliable way? Q8 – NON-CRITICAL: Was appropriate statistical analysis used?

Supplementary Appendix 4. Data collection from studies evaluating host collagenases, presented according to the method used to collagenase identification/measurement.

Collagenase assay

Author, year	Country	N (caries group)	N (control group)	*Ex vivo* specimen collected	Methods	Collagenase assay	Clinical characteristics of the sample	Type of collagenase	Main Conclusions
Charadram et al, 2012	Australia	30 permanent molar teeth carious	15 sound	Extracted teeth	Realtime qPCR and gelatinase assay	The protein extracted from reactionary dentin (layer 4) contained specific MMP-2 activity of 19.36 ± 5.13 mU/µg while protein extracted from healthy dentin samples contained specific MMP-2 activity of 3.50 ± 0.12 mU/µg	Were obtained from male and female patients aged 20 to 35 years	MMP – 2	MMP-2 activity in the Reactionary dentin (layer 4) was significantly higher than the healthy sample. All values depict means ± SD (n=14). * P≤ 0.05; ** P≤ 0.02
Gomes-Silva et al, 2017b	Brazil	19 Irradiated carious dentin (15 male and 4 female; mean age: 58-60 years)	17 Non Irradiated carious dentin	Extracted teeth (demineralized and non-demineralized)	Immunohistochemical ; Gelatinolytic activity assay by zymography	Gelatinolytic activity analysis of specimens from subgroup 1: Irradiated x Non irradiated: DEJ 0/7 (0%) x 1/7 (14,2%) p = 0,31;	Erupted teeth (n = 36) from HNC (Head and Neck Cancer) patients. 19 post-HNRT(post Head and Neck Treatment) specimens. In subgroup 1 ten patients were male and 1 patient was female, and in subgroup 2 five patients	MMP-20	MMP-20 expression was pronounced and intense along the DEJ of all of the irradiated and nonirradiated examined specimens; No differences in MMP-20 expression in the

						Caries (I/O) 4/7 (57,1%) x 4/7 (57,1%); Caries (C) 5/7 (71,4%) x 2/7 (28,5%); Dentinal tubules 4/7 (57,1%) x 5/7 (71,4%); Pre-dentin 1/7 (14,2%) x 2/7 (28,5%); Tertiary dentin 2/4 (50%) x 1/4 (25%) (I = incisal, O = occlusal, C = cervical)	were male and 3 were female. The mean age was 58 years (range 36–74) and 60 years (range 52–75) in subgroups 1 and 2, respectively. Smoking habit, as well as alcohol abuse, was recorded in 9 and 6 patients. 17 nonirradiated controls.		DEJ, dentin-pulp complex components, and carious dentin of post-HNRT patients
Lee et al, 2013	South Korea	7 Carious dentin	Sound teeth	Extracted human teeth (protein extraction)	Western Blot	MMP-13 antibody was recognized in both its latent and active forms, in the crown in normal e decayed teeth; The root in normal teeth, C1 and C2 grade caries: white bands are observed at ~50 kDa. A root with C3 grade caries; strong expression is observed at ~50 kDa.	NA	MMP-13	MMP-13 was not expressed in the coronal dentin, expressed weakly in the sound root dentin, and markedly expressed in roots with a wide range of pulp-invading caries

Tjaderhane et al, 1998	Canada; Finland; England	37	NA	Samples of active human coronal dentinal caries lesions were collected from extracted teeth.	Enzymography and Western blot	Both MMP-2 and 9 were detected in their non-active and active forms and MMP-9 appeared to be the predominant gelatinolytic enzyme in dentin caries lesions.	NA	MMP-2, MMP-8 and MMP-9	MMP-2,8 and MMP-9 were identified in the soft dentin lesions by Western immunoblots, and the gelatinase activity was confirmed by enzymography

Immunohistochemistry

Author, year	Country	N (caries group)	N (control group)	Specimen collection	Clinical characteristics of the sample	Type of collagenase	Main Conclusions
Boushell et al, 2011	USA	10 erupted 3rd molars and premolars with caries	6 erupted 3rd molars and premolars without caries	Extracted	NA	MMP-2 and BSP	MMP-2 and BSP detected throughout the caries-free dentin with increased staining of the odontoblastic processes in the inner; Sound vs. Carious = no differences; The level of MMP-2 e BSP detection did not change with the level of caries severity.
Chibinski et al, 2014	Brazil	25 carious dentin of primary teeth at baseline	25 carious dentin of primary teeth after cavity sealing (60-day sample)	Dentin excavator	N=33 patients of both genders, with age ranging from 3 to 10 years (average 6.0 ± 2.1)	MMP-2, MMP-8 and MMP-9	Presence of the MMPs at baseline and after cavity sealing; The expression of the MMPs increased after sealing, but statistical differences were observed only for MMP-8.

Charadram et al, 2012	Australia	30 permanent molar teeth carious	15 healthy	Extracted teeth	Were obtained from male and female patients aged 20 to 35 years	MMP-2	MMP-2 was detected within dentinal tubules containing odontoblastic processes. More intense reactivity for MMP-2 was detected in the Reactionary dentin compared to the comparable layer of dentin from healthy teeth.
Gomes-Silva et al, 2017	Brazil	19 Irradiated carious dentin (15 male and 4 female) Mean age: 58-60 years	17 Non Irradiated carious dentin	Extracted teeth (demineralized and non-demineralized)	Erupted teeth (n=36) from HNC patients. 19 post-HNRT. Smoking habit, as well as alcohol abuse, was recorded in 9 and 6 patients.	MMP-2 and 9	The MMP-2 and MMP-9 expression levels were pronounced and intense along the DEJ in all specimens; MMP-2 and MMP-9 highly positive in carious dentin; Tertiary dentine, pre-dentine and pulp were variably positive, and MMP-9 was predominantly positive in the non-irradiated specimens.
Kuhn et al, 2016	Brazil	23 Carious dentin	23 mesial portions from carious lesion	Dentin excavator	Students of both genders with ages ranging from 7 to 15 years (11.0 +-2.7 years).	MMP-2, 8 e 9	MMP-8 was reduced after 60 days of sealing, and no difference was observed for MMP-2 and MMP-9; The MMPs' distribution was generalized in the intertubular dentin and absent or located in the intratubular dentin, regardless of the period.
Loreto et al., 2014	Brazil	10 Carious dentin	2 Sound 3rd molars	Extracted teeth (specimens were demineralized)	NA	MMP-13	Sound dentin exhibited very weak immunoreactivity that was detected only at the peritubular level; On the contrary dilated dentinal tubuli close to the caries process showed very strong immunoreactivity; MMP-13 immunostaining diminished with increasing distance from the caries process.
Nascimento et al, 2011	Brazil	8 Carious dentin	4 Sound third molar	Extracted teeth	Adults (20 to 30 years)	Cathepsin B	Intense and consistent cathepsin B immunostaining was observed in odontoblasts and dentinal tubules of caries lesions with less intensity in healthy teeth, and no staining in negative controls.

