Secondary caries

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“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.”

(Albert Einstein)

Original Publications

The present PhD thesis is based on the following original publications.

1. Maske TT, van de Sande FH, Arthur RA, Huysmans MCDNJM, Cenci MS (2017). In vitro biofilm models to study dental caries: a systematic review. *Biofouling 38(8):* 661-675.
2. Maske TT, Kuper NK, Cenci MS, Huysmans, MCDNJM (2017). Minimal gap size and dentin wall lesion development next to resin composite in a microcosm biofilm model. *Caries Research* 51(5):475-481
3. Maske TT, Kuper NK, Hollanders A, Bronkhorst EM, Cenci MS, Huysmans, MCDNJM (2018). A threshold gap size for in situ secondary caries lesion development?
4. Maske TT, Kuper NK, Cenci MS, Huysmans, MCDNJM (2018). Matrix metalloproteinase inhibitor and secondary caries wall lesion development in a microcosm biofilm model.
5. Maske TT, Kuper NK, Hollanders A, Bronkhorst EM, Cenci MS, Huysmans, MCDNJM (2018). Secondary caries development and the role of a matrix metalloproteinase inhibitor: a clinical in situ study. *Journal of Dentistry 71:49-53.*

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CHAPTER 1

General introduction

1.1 What is secondary caries?

Dental caries has been described as a chronic and carbohydrate-biofilm-dependent disease which causes demineralization and destruction of dental hard tissues. Tooth demineralization can occur at any location of a dental surface when a mature and cariogenic biofilm is present (Selwitz et al., 2007; Takahashi and Nyvad, 2011). When caries develops adjacent to a restoration, the phenomenon is defined as secondary caries (Mjor and Toffenetti, 2000; Mjor, 2005).

The secondary caries lesion has been described by two locations named as the outer and the cavity wall lesion. The outer lesion develops on the tooth surface next to the restoration margins and the wall lesion forms within the tooth-restoration interface (Hals and Nernaes, 1971), see figure 1.

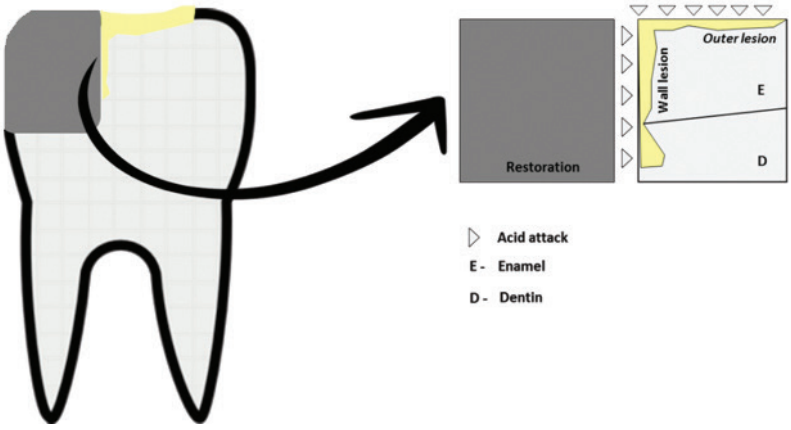

Figure 1. Schematic representation of the two locations of secondary caries lesion (outer and wall lesion).

Primary and secondary caries are not different considering the etiological factors involved in it. However, as secondary caries develops around the margins of restorations, its formation is influenced by local factors related to the restoration. It makes secondary caries lesion a special pathological entity that deserves its own focus in dental research.

A number of clinical and review studies have consistently reported that secondary caries is the main reason for failure of dental restorations (Demarco et al.,

2012; Opdam et al., 2014; Pallesen and van Dijken, 2015; van de Sande et al., 2016) and that patients with an increased risk for developing caries have their restorations failing mainly due to this problem (Opdam et al., 2014; van de Sande et al., 2013; van de Sande et al., 2016). Although there might be some specific diagnostic problems for secondary caries such as the difficulty to detect wall lesions, or the question whether a defective margin indicates secondary caries (Kidd, 1990), the above-mentioned findings highlight the importance to investigate the factors involved in secondary caries lesion formation to allow an adequate clinical management of this condition.

1.2 How to study secondary caries

When considering secondary caries as a special pathological entity this affects the ways the condition should be studied. Several caries models are available and each research method has its advantages and limitations.

Clinical studies investigating secondary caries could provide high validity and the results would be most relevant for daily clinical practice. However, these studies are limited by the need for long follow-up times for caries formation that can be detected clinically, and by the lack of lesion standardization related to a high individual variability (Askar et al., 2017). Unless the tooth is scheduled to be extracted (e.g. orthodontic reasons) and subsequently analysed using destructive methods yielding detailed information on lesion depth and/or mineral loss, secondary caries development must be evaluated by clinical diagnostic methods that are less precise and more likely to be biased. Moreover, for obvious ethical reasons it is not possible to conduct clinical experiments monitoring initial secondary caries lesion development with a focus on identifying etio-pathological factors and potential prognostic factors (Jokstad, 2016). Clinical studies may be conducted only when there is no risk to the subject or when direct benefit to the subject is anticipated (Horowitz, 1976). Therefore, the findings related to secondary caries lesions have been mainly derived from test models (Ferracane, 2017).

The test models vary from highly simplified to more complex ones. The simplest models to study secondary lesions are represented by those using demineralization solutions (cycling or static) to create caries at the margins of restorations (Hara et al., 2002; Kuper et al., 2013; Peris et al., 2007). These models, however, fail in mimicking the biological biofilm necessary for caries lesion development. This

may severely limit their simulation of pathological processes and their capability to evaluate preventive measures.

More complex test models using a biofilm to create a cariological challenge have been also been used to study secondary caries. In vitro biofilms have been derived from a simple inoculum such as a monoculture, but more complex models using consortium biofilms (using a limited number of bacterial species) or microcosm biofilms (using unlimited numbers of species, from a relevant clinical source) have been reported (Cenci et al., 2009; Khvostenko et al., 2015; Kuper et al., 2015; Seemann et al., 2005). Although consortium biofilm models consist of the use of more than one bacterial species, they may be limited because they don't represent the microbial complexity/interaction that occurs in the oral ecosystem. Microcosms originated from saliva or dental plaque may best represent the clinical biofilm. Microcosms are able to maintain much of the complexity and heterogeneity of the original sample enabling oral bacterial community dynamics to be replicated within the laboratory environment and with the possibility of manipulating the variables of interest (McBain, 2009).

In general, various in vitro biofilm models have been described, but many of them have not been completely validated regarding dose-response to antimicrobials / anti-caries agents or reproducibility. This lack of validation makes it difficult to compare among studies and the reliability of data provided by them is therefore uncertain.

In situ models represent the next level of clinical simulation and they have been also used to study secondary caries (Barata et al., 2012; Hara et al., 2006; Kuper et al., 2014; Kuper et al., 2015a; Montagner et al., 2015; van de Sande et al., 2014). In these studies, oral appliances containing dental substrate are worn by volunteers, and a natural biofilm is formed over the samples. Usually, subjects enrolled in these studies are instructed to frequently expose the dental substrate to sucrose solution to simulate a highly cariogenic environment. In situ models may serve as a bridge between the natural uncontrolled clinical situation and the highly controlled laboratory situation (Zero, 1995). They also represent an ethical clinical model to study (secondary) caries lesion formation and related factors.

1.3 Which are the factors related to secondary caries development?

Approximately half of all restorations placed in general dental practice worldwide are a replacement of a failed or defective restoration (Deligeorgi et al., 2001; Gordan et al., 2012; Mjor, 2005). One of the main reasons of restoration failure is secondary caries (Demarco et al., 2012; Opdam et al., 2014). Several factors have been reported to be associated with secondary caries lesion formation, including the marginal defect / gap size, tooth-restoration interface, dentin enzymatic degradation and the overall caries risk. These factors will be discussed in the following sections.

1.3.1 Marginal defect and gap size

Similarly to primary caries, the most important aspect to secondary caries is the presence of a cariogenic biofilm, but in association with a discontinuity or a gap between the tooth and restorative material (Ferracane, 2017).

The presence of a gap at the tooth-material interface can be related to an initial defect in the restoration margin or can develop due to interface degradation during clinical service (Carvalho and Manso, 2016). In either case, the cause of tooth demineralization has been attributed to leakage of bacteria or their acid products into the space /gap formed.

For a long time, the microleakage between tooth and restoration was considered as a risk factor for wall lesion initiation and progression. The presence of a microspace would allow bacterial invasion and subsequently lead to caries lesion development (Gonzalez-Cabezas et al., 1995; Gonzalez-Cabezas et al., 1999; 2002). This theory implied that secondary caries could be developed in any gap, but the wider the gap the higher the risk of lesion development (Totiam et al., 2007).