		42 chronic or active caries lesions	NA	Sterile spoon excavators		Cysteine cathepsin proteinases /Does not specify the MMP group	A statistically significant increase in cysteine proteinase activity was observed with the increasing depth of the dentinal caries lesions. The negative correlations between the age and enzyme activities in active caries lesions were strong for both the cysteine cathepsins and MMPs. A strong positive correlation was observed with cysteine cathepsin and MMP activities
Shimada, Y 2009	Japan	5 third molar with Carious dentin	NA	Extracted	NA	MMP-2, MMP-8, MMP-9 e MMP-20	MMP-2 was distributed in both carious and sound dentin; the level of MMP-2 showed no significant difference among the outer carious, inner carious, and sound dentine; Other MMPs showed a significant difference of distribution between different dentine regions: MMP-8 and MMP-9 increased at the outer caries may be from saliva and might cause the breakdown of dentine matrix in the outer caries lesion

Toledano, M. 2010	Spain	10 Carious dentin	Sound dentin	Extracted	Teeth were collected from patients aged from 18 to 20 years	MMP-2	MMP-2 was present in both coronal and radicular dentin of all 10 specimens, but the immunoreactivity to MMP-2 varied markedly within the different dentin regions; More intense immunoreactivity was coincident with areas surrounding wider dentinal tubules that resulted from the mineral dissolution during the carious process; Adjacent to the “caries-infected” dentin, a zone of “caries affected” dentin could be identified that exhibited a low intensity of MMP-2 expression than the caries-infected dentine, but with much higher immunoreactivity than sound dentine
Vidal et al, 2014	Brazil	5 Carious dentin	5 Sound dentin	Extracted	3rd molars from individuals that were aged from 25 to 38 years.	Catepsina B e K e MMP-2 e 9	CTs and MMPs, were more intensely localized in regions that correspond to the pulp chamber, predentin, and/or inner dentin; Immunodetection of proteases was markedly higher in caries than in sound dentin; Abundance of CT-B and CT-K was six- and seven-fold higher, respectively, in caries than in sound dentin; abundance of MMP-2 and MMP-9 was, respectively, 5- and 15-fold higher in caries affected dentin than sound dentin

ELISA

Author, year	Country	N (caries group)	N (control group)	Specimen collection	Clinical characteristics of the sample	Main Conclusions
Ballal et al, 2017	Switzerland	33 teeth	NA	Paper point	Clinically healthy patients, 13 females and 17 males, aged 18–47 years (mean = 25 years, median = 27 years).	Significantly more MMP-9 in deep carious lesions; No difference for MMP-2

Supplementary Appendix 5. Data collection from studies evaluating bacterial collagenases

Author, year	Country	N (caries group)	N (control group)	Specimen collection	Methods	Collagenase assay OD or gene expression	Clinical characteristics of the sample	Type of collagenase	Main Conclusions
Arroyo and Simón Soro, 2015	Spain	3	NA	Drill	Collagenase activity assays for selected isolates (ELISA)	Fluorescence measurements provided by Tecan: *Porphyromonas gingivalis (11700)* *Lactobacillus delbrueckii (3543.6)* *Lactobacillus gasseri (3861.5)* *Lactobacillus rhamnosus (4339.8)* *Lactobacillus salivarius (3634.5)* *Lactobacullus zeae (3720.9)* *Prevotella denticola (3575.0)* *Intermediate prevotel (3542.5)* *Pseudoramibacter alactolyticus (2752.4)* *Streptococcus mutans (2968.0)* *Streptococcus salivarius (3033.1)*	NA	NA	No collagenolytic activity, due to the low concentration of collagen in the culture media used.
Hashimoto et al, 2011	Japan	6 subjects/ samples	NA	Curette	SDS-PAGE	The SDS-PAGE analysis of collagen degradation by representative protein-degrading bacteria (*P. acnes, Actinobaculum sp. oral clone EL030 and T. denticola*) showed that the collagen bands (ca. 200 and 130 kDa) were faded out during a 6-hour incubation, and several small peptide bands appeared (ca. 70, 25 and 10 KDa in *P. acnes*, ca. 17 KDa *in Actinobaculum sp*. oral clone EL030, and smear bands in T.*denticola*).	Two females and four males, age; 48-73 years; mean age 65.5 years.	NA	The proportion of protein-degrading in root caries was 7%. *Prevotella, Actinobaculum* and *Propionibacterium* were predominant in protein-degrading isolates. SDS-PAGE = protein-degrading bacteria isolated from plaque on root caries lesions were capable of degrading collagen; Protein-coagulating bacteria did not degrade collagen, but produced enough organic acids to denature proteins, i.e., alter protein conformation.
Damé-Teixeira et al, 2018	Brazil and United Kingdom	30 Root caries	10 Biofilms from sound root surfaces	Curette	Metatranscriptomics	The genes with the highest expression in RC were: *S. mutans* [SMU_761 and SMU_759] from and *V. parvula* [RS05935], *P . alactolítico* [HMPREF0721_RS02020], *S. inopinata* JCM 12537 [SCIP_RS02440], *P. alactolyticus*	NA	Peptidase U32 Collagenase-like protease, PrtC family	201 genes coding for bacterial collagenolytic proteases were identified in 113 bacterial species; 24 from *Prevotella* spp. and 20 from *Streptococcus* spp.

						[HMPREF0721_RS04640], and *O. uli* DSM7084 [OLSU_RS02990] In SRC: *L. buccalis [*LEBU_RS10190 and LEBU_RS05040] Overexpressed proteases in RC: *P. alactolyticus* [HMPREF0721_RS02020], *S. inopinata* JCM 12537 [SCIP_RS02440], *P. alactolyticus* [HMPREF0721_RS04640], and *O. uli* DSM7084 [OLSU_RS02990]			42 bacterial collagenolytic proteases with significant differential expression: 24 were overexpressed in SRS and 18 in RC
Simon-Soro et al. 2013	Spain	3 dentin caries	3 supragengival dental plaque from enamel lesions	Excavator	Metagenomics	NA	NA	NA	Genes coding for collagenases and other proteases enabling dentin degradation are significantly overrepresented in dentin cavities.

NA=Not available

Supplementary Appendix 6. Certainty of body of evidence assessed through the GRADE approach (GRADEpro tool) for reviews without metanalysis (MMP-2, MMP-8, MMP-9, MMP-13, MMP-20, and CT-B).

6.1. Question: Caries in dentin and/or root surface compared to sound dentin for presence of **MMP-2**

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Sound vs. Carious									
7[1,2,3,4,5,6,7]	observational studies	serious[a]	not serious	not serious	serious[b]	none	MMP-2 may be increased in carious dentin when compared to sound dentin, but the evidence is very uncertain.	⊕◯◯◯ Very low	
Coronal vs. root dentin									
1[2]	observational studies	serious[c]	not serious	not serious[d]	serious[e]	none	There is no difference of MMP-2 expression/activity in root versus coronal carious dentin, but evidence is very uncertain.	⊕◯◯◯ Very low	
Lesion depth and location									
5[1,3,4,6,8]	observational studies	not serious[f]	serious	not serious	serious[b]	none	The evidence is very uncertain about the MMP-2 location in dentin, as the studies show conflicting results and no effect direction was found.	⊕◯◯◯ Very low	
Before and after cavity sealing									
2[9,10]	observational studies	not serious	not serious	not serious	serious[e]	none	There is no difference of MMP-2 expression/activity before and after cavity sealing, but the evidence is very uncertain.	⊕◯◯◯ Very low	

Explanations

a. Low methodological quality of 3 out of 6 studies considering the Joanna Briggs Institute instrument.

b. Did not reach the optimal information size (OIS) (N=244 samples for sound vs. carious and N=440 for lesion depth and location, calculated using the OpenEpi.com tool, based on averages from Shimada et al. 2009 – labelling index differences of 0.92 - and median values from Ballal et al. 2017 – labelling index differences of 1.5 –; confidence intervals of 95%, and a power of 80%).

c. Low methodological quality of the study considering the Joanna Briggs Institute instrument.

d. Lack of evidence about generalization and external validity.

e. Did not reach the OIS of the GRADE (less than 800 samples/N; no information in the literature to calculate a specific OIS).

f. Low methodological quality of 1 out of 2 studies considering the Joanna Briggs Institute instrument.

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6.W, Gomes-Silva, AC, Prado,Ribeiro, G, de,Castro,Junior, JV, Salvajoli, N, Rangel,Palmier, MA, Lopes, MM, Rocha, MF, de,Goes, TB, Brandão, AR, Santos-Silva. Head and neck radiotherapy does not increase gelatinase (metalloproteinase-2 and . Oral surgery, oral medicine, oral pathology and oral radiology; 2017.