Later on, some studies started to show that microleakage was not associated with clinical secondary caries (Kidd et al., 1995; Thomas et al., 2007). It was assumed that secondary caries wall lesions could not develop without cariogenic biofilm growth along the tooth-restoration interface. The width needed for biofilm development inside the gap was estimated to be larger than 225 μm (Kidd et al., 1995; Thomas et al., 2007). This new theory was named macroleakage and it is accepted nowadays by cariologists and the majority of operative dentists (Nedeljkovic et al., 2015).

Recently, in vitro and in situ studies have been published showing that the width of gap to allow secondary caries wall lesion development may be smaller than originally thought by researchers in the field (Khvostenko et al., 2015; Kuper

et al., 2014; Turkistani et al., 2015). However, the new literature still provides no conclusive answer to the question if there is a relationship between gap size and wall lesion development, and if so, what the threshold is for the gap size needed.

Thomas et al. (2007) using a clinical in situ study found that wall lesions could develop in gaps ranging from 80 to 225 μm. Kuper et al. (2014) showed that a gap size of around 70 μm could enable secondary caries lesions development in a high cariogenic challenge, but a threshold for the wall lesion was not yet established. More recently, Khvostenko and collaborators (2015) showed that spaces varying from ~15-30 μm were able to allow biofilm growth in the tooth-restoration interface. If small gaps can allow biofilm formation, it is assumed that wall lesions could be initiated in such space. Thus, considering the above-mentioned, if a threshold for the gap size exists, it could lie within the range of 0-70 μm.

Although all subjects enrolled in the Kuper et al. (2014) study were exposed to a highly cariogenic protocol which would make all of them high caries risk subjects, not all of them had the same pattern of secondary caries wall lesion development. These findings indicate that probably caries lesion progression is influenced by patient factors even in the presence of a standardized and very high cariogenic challenge. This may imply that the minimal gap size for developing secondary caries could also be related to the caries risk of patient.

1.3.2 Tooth-material interface

When a restoration is placed, a new interface is formed between the dental substrate and the restorative material. Ideally, when a good restoration is made and a good sealing is achieved, this protects against the development of secondary caries, at least within the interface. However, especially with composite restorations, the quality of interface is negatively affected by physiological conditions of the oral environment such as masticatory forces, biofilm, and enzymatic degradation leading to marginal gaps or defects that could predispose to a secondary caries lesion (Carvalho and Manso, 2016).

Not only gaps can be related to wall lesion formation, but also other factors, for example, the dental material used (amalgam, composite or adhesive) and the failure type of the bonding (Kuper et al., 2015a; Kuper et al., 2015b; Montagner et al., 2015; Montagner et al., 2016a; van de Sande et al., 2014).

It is reported that restorative materials can either reduce or increase caries lesion formation in several ways. Dental amalgam, for example, is able to release ions with cariostatic proprieties (Ag, Cu and Zn) decreasing adhesion of bacteria

(Glassman and Miller, 1984; Morrier et al., 1998). Moreover, amalgam restorations have the capacity to reduce existing gaps by corrosion products or creep-expansion, thus sealing restoration margins and reducing the risk of the caries formation (Ben-Amar et al., 1995; Osborne, 2006). It has been reported that resin composite restorations result in more biofilm accumulation than amalgam and glass ionomer restoration (Svanberg et al., 1990), enhancing bacterial growth due to its surface-topography (Beyth et al., 2008). Despite these observations, there is no conclusive evidence for either material in itself promoting or reducing secondary caries (Kuper et al., 2015a; Kuper et al., 2015b; van de Sande et al., 2014).

Not only the restorative material, but also the adhesive system used to bond the restoration may be involved in secondary caries development. Studies evaluating wall lesion development in restorations with gaps usually did not include the adhesive in their analysis (Diercke et al., 2009; Totiam et al., 2007). Recent studies suggested that antimicrobial components in the adhesive may influence caries progression (Kuper et al., 2015b; Montagner et al., 2015).

Clinically, bonded restorations, usually composite restorations, can suffer mainly 2 types of interface failures: cohesive and adhesive (figure 2). Cohesive failure occurs when the interface is broken within the adhesive and this adhesive covers both side of interface: both enamel/dentin and composite. Adhesive failure occurs when the adhesive is broken at the interface itself. In this case, adhesive material can be located either on the tooth tissue or on the composite. Depending on this failure type, the failed restoration may present different characteristics and reactions to the secondary caries process.

It has been reported that an adhesive failure with the adhesive on the composite may favour wall lesion progression, possibly by promoting biofilm growth (Montagner et al., 2015). On the other hand, the presence of adhesive material on the dentin side of the interface was shown to partially protect the dentin against demineralization in a simplified test model (Montagner et al., 2016b). However, this relationship was not confirmed in an in situ study (Montagner et al., 2015).

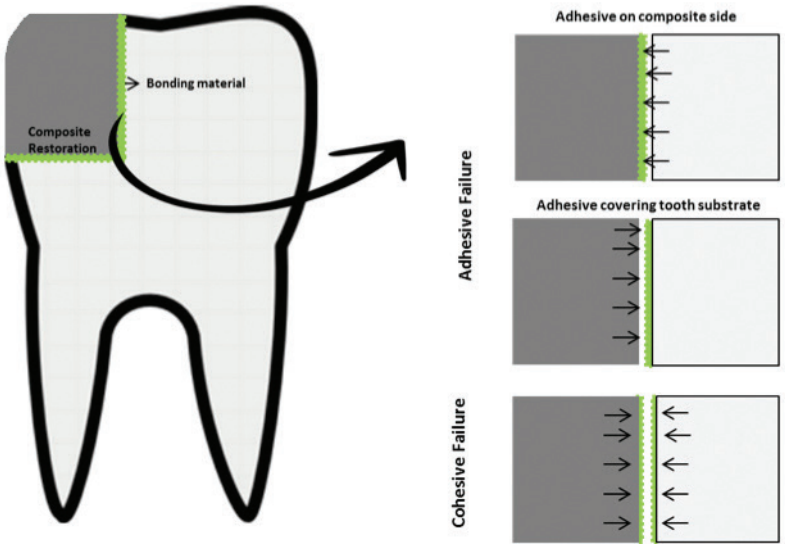

Figure 2. Schematic representation of failure types of the composite-tooth interface: adhesive and cohesive.

1.3.3 Dentin enzymatic degradation

Enzymatic degradation of dentin, promoting dentin adhesive interface failure, may be important in secondary caries formation. Moreover, dentin caries, while mainly characterized by a loss of mineral components similar to enamel caries, has some additional features not shared with enamel, due to its considerable amount of organic material: the collagen matrix. It is assumed that protein degradation plays an important role in dentin caries development, known as the proteolysis theory (Takahashi and Nyvad, 2016; Tjaderhane et al., 2015).

Recent studies have suggested that host-derived matrix metalloproteinases (MMPs) and cysteine cathepsins are directly involved in dentin matrix degradation during caries formation. MMPs are enzymes, present in dentin, dentinal fluid and saliva which are capable of degrading almost all extracellular matrix components (Visse and Nagase, 2003). Besides dentin caries pathogenesis, MMPs are also claimed to be involved in adhesive hybrid layer degradation of bonded restorations (Mazzoni et al., 2015).

MMP activity in the dentin organic matrix can be reduced by endogenous and exogenous inhibitors. Endogenous inhibitors also known as tissue inhibitor of

proteinases (TIMPs, TIMP-1 to 4) originate from different human cells exhibiting variable and non-specific action against the different MMP members (Chaussain-Miller et al., 2006). Exogenous inhibitors are artificially synthesized and are available as therapeutic agents (e.g. chlorhexidine, tetracycline, galardin, etc.) (Mazzoni et al., 2015). Most of these inhibitors chelate calcium or replace the zinc ions at the active site and/or interact with the MMP propeptide fragment, while others may prevent MMP access and inhibit activity by coating the substrate (Mazzoni et al., 2012; Mazzoni et al., 2015; Tjaderhane et al., 2013).

The most tested MMP inhibitor is chlorhexidine (CHX) which reduces the activity of MMPs and shows bond strength preservation and reduced interfacial degradation, even at concentrations as low as 0.2% (Breschi et al., 2010; Montagner et al., 2014). MMP inhibitors are also claimed to be effective for dentin caries prevention. (Sulkala et al., 2001; Tjaderhane et al., 2015).