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8.V, Ballal, S, Rao, A, Bagheri, V, Bhat, T, Attin, M, Zehnder. MMP-9 in Dentinal Fluid Correlates with Caries Lesion Depth.. Caries research; 2017.

9.E, Kuhn, A, Reis, EB, Campagnoli, AC, Chibinski, MR, Carrilho, DS, Wambier. Effect of sealing infected dentin with glass ionomer cement on the abundance and . International journal of paediatric dentistry; 2016.

10.AC, Chibinski, JR, Gomes, K, Camargo, A, Reis, DS, Wambier. Bone sialoprotein, matrix metalloproteinases and type I collagen expression after . Caries research; 2014.

6.2. Question: Caries in dentin and/or root surface compared to sound dentin for **MMP-8**

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Sound vs. Carious dentin									
2[1,2]	observational studies	not serious	not serious	not serious	serious[a]	none	The evidence is very uncertain about the MMP-8 expression in sound versus carious dentin, as the included studies show conflicting results and no effect direction was found.	⊕◯◯◯ Very low	
Before vs. after cavity sealing									
2[3,4]	observational studies	not serious	not serious	not serious	serious[b]	none	MMP-8 may be increased in the dentin before when compared to dentin after cavity sealing, but the evidence is very uncertain.	⊕◯◯◯ Very low	

Explanations

a. Did not reach the OIS (N=26 samples, calculated using the OpenEpi.com tool, based on averages from Shimada et al. 2009 – labelling index differences of 3.42; confidence intervals of 95%, and a power of 80%).

b. Did not reach the OIS of GRADE (less than 800 samples/N; no information in the literature to calculate a specific OIS).

References

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6.3. Question: Caries in dentin and/or root surface compared to sound dentin for **MMP-9**

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Sound vs. Carious dentin									
4[1,2,3,4]	observational studies	serious[a]	serious[b]	not serious	serious[c]	none	MMP-9 may be increased in carious dentin when compared to sound, but the evidence is very uncertain.	⊕◯◯◯ Very low	
Lesion depth and location									
2[3,5]	observational studies	serious	serious	not serious	serious[d]	none	The evidence is very uncertain about the MMP-9 expression in different locations and lesion depth, as the studies show conflicting results and no effect direction was found.	⊕◯◯◯ Very low	
Before vs. after cavity sealing									
2[6,7]	observational studies	not serious	not serious	not serious	serious[e]	none	There is no difference of MMP-9 expression/activity before and after cavity sealing, but the evidence is very uncertain.	⊕◯◯◯ Very low	

Explanations

a. Low methodological quality of the study considering the Joanna Briggs Institute instrument.

b. Of the 4 included studies, 1 found significantly more MMP-9 in carious dentin than sound, 2 observed its presence also in carious dentin, but without statistical calculation and without comparison with sound tissue, and 1 found more in sound tissue ($p<0.05$).

c. Did no reach the OIS (N=62 samples, calculated using the OpenEpi.com tool, based on averages from Shimada et al. 2009 – labelling index average difference of 2; confidence intervals of 95%, and a power of 80%).

d. Reached the OIS (N=14 samples, calculated using the OpenEpi.com tool, based on averages from Shimada et al. 2009 – labelling index average difference of -3.5; confidence intervals of 95%, and a power of 80%).

e. Did not reach the OIS of GRADE (less than 800 samples/N; no information in the literature to calculate a specific OIS).

References

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5.V, Ballal, S, Rao, A, Bagheri, V, Bhat, T, Attin, M, Zehnder. MMP-9 in Dentinal Fluid Correlates with Caries Lesion Depth.. Caries research; 2017.

6.AC, Chibinski, JR, Gomes, K, Camargo, A, Reis, DS, Wambier. Bone sialoprotein, matrix metalloproteinases and type I collagen expression after . Caries research; 2014.

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6.4. Question: Caries in dentin and/or root surface compared to sound dentin for **MMP-13**

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Sound vs. Carious dentin									
1[1]	observational studies	not serious	not serious	not serious	serious[a]	none	MMP-13 may be increased in carious dentin when compared to sound, but the evidence is very uncertain.	⨁◯◯◯ Very low	
Coronal vs. Root									
1[2]	observational studies	not serious	not serious	not serious	serious[a]	none	MMP-13 may be increased in root caries when compared to coronal, but the evidence is very uncertain.	⨁◯◯◯ Very low	

Explanations

a. Did not reach the optimal information size of GRADE (less than 800 samples/N; no information in the literature to calculate a specific OIS).

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6.5. Question: Caries in dentin and/or root surface compared to sound dentin for **MMP-20**

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Lesion depth and location									
1[2]	observational studies	not serious	not serious	not serious	not serious[a]	none	MMP-20 probably reduces in carious outer dentin when compared to carious inner dentin.	⨁⨁⨁◯ Modarate	
Irradiated vs. non irradiated									
1[1]	observational studies	not serious	not serious	not serious	serious[b]	none	There is no difference about the MMP-20 expression in irradiated vs. non irradiated carious dentin, but the evidence is very uncertain.	⨁◯◯◯ Very low	

Explanations

a. Reached the OIS (N=4, sample size calculated using the OpenEpi.com tool, based on from Shimada et al. 2009 – difference in labeling index differences of -5.72, confidence interval of 95%, and a power of 80%).
b. Did not reach the optimal information size of GRADE (less than 800 samples/N; no information in the literature to calculate a specific OIS).

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6.6. Question: Caries in dentin and/or root surface compared to sound dentin for CT-B

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Sound vs. Carious dentin									
2[1,2]	observational studies	serious[a]	not serious	not serious	not serious[b]	none	CT-B expression may be increased in carious dentin when compared to sound dentin, but the evidence is very uncertain.	⊕◯◯◯ Very low	

Explanations

a. Low and moderate methodological quality considering the Joanna Briggs Institute instrument.

b. Reached the optimal information size (sample size calculated using the OpenEpi.com tool, based on approximate averages of labeling index from Nascimento et al., 2011, confidence interval of 95%, and a power of 80%).

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CHAPTER 3 – COLLAGENOLYTIC POTENTIAL OF *STREPTOCOCCUS MUTANS* AT DIFFERENT pH LEVELS AND SUBSTRATES

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ABSTRACT

Background: It has been reported that bacterial collagenases are highly sensitive to pH and would not resist to an acidic challenge (pH 4.3) during the demineralization phase of dentinal caries process. However, much uncertainty still exists as about the mechanism of action of these microbial enzymes. **Aim:** The aim of this study was to evaluate the collagenolytic capacity of *Streptococcus mutans* at different pH levels, substrates, and cell concentrations. **Methods:** *S. mutans* UA159 (ATCC 700610) was cultured in BHI medium for 24 hours. Two cells concentration were evaluated (OD 0.7 and 1.0). FALGPA, collagen type I and gelatin substrates were evaluated. *Clostridium histolyticum* collagenase, *S. mutans Clarke, S. mutans R9, Veillonella parvula*, *Veillonella dispar*, *Escherichia coli* and *Porphyromonas gingivalis* strains were used as controls. *S. mutans* was also tested in biofilms. To compare collagenase activity in acidic and neutral pHs, supernatants from the same culture were centrifuged, resuspended in PBS medium and exposed to a commercially available colorimetric assay kit (Collagenase Activity Colorimetric Assay, Sigma). **Results:** There was no significant difference in the level of collagenolytic activity under pH 4 and pH 7. Media containing cells concentrations in OD 0.7 and OD 1.0 did not exhibit a significant difference to degrade FALGPA. The greatest enzymatic activity is detected within the first few minutes after its activation. *S. mutans* and *V. dispar* had a much lower collagenase activity against type I collagen than *P. gingivalis* and the *C. histolyticum* collagenase. It is noted that the control enzyme from *C. histolyticum* shows greater collagenolytic capacity, suggesting that the enzymatic purity seems to have influenced this result, since the strains in planktonic form,

showed lower cell concentration. **Conclusion**: Our findings showed that a synthetic collagen analogue (FALGPA) is easily degraded by *S. mutans* in different concentrations and pHs. The collagenolytic activity of *S. mutans* did not change in low pHs. In terms of collagenase activity over time, the enzymatic activity seems to show an inverse relationship with activation time. However, only a minor enzymatic activity was observed for type I collagen and gellatin.