Many studies have investigated the effect of MMP inhibitors (mainly CHX) on adhesive bond stability, however there is no literature showing the influence of MMP inhibitors on caries development when the marginal seal is lacking due to defects or gaps at the interface. Considering the potential use of MMP inhibitors for caries prevention and the extensive literature about the use of MMP inhibitor as a technique to prolong bond strength of restoration, the use of MMP inhibitor would also be of interest for secondary caries prevention.

1.4 Aims of this PhD Research

Considering the variability of in vitro biofilm models to study dental caries, the uncertainty of a threshold for small gap sizes and the potential role of MMP inhibitors in secondary caries development, the aims of this PhD research are:

- Systematically review the literature concerning in vitro biofilm models studying dental caries and verifying the reproducibility and dose-response to anti-caries and / or antimicrobial substances (Chapter 2)
- Investigate dentin wall lesion development next to resin composite containing very small gaps and evaluate if a threshold for the gap size could be established in vitro (Chapter 3)

- Investigate in situ the dentin wall lesion development next to resin composite containing very small gaps and to evaluate if the threshold gap size is related to the caries risk of the patient (Chapter 4)
- Evaluate the effect of using an MMP inhibitor (CHX) in the adhesive procedure on secondary caries lesion progression in vitro (Chapter 5) and in situ (Chapter 6)

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CHAPTER

2

In vitro biofilm models to study dental caries: a systematic review

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Abstract

The aim of this systematic review is to characterize and discuss key methodological aspects of *in vitro* biofilm models for caries-related research and to verify the reproducibility and dose-response of models considering the response to anti-caries and/or antimicrobial substances. Inclusion criteria were divided into Part I (PI): an *in vitro* biofilm model that produces a cariogenic biofilm and/or caries-like lesions and allows pH fluctuations; and Part II (PII): models showing an effect of anti-caries and/or antimicrobial substances. Within PI, 72.9% consisted of dynamic biofilm models, while 27.1% consisted of batch models. Within PII, 75.5% corresponded to dynamic models, whereas 24.5% corresponded to batch models. Respectively, 20.4 and 14.3% of the studies reported dose-response validations and reproducibility, and 32.7% were classified as having a high risk of bias. Several *in vitro* biofilm models are available for caries-related research; however, most models lack validation by dose-response and reproducibility experiments for each proposed protocol.

2.1 Introduction

Dental caries is one of the most prevalent oral diseases (Selwitz et al. 2007; Kassebaum et al. 2015) and is related to undisturbed microbial biofilm growth on the surface of the tooth in response to a sucrose-rich diet (Zero et al. 2009).

The etiology, diagnosis, treatment, and control of dental caries have been extensively studied over the years. The oral cavity is able to influence the microbial diversity and growth of dental biofilms due to a variety of distinct and selective environmental conditions such as tooth location, pH, pO2, and type of substratum (Bradshaw and Marsh 1998). Environmental changes, physiological disturbances, or selective pressures that might occur during dental biofilm growth are able to stimulate the proliferation of certain specific microorganisms that may lead to the development of a potential cariogenic biofilm (Parisotto et al. 2010).

The physiological complexity associated with the huge microbial diversity of the oral cavity and the ethical issues related to experimentation in clinical studies has led to the development of laboratory models in an effort to simulate those clinical conditions under well-controlled in vitro conditions (Tang et al. 2003; Keijser et al. 2008; McBain 2009; Sim et al. 2016). However, the microbial complexity of the oral flora and the intricate metabolic/ interactions chains, as well as the incapacity to cultivate some fastidious microorganisms in the laboratory, present some challenges to the development of in vitro biofilm models.

Simplified or complex in vitro microbial models have been extensively used to produce artificial caries lesions. Artificial biofilms have been cultivated from monoculture, defined-multispecies consortia or from complex microbial microcosms using different approaches, such as 24-well plates (Filoche et al. 2007; Ccahuana-Vásquez and Cury 2010; van de Sande et al. 2011; Arthur et al. 2013; Cavalcanti et al. 2014), Petri dishes (Totiam et al. 2007), multiplaque artifcial mouth (MAM) (Sissons et al. 1991), constant depth film fermenter (CDFF) (Peters and Wimpenny 1988), chemostats (Bradshaw and Marsh 1994), center for disease control (CDC) biofilm reactor (Rudney et al. 2012), flow chambers and flow cell (Seemann, Bizhang, et al. 2005; Seemann et al. 2006; Lynch and Ten Cate 2006b), artificial mouth computer controlled (AMCC) (Mei, Chu, et al. 2013; Mei, Li, et al. 2013), or the multi-station continuous-culture biofilm model (MSCBM) (Schwendicke, Doerfer, et al. 2014).

Each of these approaches allows for the control of specific parameters related to biofilm growth, such as the time and frequency of exposure to carbohydrates, carbohydrate source, type of substratum, diversity of the microbial inoculum, culture

medium, and days of biofilm formation (Tang et al. 2003; McBain 2009). However, it is important to consider the shortcomings of modeling clinical aspects under in vitro conditions. Physical and physiological variables should be incorporated to modulate microbial shifts during biofilm development and the cariogenic potential of the biofilm; the biofilm must also respond to preventive interventions currently used in clinical practice (Sissons 1997; Sim et al. 2016). Additionally, the strain culture conditions must be standardized to reduce variations in outcomes due to distinct microbial physiological adaptation. Moreover, it has been suggested that a good biofilm model should include pH oscillations and microbiological diversity to allow the simulation of oral conditions to develop caries-like lesions or to test antimicrobial treatments (Maltz and Beighton 2012; Buzalaf et al. 2015).

2.2 Materials and methods

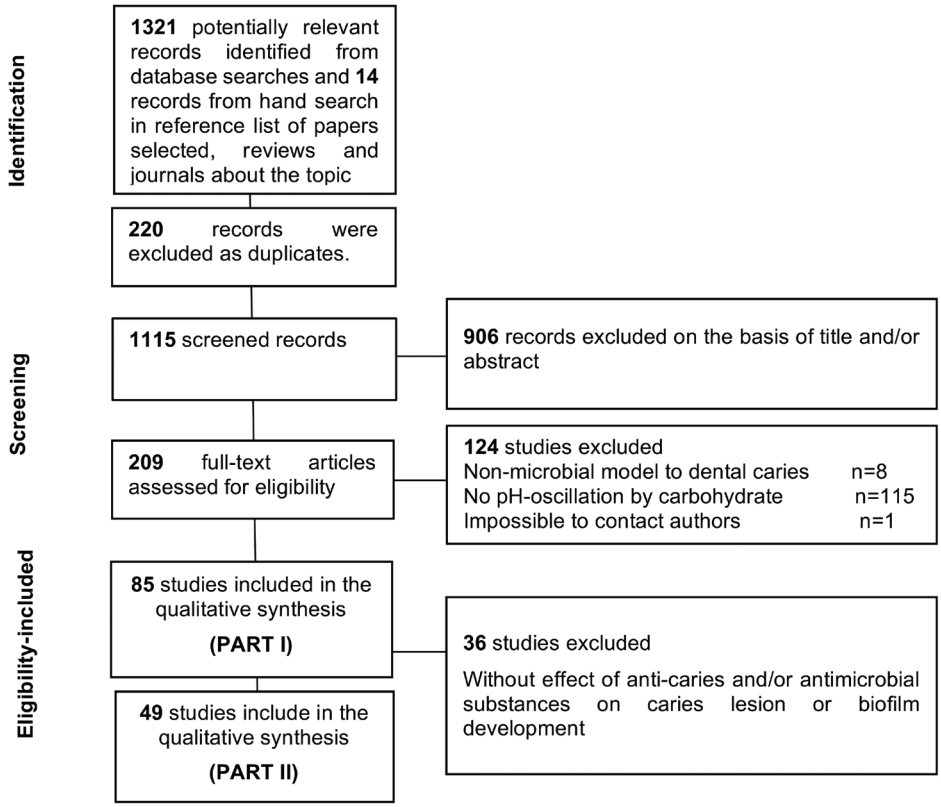
Eligibility criteria – Parts I and II

This systematic review was compiled following the PRISMA statement (Liberati et al. 2009). For Part I, evaluating methodological features of in vitro biofilm models, the inclusion criteria were defined as follows: the study should (1) use an in vitro biofilm model; (2) allow the development of potential cariogenic biofilms and/or artificial caries lesions; and (3) allow pH fluctuations (by intermittent sucrose or other carbohydrate exposure in the model). To be included in Part II, evaluation of the ability of the models to test anticariogenic substances, studies should have fulfilled the above criteria by testing the effect of anti-caries and/or antimicrobial substances. Only studies published as full-text articles were included in this review. Studies reporting in situ or in vivo investigations, non-microbiological models, microbiological models dealing with planktonic cultures, and biofilm models that did not present pH oscillations were excluded (Appendix Figure 1 in Supplemental material)

Information sources and search strategy

Tree databases (PubMed, Scopus, and Isi Web of Science) were used, with a search strategy including controlled vocabulary and free terms around the terms ‘dental caries’, ‘dental plaque’, ‘biofilm models’, and ‘in vitro studies’. First, the search strategy was developed for PubMed and then adapted to the other databases (Appendix Table 1 in Supplemental material). References of eligible articles,

scientific journals in the field, and narrative reviews on biofilm models were hand-searched to detect other potential studies of interest. The search was limited to language (English, French, German, Portuguese, and Spanish) and not limited to date.



Appendix Figure 1. Search flowchart of articles as described in the PRISMA statement. Part I: *in vitro* biofilm models that produce a potential cariogenic biofilm and/or artificial caries lesion and that allow pH fluctuations. Part II: models showing an effect of anti-caries and/or antimicrobial substances on caries lesion or biofilm development.

Appendix Table 1. Search strategy

	Search Terms in Pubmed
#5	(((((“In Vitro Techniques”[Mesh] OR in vitro technique OR technique, in vitro OR techniques, in vitro OR in vitro as topic OR in vitro))) AND ((flow cell OR flow cells OR continuous model OR batch model OR biofilm model OR biofilm models OR cdff OR constant depth film fermenter OR MAM OR multiplaque artificial mouth OR chemostats OR chemostat OR cdc reactors OR cdc reactor OR reactor OR reactors OR biochemical reactors OR biochemical reactor OR chemostat system OR artificial mouth OR bioreactor OR bioreactors OR fermentor OR fermentors OR multi-well plate OR multi-well plates OR microplates OR microplate))) AND ((“Biofilms”[Mesh] OR biofouling OR bacterial adhesion OR adhesins, bacterial OR “Dental Plaque”[Mesh] OR plaque, dental OR microcosm OR microcosms OR multi-species OR defined-multispecies OR multispecies OR consortium OR “Microbial Consortia”[Mesh] OR consortia, microbial OR microbial consortium OR consortium, microbial))) AND ((“Dental Caries”[Mesh] OR dental decay OR caries, dental OR decay, dental OR carious dentin OR carious dentin OR dentin, carious OR dentin, carious OR dental OR white spot OR white spots, dental OR white spots OR spot, white OR spots, white OR white spot OR dental white spots OR white spot, dental OR “Tooth Demineralization”[Mesh] OR tooth hypomineralization OR hypomineralization, tooth OR hypomineralization, tooth OR tooth hypomineralization OR demineralization, tooth OR enamel demineralization OR dentin demineralization OR integrated mineral loss OR mineral loss OR percentage surface hardness change OR caries-affected dentin OR caries-like lesion)) Search #1 AND #2 AND #3 AND #4
#4	Search (“Dental Caries”[Mesh] OR dental decay OR caries, dental OR decay, dental OR carious dentin OR carious dentin OR dentin, carious OR dentin, carious OR dental OR white spot OR white spots, dental OR white spots OR spot, white OR spots, white OR white spot OR dental white spots OR white spot, dental OR “Tooth Demineralization”[Mesh] OR tooth hypomineralization OR hypomineralization, tooth OR hypomineralization, tooth OR tooth hypomineralization OR demineralization, tooth OR enamel demineralization OR dentin demineralization OR integrated mineral loss OR mineral loss OR percentage surface hardness change OR caries-affected dentin OR caries-like lesion)
#3	Search (“Biofilms”[Mesh] OR biofouling OR bacterial adhesion OR adhesins, bacterial OR “Dental Plaque”[Mesh] OR plaque, dental OR microcosm OR microcosms OR multi-species OR defined-multispecies OR multispecies OR consortium OR “Microbial Consortia”[Mesh] OR consortia, microbial OR microbial consortium OR consortium, microbial)
#2	Search (flow cell OR flow cells OR continuous model OR batch model OR biofilm model OR biofilm models OR cdff OR constant depth film fermenter OR MAM OR multiplaque artificial mouth OR chemostats OR chemostat OR cdc reactors OR cdc reactor OR reactor OR reactors OR biochemical reactors OR biochemical reactor OR chemostat system OR artificial mouth OR bioreactor OR bioreactors OR fermentor OR fermentors OR multi-well plate OR multi-well plates OR microplates OR microplate)
#1	Search (“In Vitro Techniques”[Mesh] OR in vitro technique OR technique, in vitro OR techniques, in vitro OR in vitro as topic OR in vitro)

Screening and study selection

All retrieved references were managed and stored in EndNote X7® software (Thomson Reuters, San Francisco, CA, USA). Duplicates were identified and excluded. Two independent examiners (TTM and FHV) assessed all identified studies in the electronic search of the abovementioned databases. No blinding was carried out regarding author names and journals. After title screening, the abstracts of the studies were carefully evaluated. All eligible studies were identified, and the full text was read. In the case of doubt during abstract evaluation, the full text of the article was also read. In the case of disagreement, a third reviewer (MSC) was consulted, and the decision was made by consensus. During the process of selection, studies were included or excluded using pilot-tested tables addressing the following: (1) microbiological model (yes or no); (2) development of cariogenic biofilms and/or artificial caries lesions (yes or no); (3) pH oscillation by carbohydrate (yes or no); and (4) evaluation of anticariogenic and/or antimicrobial substances (yes or no).

Data collection and risk of bias assessment

For Part I, the following data were extracted from all selected articles: apparatus used to allow biofilm growth; surface coating method; inoculum source; substratum for biofilm growth; length of the experiment; culture medium and flow rate; carbohydrate source; and time-frequency of exposure of biofilms to carbohydrates. For Part II, the collected data involved the evaluation methods, anticariogenic/antimicrobial substances used, dose-response and reproducibility of the model, and significance of the effect within the response variables. Two reviewers extracted all the data simultaneously under a standardized outline for both Part I and II. A brief description of each biofilm model was carried out, and the models were divided according to the presence or absence of the flow-rate of a culture medium (batch or dynamic biofilm models).

The quality of the studies included in Part II was assessed using the criteria of Sarkis-Onofre et al. (2014) with modifications. The following parameters were used for risk of bias assessment: description of the sample size calculation, randomization of treatment allocation, presence of a control group, blinding, standardization of samples (teeth or other surfaces), and control of contamination (sterilization before starting the experiment). If the authors reported the parameters listed above, the article received 'Y' (yes); if the information was not found, the article received 'N' (no). The articles that obtained 'Y' for one to two items were considered to have a

high risk of bias, those with three to four were considered to have a medium risk of bias, and those with five to six were considered to have a low risk of bias.

2.3 Results and discussion

Study selection

The search retrieved 1,335 records, and after the removal of duplicates, 1,115 titles were screened. The appraisal of titles and abstracts resulted in the selection and assessment of 209 articles for eligibility. Eighty-five studies met the inclusion criteria for Part I, and 49 of the selected studies evaluated the effect of antimicrobial and/or anticariogenic substances and, therefore, were selected for the Part II analysis (Appendix Figure 1 in Supplemental material). The reasons for exclusion of articles included the following: not being a microbial model for dental caries (n = 8), not presenting pH oscillation by carbohydrate (n = 115), and the inability to retrieve the full-text article after attempting to contact the authors twice (n = 1).

Methodological features – Part I

Narrative reviews on the historical and general characteristics of microbial models are well documented in the literature (Sissons 1997; Tang et al. 2003; McBain 2009; Salli and Ouwehand 2015; Sim et al. 2016); however, detailed information on the experimental conditions for biofilm growth have not been addressed in previous reviews.

Several studies focusing on dental caries biofilm models were excluded from this systematic review because they did not satisfy the proposed inclusion criteria (allow pH fluctuations by intermittent sucrose or other carbohydrate exposure). Although they have contributed significantly to the understanding of dental caries as part of a complex and dynamic process (Exterkate et al. 2010; Guggenheim et al. 2001), the present review only reported those biofilm models that applied pH shifts during biofilm growth, since they may simulate more appropriately a clinical condition for caries development. The focus on this systematic review was not to revise all biofilm models available to study dental caries but to show in detail those dental caries biofilm models with similar characteristics. Therefore, this is the first review to describe and discuss the methodological parameters related to the different in vitro biofilm models for the development of artificial caries lesion with characteristics closer to clinical conditions.