3.1 INTRODUCTION

A set of virulence factors is present in oral microorganisms during the development of oral diseases. Among them, the action of bacterial proteases could be determinant to dentinal caries and periodontitis development. Studies indicate that the primary and most important function of bacterial proteases would be the acquisition of nutrition for growth and proliferation by digesting the host tissue (Maeda 1996, Takahashi and Nyvad 2016). Their role in the degradation of the collagenolytic matrix through proteases could be fundamental in the progression of oral diseases, such as dental caries (Simon-Soro, Belda-Ferre et al. 2013). *Treponema denticola, Fusobacterium, Veillonella, Porphyromona. gingivalis* and *Actinobacillus actinomycetemcomitans*, for example, are capable of digesting host collagen, releasing amino acids that will later be used as bacterial nutrients (Robertson, Lantz et al. 1982). Bacterial proteases are also substantial part of *S. mutans* infective capabilities (Dubin, Koziel et al. 2013, Zhang, Ran et al. 2015). However, this collagenolytic potential has not yet been fully elucidated and there is a lack of studies dedicated to the characterization of these proteases (Birkedal-Hansen 1993).

Previous studies indicated that cariogenic bacteria did not have any collagenolytic or gelatinolytic activity, based on *in situ* models (Katz, Park et al. 1987). And, in addition, the *in vitro* experiments existing until then were carried out with purified enzymes, isolated from bacteria that were not present in caries lesions (Kleter, Damen et al. 1994, van Strijp, van Steenbergen et al. 1994, Chaussain-Miller, Fioretti et al. 2006). However, this concept has been weakened by recent findings that indicate the opposite. Two studies utilizing omics data have identified gene expressions of bacterial collagenases, with both studies

revealing significant expression of genes encoding collagenolytic proteases in coronal (Simon-Soro, Belda-Ferre et al. 2013) and root caries (Damé-Teixeira, Parolo et al. 2018). Furthermore, it has been observed that other microorganisms, such as *Veillonella* spp and *Leptotrichia buccalis*, also possess genes related to collagenases, indicating their potential involvement in protein degradation (Damé-Teixeira, Parolo et al. 2018).

Understanding the characteristics of these proteolytic bacteria is crucial for comprehending the various oral pathologies influenced by these microorganisms. Notably, their activation capability at low or neutral pH, along with their ability to degrade diverse substrates, are significant features. Most proteolytic bacteria exhibit growth potential across a pH range of 6 to 8. However, some streptococci such as *S. mutans* are not only able to withstand a lower pH of 5.5, as they are able to produce acids at low pH values (aciduric capacity) (Harper and Loesche 1984, de Soet, Toors et al. 1989, Sorsa, Tjäderhane et al. 2004). Therefore, the pH of the oral cavity is an ecological determinant that influences the events that affect the oral cavity.

Regarding the environmental characteristics for protease activation, host proteases that have already been elucidated, such as matrix metalloproteinases, are activated automatically under acidic conditions (around pH 4.5), but this characterization has not yet been done for bacterial proteases (Chaussain-Miller, Fioretti et al. 2006). This means that different pH levels would directly influence the activation and initiation of the process of proteolytic action (de Soet, Toors et al. 1989). After activation of bacterial proteases, protein substrates may be degraded. This ability to degrade protein substrates has also been seen in synthetic collagen. Two extracellular proteases from *S. mutans* were able to hydrolyze synthetic collagen substrates, PZ-Pro-Leu-Gly-Prop-Arg (PZ-PLGPA) and furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) isolated in a previous study indicating a collagenolytic enzymatic potential (Harrington and Russell 1994, Harrington 1996). Hence, the aim of this study is to evaluate the collagenolytic capacity of *Streptococcus mutans,* at different pH levels, cells concentrations, and different substrates (synthetic collagen, collagen type I, gellatin, and comparing to other oral strains).

3. 2 METHODS

3.2.1 Assay to study activity in different pHs, cells concentration, time, and different strains using furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) as a collagen substrate

S. mutans UA159 (ATCC 700610) was cultured in BHI medium for 24 hours. Cell concentration was measured by absorbance until reaching the respective optical dentisty (OD 600) 0.7 and 1.0. To measure their collagenase activity, the OD 0.7 inoculum was centrifuged at 5000x RPM for 4min and then the pellet was resuspended in 100µl of PBS1x (Phospate buffered saline). Soon after, it returned to the incubator until it reached OD 1.0 and went through the same process.

The Collagenase Activity Colorimetric Assay Kit (Sigma-Aldrich, Merck) was used to determine activity of collagenase, according to the supplier's instructions. The kit measures collagenase activity using a synthetic peptide (FALGPA) that mimics collagen′s structure - FALGPA (N-[3-(2-Furyl) acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine). Absorbance readings were collected at 0, 5 and 15 minutes after the end of the experiment.

For each reaction, 200 µl of mix was prepared, containing 100 µl of collagenase substrate (FALGPA), 90 µl of collagenase assay buffer, and 10 µl of the previously prepared *S.mutans* inoculum. For the positive control, a *Clostridium histolyticum* pure collagenase was used in the same volume as the inoculum. Each reaction was resuspended and measured immediately in a spectrophotometer with absorbance (A) at 345 nm at times 0 to 15 minutes. The assay was performed in triplicate for each saturation condition (neutral pH, acidic pH) and for different cell concentrations (OD 0. 7 and OD 1.0). In addition, the duplicate of the experiment was performed on alternate days in order to guarantee the faithful reproducibility of the results.

For each of the reactions, absorbance values (A) at 345 nm were recorded at times 0 to 15 minutes. The results obtained were calculated according to the formula (Supplementary Appendix 1 attached) indicated by the manufacturer, where ∆A345 = Difference between A2 and A1; ∆T = Difference between T2 and T1; 0.2 = Reaction volume (mL); DF = Dilution Factor; 0.53 = millimolar extinction coefficient of FALGPA; V = Enzyme volume (mL).

In order to verify the influence of pH on collagenolytic activity of *S. mutans*, the same experiment described above was carried out in two different saturation environments (neutral pH – 7.0 and acidic pH 4.0). For the second case, 3 µl of HCL was added in 1.5 ml eppendorff until reaching an acidification of 4.0, confirmed by colorimetric pH strips.

Using another *S. mutans* strain (*S. mutans Clarke* ATCC 25175*)* and other microorganism as controls, the same experiment described above (Collagenase Activity Colorimetric Assay Kit - Sigma-Aldrich, Merck), was carried out with the following strains: *S. mutans Clarke, S. mutans R9, Veillonella parvula* (ATCC 17745), *Veillonella dispar* (ATCC 17748), *Escherichia coli and Porphyromonas gingivalis* W83. Pellets were stored for 24 hours at -20^{o}C and resuspended in 100 µl of cold PBS medium. Soon after, 10 µl of each suspension was used in the experiment.

3.2.2 Assay to study activity using collagen type I substrate

Overnight cultures of strains (10 mL) were adjusted to (OD 600) 1.0, pellets washed twice with PBS, and resuspend into 1 mL of the 1x reaction buffer. The *P. gingivalis* W3 strain was used as a model microorganism due to its high collagenolytic capacity. It was anaerobically cultured in BHI broth (supplemented with hemin and menadione). Cultures were then assayed by the EnzChek® Gelatinase/Collagenase Assay Kit using the DQ collagen type I as the collagen substrate (from bovine skin, fluorescein conjugate) (Molecular Probes, Inc.), following the supplier protocol. The final collagen concentration was 100 µg/mL, and the volume of the bacteria inoculum was 100 µL. The collagenolytic activity of mature biofilms of *S. mutans* biofilms was tested using the Calgary Biofilm

Device (CBD; MBECTM Assay System, MBEC Biofilms Technology Ltd., Calgary, Alberta, Canada), as described elsewhere (Dame-Teixeira, El-Gendy et al. 2022).