Descriptive data of the methodological features for each included study are presented in Appendix Table 2 in Supplemental material, and a comparative discussion of each element is presented under the sub-topics below.

Batch and dynamic biofilm models

For this systematic review, the models were divided according to the presence or absence of any kind of flow during biofilm growth (dynamic and batch models, respectively). A brief description of the biofilm models is shown in Table 1, and positive and negative points of dynamic and batch models are summarized in Appendix Table 3 in Supplemental material.

Both categories of models were found to produce potential cariogenic biofilms and/or artificial caries lesions (Appendix Table 2 in Supplemental material). Within the studies reporting dynamic models, 37.10% used a flow chamber/cell, 25.81% used MAM, 16.13% used CDFF, 9.68% used chemostats, 4.84% used MSCBM, and 3.22% used AMCC and CDC biofilm reactors. In the batch model approaches, 91.3% were carried out in 24-well plates and 8.7% in Petri dishes.

In the dynamic models, the output is controlled by peristaltic pumps, which provide a precise flow of medium or carbohydrate to the system according to the study protocol. In general, models simulate the salivary flow rates, which vary from 0.04 to 2.5 ml min–1 (mimicking an unstimulated or a stimulated condition), whereas some systems, such as flow cells or chambers, presented higher flow-rates (60 ml min–1). Possible changes in methodological parameters make dynamic in vitro models adaptable and flexible to simulate the complexity of the oral environment. However, the majority of these models do not provide independent biofilm growth and require more than one experimental run, making them costly and time-consuming.

In the CDFF model, biofilms are grown in a set of 15 pans, but the samples are not grown in an independent manner. Modifications of this approach (by rotating 180° back and forward) allows the use of that model in a reciprocal mode in two simultaneous growth conditions (representing two treatment modes) (Deng et al. 2005; Zaura et al. 2011), yet within only one CDFF run. In general, the flow cell/chambers also have this limitation. In each cell or chamber, the biofilms formed are dependent on the same growth condition and either multiple runs or multiple chambers are needed to produce independent biofilms.

New settings providing independent units for the simultaneous accommodation of experimental samples and/or biofilm formation seem to be desirable to overcome

this limitation (Maske et al. 2016). Model approaches such as MAM, MSCBM, and AMCC also allow independent biofilm development but the complex assembly of these models also seems to be a limitation to their use. Considering the capability of reproducing clinical oral conditions, multi-well plates, as batch culture-based models, seem to be less effective as dynamic models in mimicking clinical conditions in a laboratorial environment (Salli and Ouwehand 2015). In these models, bacterial growth occurs in the presence of a limited availability of nutrients. Under this condition, the bacterial growth is faster than in the oral cavity, representing an unusual behaviour in nature (McBain 2009). Tis phenomenon may explain why experiments based on batch culture are on average of a shorter duration than those in more complex models.

Culture medium

Eleven different types of artificial saliva solutions and eight culture media are reported in this review (Appendix Table 2 in Supplemental material). Artificial saliva (AS) compositions are presented in Appendix Table 4 in Supplemental material.
The choice of the proper culture medium seems to depend more on the complexity of the inoculum rather than the complexity of the model. Deng et al. (2004) and Deng and Ten Cate (2004) used a simplified medium (BHI) for biofilm growth in a CDFF approach using a pure suspension of S. mutans as a microbial source, while van de Sande et al. (2011) used the most complex artificial saliva medium (defined medium mucin, DMM) (Wong and Sissions 2001) in a 24-well plate model using saliva as an inoculum for biofilm growth.

In general, the nutritional needs of biofilm cultivated from the monoculture of pure microorganisms allows for the use of a simplified culture medium based on tryptone, peptone, yeast extract, casein, and a source of dextrose, such as a TSB(S), BHI, and UTEYEB medium (Deng et al. 2005; Seemann et al. 2006; Fernández et al. 2016). However, the complexity of biofilms grown from microbial consortium or a microcosm requires a more enriched culture medium in which the chemical composition tends to be similar to natural saliva (Wong and Sissions 2001).

Artificial saliva media (Appendix Table 4 in Supplemental material) are based on a low carbohydrate solution that contain proteins, electrolytes, amino acids, and supplements other than the low-molecular-weight compounds found in simplified media, supporting the growth of microorganisms with different and specific nutritional needs. Wong and Sissons (2001) compared an undefined chemical culture medium (BMM) to a chemically defined analogue to saliva (DMM) to

grow microcosm biofilms. They found that the medium with more similarity to natural saliva allowed more realistic biofilm growth rates and the investigation of nutritional variables on plaque. Moreover, the reduced carbohydrate availability on AS medium stimulated synergistic and antagonistic metabolic interactions among microorganisms such as those found in the oral cavity (Elias and Banin 2012).

In addition to artificial saliva and simplified culture media, some studies have also described the use of fresh processed saliva as a method to cultivate dental biofilm (van der Ploeg and Guggenheim 2004; Thurnheer et al. 2006; Giertsen et al. 2011). In this method, whole saliva is processed by centrifugation and pasteurization methods (Guggenheim et al. 2001) and is used to reproduce fastidious periods of plaque development. Although shorter experimental times (~64 h) have been reported, a very large amount of saliva must be collected to conduct the experiment, and thus the protocol is difficult to perform.

Inoculum source

While the choice of a culture medium seems to depend on the type of inoculum, the selection of an inoculum source depends on the needs of the study or on the research question to be addressed. Monocultures are better indicated for studies aiming to evaluate specific physiological aspects of the biofilm through the response of the test inoculum to some specific experimental conditions. Streptococcus mutans has been the microorganism of choice for monoculture biofilms (Zanin et al. 2005; Totiam et al. 2007; Diercke et al. 2009; Fernández et al. 2016). Defined microbial consortia-based models, however, may be chosen to study ecological changes that would otherwise be difficult to measure for reasons of complexity in a more diverse microbial community (McBain 2009).

The studies included in this review have reported microbial consortia from two to 10 species for cariogenic biofilm development (Bradshaw et al. 2002; Yip et al. 2007; Mei, Li, et al. 2013; Schwendicke, Horb, et al. 2014; Fernández et al. 2015). This model may pose an advantage over monoculture models since the biofilm is grown as a result of the interaction among different microorganisms and not only as a result of the role of a specific strain. Additionally, since the proportions and types of microorganisms are adjusted at the start of the experiment, it is possible to monitor the effect of treatments or conditions on the competition or synergism among the players in the consortium. However, the disadvantage is related to its poor representativeness in comparison to natural biofilms.

Microcosm-based models seem to more closely mimic the natural dental biofilm (McBain 2009). By using saliva or dental plaque as an inoculum, thousands of different strains may compete among each other to establish a microbial community (Angker et al. 2011; Mei, Chu, et al. 2013; Li et al. 2014; Maske et al. 2016; Signori et al. 2016).

Dental caries is then a result of metabolic interactions of such diverse communities. However, it is important to point out that an oral cavity is composed of more than 700 species (Dewhirst et al. 2010), and some of these species can be lost when in vitro microcosm biofilm models are used to mimic the oral environment. This limitation may be inherent to laboratory biofilm models and can be explained by the use of anaerobic conditions other than those found in the oral cavity and also by a different composition of growth medium in comparison to natural saliva (Rudney et al. 2012).

The frequency in which saliva is inoculated varies among experimental models (Cutress et al. 1995; Sissons et al. 1995, 1998; Wong and Sissions 2001; van de Sande et al. 2011). Wong and Sissons (2001) compared the frequency of inoculation procedure, viz. one and three times, in the MAM apparatus, and there were no differences regarding biofilm patterns of growth. However, this procedure was not tested in other biofilm approaches.

When biofilms originate from different types of inocula (dental plaque or saliva), from caries-active and caries-free individuals, the cariogenic potential of these microcosm biofilms seems to be similar (demineralization and selection of acid-tolerant bacteria) regardless of the differences present in the baseline source of microorganisms (Azevedo et al. 2011, 2014; Signori et al. 2016). This is usually explained by the observation that these in vitro biofilms respond more to ecological pressures from the environment rather than the source or type of inoculum (Signori et al. 2016). Although caries lesion formation may respond similarly according to ecological pressures, Rudney et al. (2012) demonstrated that microcosm biofilms derived from saliva or dental plaque seems to be relatively stable within the subject over time, but there are clearly differences in the species composition between subjects. Even with some species in common, each biofilm derived from a microcosm seems to be unique in microbial composition and with individual characteristics, which should be considered in models using microcosms.

Surface coating

The biofilm formation on the tooth surface involves three main steps: (1) formation of the conditioning film, (2) cell-to-surface attachment of the primary colonizer, and (3) cell-to-cell interaction between late and new colonizers (Davey and O'Toole 2000). The first step in oral biofilm formation is represented by the adsorption of salivary molecules such as proteins, carbohydrates and lipids to the tooth (Lindh et al. 2014). The acquired pellicle formation serves as a substratum for the first bacteria (primary colonizers) and thus plays a crucial role in biofilm initiation (Marsh 2006).

Considering the studies included in Part I, > 55% of bacteria used natural saliva (processed or not) as a source for acquired pellicle formation, and 7.1% of the studies used artificial saliva to produce the coated surface. However, in most of the other studies, the methods employed to produce a coated substratum or the sources were not clear or not mentioned.

In general, when microcosm models were used, saliva was the simultaneous source for the acquired pellicle and inoculum (Cenci et al. 2009; van de Sande et al. 2011; Maske et al. 2016). However, in the MAM device, the surface coating method and inoculum process were performed in independent steps (Sissons et al. 1991). Some flow cell/chamber approaches reported that the inoculum (by consortium or monoculture) was maintained in contact with the substratum for up to 4 h to allow bacterial adhesion (Zampatti et al. 1994; Fontana et al. 1996, 2000, 2002; Thneibat et al. 2008). In this case, the inoculum and salivary pellicle formation seem to have been performed concomitantly. The bacteria per se could have secreted some products/molecules that served as the substratum coating method (Busscher and van der Mei 2000). In contrast, most of the microplate models used saliva for substratum coating (HA or tooth material) when consortia or monoculture was the chosen inoculum (van der Ploeg and Guggenheim 2004; CcahuanaVásquez and Cury 2010).

The findings from this review indicate that several surface coating methods were used for several available biofilm model approaches. These methods were neither the same for each kind of inoculum nor for each kind of protocol used to support biofilm growth (source of carbohydrate and culture medium). This fosters the diversity of biofilm caries modeling and highlights the need to validate these methods. The impact of these different methods on surface coating of carious lesion outcomes remains unclear.

Time and frequency of exposure to dietary carbohydrates

A cariogenic diet is an indispensable factor for the development of cariogenic biofilms and caries lesion formation. The effect of carbohydrate intake has been shown to be selective and demonstrates microbiological shifts in the presence of a low pH (Marsh 2006).

To reproduce the pH oscillation commonly found in the oral cavity, dynamic models deliver carbohydrates in small volumes and at high dilution rates. Under this condition, it is possible to maintain a degree of control over the cariogenic challenge provided to the biofilm (Hodgson et al. 2001). The time and frequency of exposure are controlled by peristaltic pumps that provide a precise flow of carbohydrates to the system according to the study protocol.

In the CDDF models, the frequency and time of exposure varied between three to eight times daily, for 5–30 min (Pratten and Wilson 1999; Seemann, Bizhang, et al. 2005; Deng et al. 2004; Cenci et al. 2009; Zaura et al. 2011).

The artificial mouths described by Mei, Chu, et al. (2013) and Sissons et al. (1991), and the subsequent publications using these approaches, showed that the frequency of sucrose exposure varied from three to 12 times daily, with exposure for 6 min in each pulse. For the flow chamber and flow cell models, several exposure protocols were also reported, varying from two (Hodgson et al. 2001; Lynch and Ten Cate 2006b; Xie et al. 2008) to > 12 times daily (Seemann, Bizhang, et al. 2005; Seemann, Klück, et al. 2005; Seemann et al. 2006; Diercke et al. 2009); and from 1 min (Lynch and Ten Cate 2006b; Xie et al. 2008) to 1 h (Perrons and Donoghue 1990; Donoghue and Perrons 1991). For the chemostat models included in the present review, carbohydrate was delivered from one to four times daily, whereas in the CDC reactor, exposure occurred five times a day for 1 min (Rudney et al. 2012; Li et al. 2014).

In batch models, pH oscillations are reproduced with an intermittent regimen of culture media (containing a source of sugar or no source). Microplate models report the use of several intermittent sucrose regimens varying in concentration and time of exposure. In microcosm biofilm models, sucrose was applied once in protocols with exposure for 4 or 6 h, while the sugar was applied more times per day (three to eight times daily) and with different exposure times (from 1 to 45 min) in those studies using consortia or monoculture.

Since the delivery of carbohydrate is manually performed by researchers (batch models) or mechanically by pumps (dynamic models), the frequency and exposure times can be easily altered. If and how this difference in frequency and exposure

times affects the biofilm or the formation of artificial caries have not yet been fully examined in complex biofilm models, but a dose-response effect of frequency, time and the concentration of carbohydrate should be validated (for several parameters) against in situ studies showing the ability to more accurately reproduce the clinical situation.

Even under distinct regimens of exposure to dietary carbohydrates, all studies included in the present review showed a direct relationship between a cariogenic diet and caries lesion or cariogenic biofilm development as a response to pH oscillations produced by sugar pulsing. These models are able to mimic the natural pH curves observed in plaque in vivo, one at low sugar availability (pH rise) and the other at high sugar availability (no pH rise). In this sense, the presence of pH oscillations makes the model more representative of caries lesion development once it reproduces the phenomena of de- and re-mineralization/rest periods and may be better suited to produce artificial caries-like lesions. Beyond representing the in vivo phases of dental plaque and allowing microbial shifting, in vitro models with pH oscillation avoid erosion of the tooth surface. A previous study for the development of an in vitro cariogenic biofilm model (van de Sande et al. 2011) showed that, under a continuous sucrose exposure regimen (0.5% sucrose), erosion occurred on the tooth surfaces, probably due to continuous exposure to acidogenic activity of the cariogenic biofilm. Thus, short-term in vitro studies examining initial microorganism interactions or metabolic activity (minutes or hours) may benefit from a continuous low pH, but an intermittent regimen seems appropriate for artificial caries lesion formation.

Most of the studies included in this review used sucrose as a nutrient source, and monosaccharide pulsing was only used by seven studies (Bradshaw et al. 1989, 1996, 2002; Perrons and Donoghue 1990; Giertsen et al. 2011). Although monosaccharides are potentially cariogenic (Cury et al. 2000), sucrose is especially able to provide energy for extracellular polysaccharide production that favors cariogenic plaque formation (Leme et al. 2006). Thus, sucrose pulsing seems to be more representative of in vivo biofilms and may be preferred for dental caries related studies.

Substratum for biofilm growth

Several substrata have been used for in vitro biofilm growth (Appendix Table 2 in Supplemental material). Human and bovine teeth (enamel, dentin, and root dentin) are generally used to study caries lesion development whereas inert materials (glass and plastic) or hydroxyapatite disks have been used mainly to study microbial

changes during biofilm development or in response to antimicrobial agents. Additionally, surfaces with specific designs such as grooves within dentin (Deng and Ten Cate 2004; Deng et al. 2004, 2005), with standardized gaps between the restoration and dental substratum (Cenci et al. 2009; Diercke et al. 2009; Kuper et al. 2015), and sealed fissures from natural teeth have also been reported (Seemann, Bizhang, et al. 2005; Seemann, Klück, et al. 2005). Dental materials and dental tissues have been used to investigate secondary caries development (Fontana et al. 2002; Seemann, Bizhang, et al. 2005; Cenci et al. 2009; Diercke et al. 2009; Kuper et al. 2015) or bonding to the caries affected dentin substratum (Maske et al. 2015; Peixoto et al. 2015). Thus, depending on the research question, a more representative surface may be used. However, the type of substratum may affect biofilm development and even the caries output, as previously demonstrated (Zaura et al. 2002; Lynch and Ten Cate 2006a). This phenomenon may be explained by the selective adherence of microorganisms to each surface or by the buffering of the environment caused by tooth substratum demineralization, especially dentin (Lynch and Ten Cate 2006a).