In order to mimic dentin in the experimental model, CDB pegs were coated overnight with 200 µL of collagen coating solution (Sigma, concentration of 100 µL/0.32cm^2), at 25^{o}C, 65rpm for 2h. The pegs were then washed with sterile PBS and treated with human saliva for 5h (37^{o}C; 65rpm). The saliva was previously prepared by adding 2.5 mM DL-dithiothreitol (final concentration 2.5 mM) and 50% of PBS, then sterile filtered (Naginyte 2018). CBD pegs containing the mature biofilms were dipped into the DQ collagen type I and buffer and incubated at root temperature. Planktonic cells suspensions and *S. mutans* biofilm were incubated in the experiment for 2-24h, 37^{o}C. Fluorescence emission from the released fluorescent peptides at the collagen cleaving was read at 495-515 nm.

3.2.3 Assay to study gelatinase activity

This protocol was adapted from Van Strijp et al. 1994 to determine the ability of an organism to produce extracellular proteolytic enzymes (gelatinases) that liquefy gelatin. If the gelatinases hydrolyse gelatin into polypeptides and then polypeptides are further converted into amino acids, the amino acid can be converted into energy by the cell. The presence of gelatinases is detected using a nutrient gelatin medium. When an organism produces gelatinase, the enzyme liquefies the growth medium by hydrolyzing gelatin present in the medium.

Isolates were grown anaerobically on BHI or CBA agar for 3 days. Afterwards, the colonies were harvested and transported in 2.5mL of gelatin (120g of gelatin/L); and 2.5 media (19 g/L BHI; 10 g/L proteose peptone; 10 g/L yeast extract; 19 g/L sucrose; 0.5 g/L L-cysteine; 0.6 g/L gelatin). Groups with only 5 mL of gelatin (120g of gelatin/L) were also performed. Tubes of anaerobically incubated *S. mutans* were also tested aerobically and checked for growth at 72h and placed at 4oC for 1 day to solidify the gelatin, then the tubes were placed at room temperature and examined for liquefaction every minute; if the gelatin was completely hydrolyzed – gelatinolytic activity of the culture was verified. This experiment was performed in duplicate. Negative controls without

inoculum (in one tube we added the same volume of ethanol as in the AA tests) were added under the same conditions.

3.2.4 Statistical analysis

GraphPad Prism (version 9.5.0 (525) for Mac) was used to compare averages of collagenolytic activity or fluorescences within each assay. 2-way ANOVA was used to compare time points and pHs. Kruskal-Wallis with uncorrected Dunn's/ Šídák's multiple comparisons tests were used to compare fluorescences (meaning the peptide release after collagenase activity) or collagenase activity of *S. mutans*. The significance level was set at 5%.

3. 3 RESULTS

3.3.1 Collagenase activity under low and neutral pH

Collagenase activity values were plotted in Figure 1. Acid activation of MMPs at low pH simulates a common challenge in the oral cavity, such as exposure to fermentable carbohydrates from the diet, which can lead to acidification around 5.5, causing the dissolution of dental mineral content. Interestingly, under the tested conditions of pH 4 and pH 7, no significant difference was observed in the level of collagenolytic activity of proteases. This suggests that collagenases from *S. mutans* may possess the potential to act even under low pH conditions.

3.3.2 Cell concentration

The cell concentration of the strain used did not exhibit a significant difference in the values obtained when utilizing FALGPA as a collagen substrate. In both situations (OD 0.7 and OD 1.0), the bacterial sample demonstrated sufficient quantity to demonstrate some collagenolytic potential (Figure 2), albeit to a small extent. In contrast, as anticipated, the pure collagenase control

exhibited a higher level of collagenolytic activity when compared to the bacterial sample.

3.3.3 Collagenase activity over time

Another factor to be considered is the time of action of these collagenases. In this collagenolytic assay, the greatest collagenolytic activity is seen within the first few minutes after its activation. After the first 5 minutes of bacterial activation, a gradual decrease in enzymatic activity is observed, and a significant decrease was observed after 15 min (Figure 2).

3.3.4 Comparison with other strains

Collagenolytic activity of *S. mutans* compared to other strains using FALGPA can be observed in Figure 3. On the y axis, it is possible to observe the level of collagenolytic activity exerted by the microorganism, while on the x axis, the minutes elapsed throughout the experiment. In this sense, the control strain would have the highest production, followed by *S. mutans* and *V. dispar.* It is noted that the control enzyme shows greater collagenolytic capacity, due to its greater enzymatic purity, while the tests were cells in their planktonic form. Even so, the amount was sufficient to demonstrate important collagenolytic potential of the strains.

3.3.5 *S. mutans* capacity to degrade collagen type I

In this experiment, the collagenolytic capacity of microorganisms was seen from the level of fluorescence (y axis), and the x axis corresponds to cell concentration for *S. mutans* in its planktonic form or in biofilm (Figure 4). As a control, *Clostridium* collagenase (provided within the kit) was used. For this assay (EnzCheck collagenase assay kit) collagen type I is the substrate, at a concentration of 100 µg/ml of collagen.

Figure 4A represents the result of the EnzCheck collagenase assay kit experiment, using collagen type I as substrate, at a concentration of 100 µg/ml of

collagen. *S. mutans* at different concentrations (OD 1.0 and 0.5) and in biofilm. As the bacterial concentration increases, there is a corresponding increase in the fluorescence level observed, indicating a higher utilization of collagen by the bacteria. Although the collagen degradation is minimal, the collagenolytic activity of *S. mutans* appears to be dependent on the dose. It is plausible that *S. mutans* employs collagenase more prominently in biofilms compared to its planktonic form. However, we cannot draw definitive conclusions solely from the findings of this experiment, since the exact number of cells in the biofilm for this assay is unknown.

Figure 4B shows the results of the experiment, using the same kit described above, comparing the ability of *S. mutans* to use type I collagen compared to other oral microorganisms, including *P. gingivalis*. This is known to be capable of degrading type I collagen and, as expected, demonstrated the significantly higher levels of fluorescence among the evaluated strains.

3.3.6 *S. mutans* capacity to hydrolyse gelatin

When an organism is able to produce gelatinase, the enzyme liquefies the growth medium by hydrolyzing the gelatin present. *S. mutans* did not demonstrate gelatin hydrolysis capacity, and only *P. gingivalis* showed medium liquefaction capacity (Table 1).

3.4 DISCUSSION/CONCLUSION

Bacterial proteases may play a role in the progression of oral pathogenesis (Maeda 1996). Recently, the tissue-dependent hypothesis gained strength and showed that microorganisms such as *S. mutans* and *P.* gingivalis can promote the degradation of the dentin matrix present in the oral microenvironment and, consequently, favor the establishment of periodontal disease and caries (Simon-Soro, Belda-Ferre et al. 2013). In light of this, our study aimed to assess the collagenolytic capacity of S. mutans under various conditions, including different pH levels, substrates, and cell concentrations. Interestingly, our findings showed

that FALGPA is easily degraded by *S. mutans* in different concentrations and pHs. However, we were not able to show its capacity to degrade type I collagen or to hydrolyse gelatin.

In addition to activating protein-degrading proteases, the way in which these bacteria exert their virulence factors can vary significantly. *S mutans*, for example, is capable of producing various fermentation products and to survive in low pH, forming a biofilm rich in extracellular polysaccharides (PEC), and favoring the acidification of the environment (Fitzgerald, Adams et al. 1989, Burne 1998). This ability to survive in a low pH environment is a shared characteristic among oral pathogenic bacteria. This is due to the fact that many issues affecting the oral cavity are closely linked to changes in oral pH, particularly within the biofilm fluid (Takenaka, Edanami et al. 2021). When the oral pH drops below 5.5, the demineralization process of the dental enamel surface initiates. Unlike enamel, which mainly consists of mineral content, dentin tissue is more soluble. As a result, not only acids will contribute to the mineral dissolution, but also enzymatic proteolysis will be activated (Klimuszko, Orywal et al. 2018). However, according to Kawasaki et al., bacterial collagenases would be highly sensitive to pH and incapable of resisting acid fall (pH 4.3) during the dental demineralization phase (Kawasaki and Featherstone 1997).

In line with this finding, Tjaderhane et al (Tjäderhane, Larjava et al. 1998) identified host MMP-2, MMP-8 and MMP-9 in demineralized and activated dentinal lesions at low pH (4.5), followed by neutralization, mimicking existing conditions during caries progression. However, the study mentioned did not observe any gelatinolytic or collagenolytic activity in the bacterial samples. Although these results demonstrated the pH-dependent activation mechanism of human MMPs, they did not provide evidence for a similar mechanism in bacterial enzymes. Consequently, these findings suggested, at that time, that *S. mutans* proteases might not play a substantial role or have limited contribution to the development of carious lesions. Nevertheless, our current results contradict the previous findings. We showed no differences in *S. mutans* activity when using FALGPA at acidic and neutral pH levels. This indicates that the bacteria were capable of exerting some collagenolytic function even in lower pH environments, suggesting an intrinsic collagenolytic capability of *S. mutans*.

In 1994, Van Strijp *et al*. conducted an *in situ* experiment demonstrating the capacity and gelatinolytic activity of various bacterial strains. For this experiment, decalcified dentin disks were inserted into partial dentures and exposed to acid challenges over a period of 7 weeks. Subsequently, the samples were evaluated through microscopy and bacteriological analysis (van Strijp, van Steenbergen et al. 1994). As a result, the authors showed a variation in the level of degradation of the specimens and an associated rich and diverse microbiota, with a predominance of *Streptococcus mitis, Peptostreptococcus*, *Lactobacillus casei, Propionibacterium species and Veillonella parvula.* Despite demonstrating gelatinolytic activity, no correlation was found with the severity of dentin matrix degradation. In contrast, in our assay, we could not show gelatinolytic activity in *S. mutans.* These might happen for two reasons: 1) our experimental model was not suitable of analysing these characteristics in the present conditions; or 2) this bacterium really do not utilize collagen or gelatin as substrates. As *P. gingivalis* showed medium liquefaction capacity, we believe that the second option would be true. However, it is important to consider that Van Strijp's study involved specimens exposed to a diverse and natural microbiota, which we did not precisely replicated *in vitro*. Additionally, the concentration of the microorganism in our study may not have been sufficient to exhibit protein-degrading function, as well as the substrate concentration may have played a role.

The same problem can be explained by the fact that *S. mutans* showed a very low activity against type I collagen. In our assay, *P. gingivalis* showed the highest collagenolytic activity, even higher than the positive control collagenase isolated from *C. histolyticum*. This property is already widely seen in other studies that point to this microorganism as an important protein-degrading bacteria, and its PrtC protease from ATCC 53977 has been one of the most reported produced by oral bacteria (Kato, Takahashi et al. 1992, Zhang, Ran et al. 2015). Again, the difficulty of reproducing the activity of proteolytic bacteria in planktonic conditions not in their natural site, may have an influence in the result. However, it is important to highlight the massive presence of proteolytic bacteria in root caries lesions under the gingival margin, such as *Porphyromonas,* in a recent study (Takenaka, Edanami et al. 2021), supporting our hyphotesis of the bacterial involvement in root dentin collagen matrix degradation. Maybe an *in vitro* model

using multispecies complex biofilms would show a greater proteolytic capacity than when evaluated in unispecies planktonically.

In conclusion, a synthetic collagen analogue (FALGPA) is easily degraded by *S. mutans* in different concentrations and pHs. The collagenolytic activity of *S. mutans* did not change in low pHs. However, only a minor enzymatic activity was observed for type I collagen and gellatin. Nevertheless, these findings still indicate a potential collagenolytic activity of *S. mutans*. In terms of collagenase activity over time, the enzymatic activity seems to show an inverse relationship with activation time. In other words, the longer the time elapsed after the activation of the bacteria, the lower the demonstrated collagenolytic activity. It is observed that the control enzyme exhibits higher collagenolytic capacity, implying that the enzymatic purity may have influenced this outcome, especially considering that the strains in planktonic form had a relatively low cell concentration, and not reflect their metabolism in natural biofilms. It is crucial to acknowledge that the oral environment provides a more favorable setting for the bacteria to exert its collagenolytic function within multispecies complex biofilms and target type I collagen. Reproducing this complex scenario *in vitro* for experiments may be challenging.

FIGURES

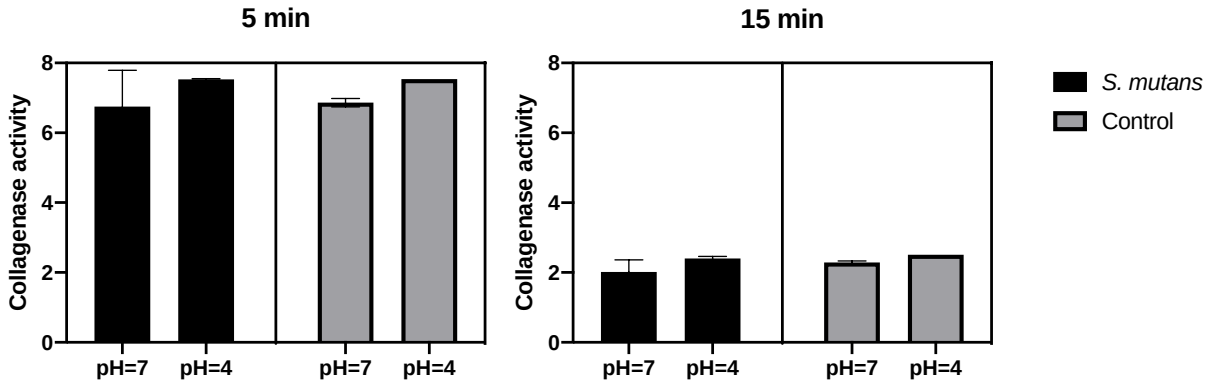

Figure 1. Collagenolytic activity of *Streptococcus mutans* UA159 at acidic and neutral pH. 2-way ANOVA. p>0.05.

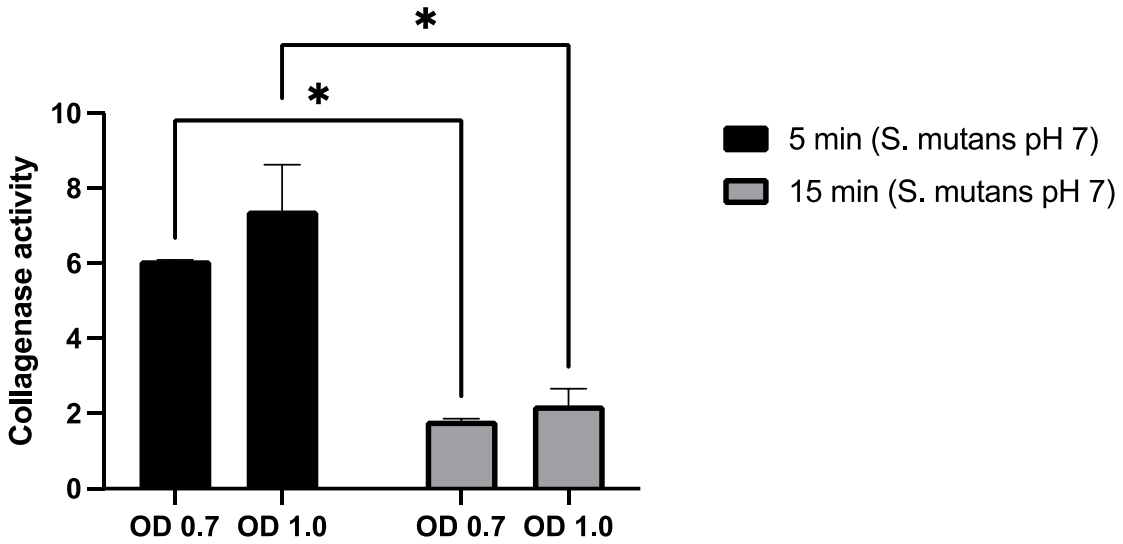

Figure 2. Collagenase activity of *Streptococcus mutans* UA159 at different cells concentrations. 2-way ANOVA and Šídák's multiple comparisons test. *p<0.05.