Table 1. Simplified description of biofilm model approaches included in this systematic review

Biofilm model approaches		Brief description
Dynamic models	**Constant Depth Film Fermenter (CDFF)**	Consists of a glass vessel with a stainless-steel end-plate, with ports for the entries of flow (medium, carbohydrate and gas) and another port for waste medium. The vessel accommodates polytetrafluoroethylene sampling pans rotating under a polytetrafluoroethylene scraper bar that smear the incoming medium over the pans and keep the formed biofilms at a constant depth. Each sampling pan has cylindrical holes and permits the sample positioning (McBain 2009). This approach can be used with a differentiated operate mode, which means that the turntable rotates 180º instead of 360º, allowing two simultaneous growth conditions (treatment modes) without a carry-over effect (Deng et al. 2005).
	Multiplaque Artificial Mouth (MAM)	It is characterized as a glass cylinder with five plaque growth stations. Each station contains a head assembly with 3 lines for flow (nutrients and experimental treatments), access for the plaque sample, an inoculation assembly and electrode access, supplementary access and access for plaque growth assembly (Sissons et al. 1991). In the MAM approach, the substance flow can be controlled by a computer (Wong et al. 1994).
	Multi-station continuous-culture biofilm Model (MSCBM)	MSCBM is a computer-controlled system and has three different chambers; nutrition and saliva are supplied via peristaltic multi-channel pumps (Schwendicke et al. 2014a).
	Artificial Mouth Computer controlled (AMCC)	The principle is similar to the Multiplaque Artificial Mouth, but with modification related to design. AMCC is composed of a glass cylinder with plaque growth stations. The stations contain places for inoculation, entrance of the flow, access to the plaque sample, and supplementary assembly, and the flow is controlled by a computer (Mei et al., 2013a).
	Flow cell /chamber	Flow chambers are apparatuses in which bacteria grow under continuous hydrodynamically controlled conditions. Biofilms can be formed and continually fed fresh nutrients. In general, the system consists of five components: a medium reservoir, peristaltic pump, bubble traps, flow cell/chamber and effluent reservoir. Flow cells are considered small chambers with transparent surfaces where submerged biofilms can also be formed. Flow cells can be used for microscopic analysis (Shunmugaperumal 2010).
	Chemostat	Consists of a glass vessel sandwiched between two stainless steel plates (Bradshaw et al. 1996) and is considered a bioreactor to which fresh medium is continuously added, while culture liquid is continuously removed to keep the culture volume constant (Novick and Szilard 1950).
	CDC Biofilm Reactor	Consists of a glass vessel with an effluent positioned to provide an operational fluid capacity. A polyethylene top supports independent and removable polyethylene rods, a medium-inlet port, and a port for gas exchange. Each rod holds a removable coupon with surfaces for biofilm growing-sampling opportunities (Goeres et al. 2005; McBain 2009).
Batch models	**Microplate and Petri Dish model**	These two approaches are considered batch culture models (McBain, 2009). Both models are developed in a plate: 24-well plate and Petri dishes. In these systems, each micro-well or the entire Petri dish receives a specific amount of culture medium, which is changed daily. Different regimens, such as sucrose exposure, can sometimes also used (Cahuana-Vasquez and Cury et al. 2010; van de Sande et al. 2011; Giacaman et al. 2013).

Appendix Table 2. Papers included in Part I and methodological features of dynamic[1] and batch[2] biofilm models

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
AMCC[1]						
(Mei, Chu, et al. 2013)	Not clear	300 µl Saliva	Human Root	7	0.06 ml min^{-1} DMM*	15 ml h^{-1}, 5% sucrose, 6 min, 3x
(Mei, Li et al. 2013)	Not mentioned	Consortium (5 species)	Human dentine	21	0.04 ml min^{-1} DMM	15 ml h^{-1}, 5% sucrose, 6 min, 3x
CDFF[1]						
Pratten and Wilson (1999)	Saliva + AS1 / pumped for 8h	1 ml saliva (10 individuals)	Bovine enamel	1 to 11	0,72 l day^{-1} AS1*	330 ml, 10% sucrose, 3x, 30 min
(McBain et al., 2003b)	± 9.6 ml h^{-1} AS2, 24 h before inoculation	2.5 ml saliva, (2 individuals)	Teflon	18	± 9.6 ml h^{-1} AS2*	19 ml h^{-1}, 0.3% sucrose, 4x, 30 min
(McBain et al., 2003a)	± 9.6 ml h^{-1} AS2, 24 h before inoculation	2.5 ml saliva (3 individual)	Teflon	5	± 9.6 ml h^{-1} AS2	19 ml h^{-1}, 0.3% sucrose, 4x, 30 min
(McBain et al., 2003c)	± 9.6 ml h^{-1} AS2, 24 h before inoculation	2,5 ml saliva (1 individual)	Teflon	28	± 9.6 ml h^{-1} AS2	19 ml h^{-1}, 0.3% sucrose, 4x, 30 min
(Cenci et al., 2009)	Saliva + DMM 2.4 ml h^{-1}, 1 h	10 ml saliva (1 individual)	Bovine dentine, gaps between restoration	18	0.5 ml min^{-1} DMM	1,2 ml min^{-1}, 10% sucrose, 8x, 5 min
(Zaura et al., 2011)	Saliva + DMM, 2.3 ml min^{-1}, 1.5 h	9 ml saliva (10 individuals)	Bovine dentine	8	0.3 ml min^{-1} DMM	1.2 ml min^{-1}, 10% sucrose, 8x, 5 min
(Deng et al., 2004)	100 ml sterile saliva, pumped for 3.5 h	15 ml *S. mutans*	Bovine dentine and polyacrylate grooves	20	0.5 ml/min BHI buffered	0.5 ml min^{-1}, 2% sucrose, 4x, 30 min
(Deng and ten Cate, 2004)	100 ml sterile saliva, pumped for 3.5 h	15 ml S. mutans	Bovine dentine grooves	20	0.5 ml min^{-1} BHI buffered	0.5 ml min^{-1}, 2% sucrose, 4x or 8x, 30 min
(Deng et al., 2005)	100 ml sterile saliva, pumped for 3.5 h	15 ml *S. mutans*	Bovine dentine grooves	20	0.5 ml min^{-1} BHI buffered	0.5 ml min^{-1}, 2% sucrose, 4x, 30 min
(Zanin et al., 2005)	100 ml AS1, pumped for 3.5 h	*15 ml S. mutans*	Hydroxyapatite	10	0.5 ml min^{-1} AS1	0.5ml min^{-1}, 2% sucrose, 4x, 30 min
MAM[1]						
(Sissons et al., 1991)	5 ml, saliva supernatant†	Saliva	Plastic	42	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 3x or 6x, 6min
(Sissons et al., 1992)	5 ml, saliva supernatant†	Saliva (re-innoculed 3 and 5 day)	Plastic	42	3.3-3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 4x or 3x, 6min

Appendix Table 2. Continued.

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
MAM[1]						
(Sissons et al., 1994b)	5 ml, saliva supernatant[†]	Saliva (re-innoculed 3 and 5 day)	Plastic	56	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 4x or 3x, 6min
(Sissons et al., 1994a)	5 ml, saliva supernatant[†]	Saliva	Plastic	49	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 4x or 3x, 6min
(Wong et al., 1994)	5 ml, saliva supernatant[†]	Saliva (1 individual/ re-innoculed 3 and 5 day)	Plastic	42	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 3x or 6x, 6min
(Sissons et al., 1995)	5 ml of whole saliva filtered through glass wool.	Saliva (1 individual/ re-innoculed 3 and 5 day)	Plastic	18	3.6 ml h^{-1} BMM	1.5 ml, 5%sucrose, 3x, 6min
(Cutress et al., 1995)	5 ml of whole saliva, 1h	Saliva (re-innoculed 2 and 5 day)	Bovine enamel	31	3.6 ml h^{-1} BMM	6 ml, 5%sucrose or 10%, 4x, 6 min
(Sissons et al., 1996)	5 ml, saliva supernatant[†]	5 ml saliva (1 individual)	Plastic	14	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 3x, 6 min
(Sissons et al., 1998)	5 ml, saliva supernatant[†]	Plaque (re-innoculled 3 and 5 days)	Plastic	56	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 3x, 6 min
(Wong and Sissions, 2001)	5 ml, saliva supernatant[†]	Saliva (re-innoculed or not 3 and 5 days)	Plastic	22	BMM and DMM 3.6 ml h^{-1}	1.5 ml, 5%sucrose, 3x , 6 min
(Pearce et al., 2002)	5 ml, saliva supernatant[†]	Saliva	Plastic	15	2.5 ml h^{-1} DMM	1.5 ml, 10% sucrose, 12, 8, 6 or 4x, 6 min
(Sissons et al., 2007)	5 ml, saliva supernatant[†]	Saliva	Plastic	19	2.5 ml h^{-1} DMM and BMM	1.5 ml, 5%sucrose, 3x , 6 min
(Angker et al., 2011)	5 ml, saliva supernatant[†]	Saliva	Human enamel	28	2.5 ml min^{-1} DMM	1.5 ml, 10% sucrose, 5x, 6 min
(Shu et al., 2000)	Not mentioned	Consortium (4 species)	Plastic, bovine enamel, root and dentine	22	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 3x or 4x, 6 min
(Filoche et al., 2004)	Not mentioned	Consortium (6 species)	Plastic	19	2.5 ml h^{-1} DMM	1.5 ml, 5%sucrose, 3x , 6 min
(Yip et al., 2007)	Not mentioned	Consortium (4 species)	Human enamel and root dentin	21	0.2 ml min^{-1} BMM	1.5 ml, 5% sucrose, 3x, 6 min

Appendix Table 2. Continued.