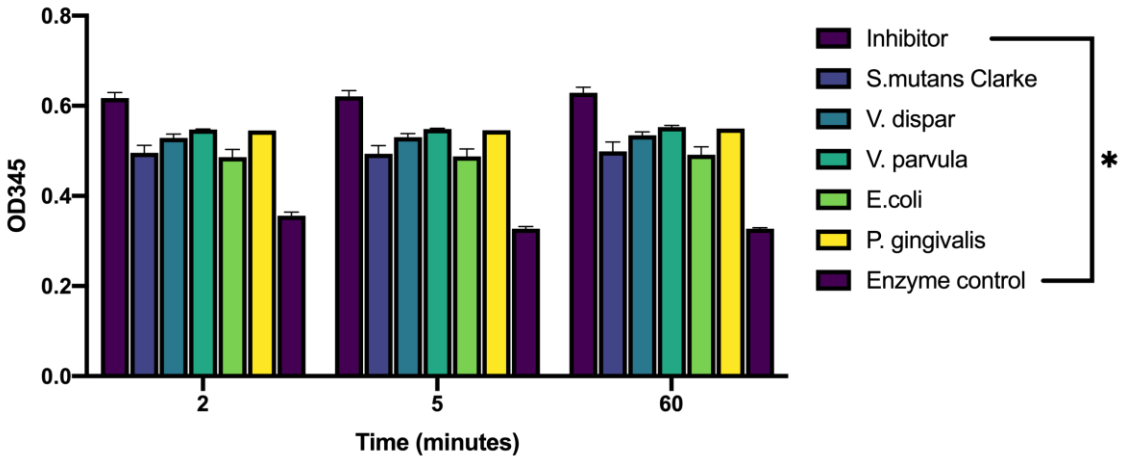

Figure 3. Collagenolytic activity of different strains using FALGPA as substrate. The FALGPA, the inhibitor and the enzyme controls are provided by the kit (Sigma). The higher the fluorescence, the higher the collagenase activity. Kruskal-Wallis with uncorrected Dunn's tests. *p<0.05.

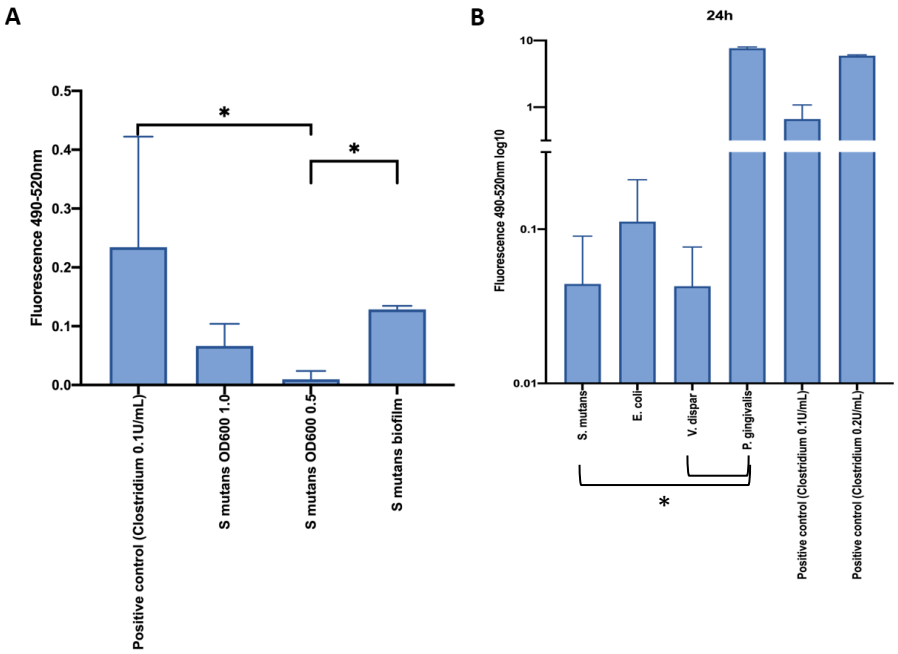

Figure 4. Collagenolytic activity of *S. mutans* and different strains using collagen type I as substrate in planktonic form and in unispecies biofilms. The higher the fluorescence, the higher the collagenase activity. Kruskal-Wallis with uncorrected Dunn's tests. *p<0.05.

Table 1. Gelatin hydrolysis (positive [++] = total liquefaction of the gelatin media; negative [--]= complete solidification of the tube at 4°C).

Group	Hydrolysis
Anaerobiosis	
S. mutans + media with sucrose	**--**
S. mutans – only gelatin	**--**
Veillonella parvula + media with sucrose	**--**
Veillonella dispar + media with sucrose	**--**
Veillonella parvula – only gelatin	**--**
Veillonella dispar – only gelatin	**--**
Porphyromonas gingivalis + media with sucrose	**++**
Aerobiosis	
S. mutans + media with sucrose	**--**
S. mutans – only gelatin	**--**

Supplementary Appendix 1. Equation to determine collagenase activity (U/mL).

Results

Calculations

Take the absorbance (A_1 and A_2) at two time points (T_1 and T_2) in the linear range. There should be at least two readings in between and at least 1 minute apart.

To determine collagenase activity (U/mL), use the following equation:

$$\frac{\left(\frac{-\Delta A_{345\ \text{Test}}}{\Delta T} - \frac{-\Delta A_{345\ \text{Reagent Background}}}{\Delta T}\right) \times (0.2) \times DF}{(0.53) \times V}$$

ΔA_{345} = Difference between A_2 and A_1
ΔT = Difference between T_2 and T_1
0.2 = Reaction volume (mL)
DF = Dilution Factor
0.53 = millimolar extinction coefficient of FALGPA
V = Enzyme volume (mL)

For inhibitor screen, calculate percent inhibition using the following equation:

$$\%\ \text{Inhibition} = \frac{\text{Activity}_{(\text{Enzyme})} - \text{Activity}_{(\text{Inhibitor})}}{\text{Activity}_{(\text{Enzyme})}} \times 100$$

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CAPÍTULO 4 – DISCUSSÃO GERAL E CONCLUSÕES

4.1DISCUSSÃO GERAL

Com base na teoria ecológica da cárie, proposta por Marsh como "hipótese da placa ecológica", em 1994, e posteriormente estendida por Takahashi e Nyvad (Marsh 1994, Takahashi and Nyvad 2016), o desenvolvimento da doença ocorre em resposta ao desequilíbrio homeostático na microbiota bucal. Propõem-se que o aumento de algumas espécies de microrganismos, antes consideradas odontopatógenas, se dá em resposta a uma mudança nas condições ambientais ocasionadas pelo alto consumo de carboidratos fermentáveis (Takahashi and Nyvad 2011, Takahashi and Nyvad 2016). Segundo Marsh, esse desequilíbrio – denominado disbiose - é o ponto chave no desenvolvimento e progressão da doença cárie (Marsh 1994).

Apesar dessa hipótese ecológica formar a base da atual compreensão da cárie, pouco se discutiu sobre suas particularidades em superfícies radiculares, enfatizando principalmente, os processos que acometem a região de esmalte. Sugere-se que, para cárie radicular, durante o desenvolvimento da lesão cariosa, além de ocorrer uma acidificação bacteriana e consequente desmineralização dentária, acontece também a degradação de material orgânico, principalmente colágeno, por meio da ação de proteases (Marsh, Do et al. 2016). Pouca informação cientifica foi destinada às proteases bacterianas. Nesse cenário, o *S. mutans* tem sido alvo de estudos ultraestuturais recentes, que encontraram, nessa bactéria, características colagenoliticas importantes.

Tendo em vista o pouco conhecimento sobre o envolvimento bacteriano e a necessidade de explorar o papel do próprio *S. mutans* na segunda fase de formação de cárie radicular, esta tese busca incrementar bases de evidências cientificas, por meio de revisões do estado da arte e sistemática, além de um estudo *in vitro* acerca do potencial colagenolítico do *S. mutans*. Há um potencial para futuras aplicações biotecnológicas e médicas, se identificarmos as colagenases como possíveis alvos para o desenvolvimento de agentes terapêuticos. Após o entendimento da função das colagenases de *S. mutans*

neste processo, colagenases de outras espécies, como as do gênero *Veillonela* poderão ser avaliadas em projetos futuros do mesmo grupo de pesquisa.

Os achados da revisão de literatura apontam para uma controversa evidência acerca do papel de proteases bacterianas em lesões cariosas dentinária, além de um número significativamente menor de estudos focados na proteólise microbiana comparadas com as colagenases da própria matriz tecidual (MMPs), representando uma resposta dos tecidos do hospedeiro ao desafio cariogênico em condições ácidas (Chaussain-Miller, Fioretti et al. 2006, Chaussain, Boukpessi et al. 2013). Os achados da revisão sistemática apontam evidência muito baixa, principalmente devido ao número amostral avaliado nos estudos primários. Isso significa que, mesmo com relação a colagenases do hospedeiro, é importante que novos estudos sejam realizados, dando mais foco para outras MMPs, principalmente a MMP-9 e MMP-13.

Os achados do estudo *in vitro* acerca da avaliação de atividades enzimáticas do *S. mutans*, apontaram um resultado positivo para seu potencial colagenolitico ao usar FALGPA como substrato. Nesse experimento, descartamos a hipótese da literatura de que as colagenases microbianas ficariam inativadas em pH baixo. Ao utilizar o colágeno tipo I, a atividade colagenolítica do *S. mutans* foi baixa, mas ainda não pode ser descartada. Acredita-se que em meio bucal com toda a complexidade do microbioma dos biofilmes, as bactérias tenham o ambiente ideal para exercer sua função colagenolítica. Para experimentos *in vitro*, é extremamente difícil reproduzir esse cenário e novos modelos experimentais precisam ser delineados.

Em conclusão, demonstramos aqui que há um potencial para degradação de colágeno a partir de bactérias bucais, inclusive do *S. mutans*. Acredita-se que isso sinaliza alguma influência bacteriana nesse processo e representar um alvo potencial para a modulação do biofilme. Mais estudos são necessários para avaliar a capacidade colagenolítica de *S. mutans* em condições laboratoriais mais próximas do que se encontra clinicamente.

4.2 LIMITAÇÕES

É importante lembrar que a tese apresentada possuía como caráter primário o desenvolvimento de estudos *in vitro*, cujo tempo de laboratório é fator primordial para a execução do trabalho proposto. No entanto, desde 2020, com o surgimento da COVID-19, grande parte das atividades laboratoriais precisou ser paralisada por período indeterminado. Em outras ocasiões, alguns experimentos foram interrompidos ou perdidos por interferências da pandemia (necessidade de isolamento, teste positivo para o vírus SARS-CoV-2).

4.3 CONCLUSÕES

- A literatura aponta para a cárie radicular como um processo de dois estágios, em que quebra do colágeno é subsequente à perda mineral;
- A presença de MMPs é indiscutível e deve estar ligada à desnaturação do colágeno;
- MMP-2, MMP-9, MMP-13 e CT-B podem estar aumentados em dentina cariada quando comparados a dentina sadia;
- MMP-13 pode estar aumentada na raiz quando comparada à dentina cariada coronária;
- MMP-8 pode estar aumentado na dentina antes do selamento da cavidade em relação à dentina após o selamento da cavidade;
- MMP-20 provavelmente reduz na dentina externa cariada quando comparada à dentina interna cariada (certeza moderada);
- Bactérias proteolíticas são prevalentes em cárie radicular, especialmente nas lesões que se estendem subgengivalmente;
- Genes que codificam proteases colagenolíticas bacterianas e bactérias degradadoras de proteínas foram detectados em lesões cariosas coronárias e radiculares.
- FALGPA é facilmente degradado por *S. mutans* em diferentes concentrações e pHs. No entanto, não fomos capazes de mostrar sua capacidade de degradar o colágeno tipo I ou hidrolisar a gelatina.

- A atividade enzimática parece ser inversamente proporcional ao tempo de ativação. Ou seja, quanto mais tempo se passa após a ativação da bactéria, menor é a atividade colagenolítica demonstrada para uso de FALGPA.
- Quando comparado, *in vitro*, à outras cepas bacterianas reconhecidamente colagenoliticas (como a *Phorphyromonas gingivalis*), o *S. mutans* demonstrou pouco potencial de degradar colágeno tipo I e incapacidade de hidrolisar gelatina.

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ANEXO 1 – PRODUÇÃO CIENTÍFICA

Anexo 1.1 *Streptococcus mutans* e seu metabolismo a nível molecular no contexto ecológico da doença cárie
Link para acesso:
https://www.seer.ufrgs.br/RevistadaFaculdadeOdontologia/article/view/118914

Anexo 1.2 Manejo de cárie radicular: um guia para o dentista brasileiro baseado na tradução e adaptação cultural do consenso internacional/ORCA E EFCD
Link para acesso:

https://seer.ufrgs.br/index.php/RevistadaFaculdadeOdontologia/article/view/116951

Anexo 1.3 Minimum intervention oral care: defining the future of caries management
Link para acesso:

https://www.scielo.br/j/bor/a/DNxwRhTYsVqW8YSbHdv9NWr/?format=pdf&lang=en

Anexo 1. 4 Coorientação e participação em banca de trabalhos de conclusão de curso
Link para acesso:

https://bdm.unb.br/bitstream/10483/30603/1/2021_BarbaraAranhaRibeiro_tcc.pdf

https://bdm.unb.br/bitstream/10483/34801/1/2023_BrunaLeisEndres_tcc.pdf

CAPÍTULO 5 – PRESS RELEASE

A doença cárie continua sendo um grande problema de saúde pública, afetando pessoas de diferentes faixas etárias. A cárie radicular é uma preocupação frequente na população idosa. Isso significa que os sinais clínicos da doença cárie atingem não somente a coroa do dente, mas também a raiz. Para que aconteça a formação da lesão, várias bactérias que estão em boca atuam acidificando o meio bucal e, consequentemente, dando início à desmineralização do dente. No entanto, para cárie radicular, esse processo parece ocorrer em duas fases distintas: perda de minerais do dente e perda da parte orgânica do dente. Nessa última, a função das bactérias ainda é pouco conhecida, mas estudos sugerem que proteases, um importante grupo de enzimas, estejam envolvidas nesse processo, com destaque para proteases da bactéria *Streptococcus mutans.* Essa bactéria exerce importante papel na doença cárie e seus fatores de virulência já foram extensamente estudados, mas pouco se fala sobre a atuação na degradação de colágeno por meio dessas enzimas. Nesse trabalho*,* essas proteases foram estudadas, assim como a atividade enzimática de uma bactéria comumente associada com cárie – o *Streptococcus mutans*, constituindo o passo inicial para entender o papel dessas enzimas em cárie radicular e possibilitar o desenvolvimento de novos tratamentos.