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
MSCBM[1]						
(Schwendicke, Doerfer, et al. 2014)	15 ml of saliva filtrated, 2h before inoculum	Monoculture or Consortium (2 species)	Bovine enamel and dentin	10	1.25 ml min^{-1} DMM	1.25 ml min^{-1} for 15 min, BHI + 2% sucrose 2x or 6x
(Schwendicke, Horb, et al. 2014)	15 ml of saliva filtrated, 2h before inoculum	Monoculture or consortium (2 species)	Bovine enamel and dentin	10	1.25 ml min^{-1} DMM	1.25 ml sterile MS-MTU supplemented with 5% sucrose for 15 min, 8x
(Schwendicke et al., 2015)	Not mentioned	Monoculture (*S. mutans*)	Bovine dentin	21	1.25 ml min^{-1} DMM	1.25 ml min^{-1} BHI + 2% sucrose, 1 x/ day, 1 min
FLOW CELL/CHAMBER[1***]						
(Sissons et al., 1985)	Not mentioned	Saliva	Bovine enamel	7	4 ml h^{-1} PYE	1.4 ml, 5% sucrose, 6x, 6 min
(Sissons and Cutress, 1987)	Not mentioned	Saliva	Bovine enamel	7	4 ml h^{-1} PYE	1.4 ml, 5% sucrose, 6x, 6 min
Sissons et al. (1988)	Not mentioned	Saliva	Bovine enamel	7	4 ml h^{-1} PYE	1.4 ml, 5% sucrose, 6x, 6 min
(Smullen et al., 2012)	Immersion in centrifuged saliva, 4h	Saliva and *S. mutans*	Bovine teeth	5	3.6 ml h^{-1} BMM	1.5 ml, 5% sucrose, 6 min, 3x
(Maske et al., 2016)	Saliva + DMM, 1h	Saliva	Bovine enamel	21	0.04 or 0.06 ml min^{-1} DMM	10% sucrose, 1.2 ml min^{-1}, 5 min, 5x 5% sucrose, 0.25ml/min, 6min, 3x
(Perrons and Donoghue, 1990)	Not mentioned	Consortium (4 species)	Human teeth (crown)	2 days and 22h	0.5 ml h^{-1} AS3 *	0.5 ml min^{-1} glucose 1% added medium, 4x, 1h
(Donoghue and Perrons, 1991)	Not mentioned	Consortium (3 species)	Human teeth (crown)	2 days and 22h	0.5 ml h^{-1} AS3	5 ml min^{-1} glucose 1% added medium, 4x, 1h
(Fontana et al., 1996)	Inoculum for 2 h on surface to bacteria adhesion	Consortium (2 species)	Human teeth	12	0,7 ml min^{-1} MW*	0.7 ml min^{-1} TSBS (5% sucrose), 3x, 30 min
(Hodgson et al., 2001)	Not mentioned	Consortium (7 species)	HA, bovine enamel	14	60 ml min^{-1} AS4*	0.34% and 1.7% sucrose, 2x
(Fontana et al., 2004)	sterile saliva for 20 min, 37° C	Consortium (5 species)	Human enamel	7	0.7 ml min^{-1} MW	0.7 ml min^{-1} TSBS (5% sucrose), 3x, 30 min
(Lynch and ten Cate 2006b)	Not mentioned	Consortium (7 species)	HA, Bovine enamel and dentine	7	60 ml min^{-1} AS4	2 ml, 0.8% sucrose, 2x, 1 min
(Xie et al., 2008)	Not mentioned	Consortium (4 species)	HA, glass, bovine enamel	5	60 ml min^{-1} AS4	10 ml, 0.8% sucrose, 2x, 1 min

Appendix Table 2. Continued.

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
FLOW CELL/CHAMBER[1]*						
(Klimek et al., 1982)	3 days, 37° C in AS5	*S. mutans*	Human enamel	2	5 ml min^{-1} AS5*	3% sucrose, 3x, 30 min
(Zampatti et al., 1994)	Immersion for 4 h in bacteria suspension to adhesion	*S. mutans*	Bovine enamel	5	0.125 ml min^{-1} AS6*	3.4% sucrose, 5x, 30 min
(Sorvari et al., 1994)	AS7, 50 ml h^{-1}, 24 h	*S. mutans*	Human enamel	10	50 ml h^{-1} AS7*	3% sucrose, 6x
(Fontana et al., 2000)	Inoculum for 2 h, on surface to bacteria adhesion, *37° C*	*S. mutans*	Human enamel	4	0,7 ml min^{-1} MW	0.7 ml min^{-1} TSBS (5% sucrose), 3x, 30 min
(Fontana et al., 2002)	Inoculum for 2 h, on surface to bacteria adhesion, *37° C,*	*S. mutans*	Human enamel	4	0,7 ml min^{-1} MW	0.7 ml min^{-1} TSBS (5% sucrose), 3x, 30 min
(Seemann, Bizhang, et al. 2005)	Not clear	*S. mutans*	Human teeth	21	20 min MS*+ TSB	10% sucrose, 5 min, hourly
(Seemann, Klück, et al. 2005)	Not clear	*S. mutans*	Human teeth	21	20 min MS+TSB	10% sucrose, 5 min, hourly
(Seemann et al., 2006)	Not clear	*S. mutans*	Human enamel	16	20 min MS+TSB	10% sucrose, 5 min, hourly
(Thneibat et al., 2008)	Inoculum for 2 h, on surface to bacteria adhesion, *37° C*	*S. mutans*	Root human teeth	5	0.7 ml min^{-1} MW	0.7 ml min^{-1} TSBS (5% sucrose), 3x, 30 min
(Diercke et al., 2009b)	Not clear	*S. mutans*	Bovine teeth	21	15 min MS+TSB	10% sucrose hourly
(Lee et al., 2010)	Not clear	Consortium (2 species)	Human enamel	5	2 ml min^{-1} Bacto ™ THB	2 ml min^{-1} 10% sucrose, 4x, 6min
MICROPLATE[2]						
(van de Sande et al., 2011)	Saliva, 1h, 23±3° *C*	0.4 ml saliva (1 individual)	Bovine enamel	10	DMM static	1% sucrose, 1x, 6h
(Azevedo et al., 2011)	Saliva, 1h, 23±3° *C*	0.4 ml saliva (1 individual healthy or caries active)	Bovine enamel	10	DMM static	1% sucrose, 1x, 6h
(Edlund et al., 2013)	Saliva supernatant, 1h, 37° *C*	Saliva	-	48h	SHI and CDM* static	SHI medium 0.5% sucrose, 16h
(Azevedo et al., 2014)	Saliva, 1h, 37° *C*	Saliva	Bovine enamel	10	DMM static	1% sucrose, 1x, 6h
(Maske et al., 2014)	Saliva, 1h, 37° *C*	Saliva	Bovine dentine	21	DMM static	1% sucrose, 1x, 4 h

Appendix Table 2. Continued.

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
MICROPLATE[2]						
(Kuper et al., 2015b)	Saliva, 1h, 37° *C*	Saliva	Bovine teeth (blocks)	20	DMM static	1% sucrose, 1x, 6h
(Peixoto et al., 2015)	Saliva, 1h, 37° *C*	Saliva	Human dentine	14	DMM static	1% sucrose, 1x, 4 h
Signori et al. (2016)	Saliva, 1h, 37° *C*	Saliva	Bovine enamel	10	DMM static	1% sucrose, 1x, 6 h
(van der Ploeg and Guggenheim, 2004)	Pasteurized saliva, 4h, room temperature	Consortium (6 species)	HA	64h	Fresh processed Saliva	mFUM + 0.30% glucose + 0.30% sucrose, 45 min, 5x
(Thurnheer et al., 2006)	Pasteurized saliva, 4h, room temperature	Consortium (6 species)	HA	64h	Fresh processed Saliva	mFUM with 0.30% glucose + 0.30% sucrose, 45 min, 5x
(Giertsen et al., 2011)	Pasteurized saliva, 4h, room temperature	Consortium (6 species)	HA	64h	Fresh processed Saliva	mFUM + 0.30% glucose, 45 min, 5x
(Cavalcanti et al., 2014)	Saliva filtered, 30 min, 37° *C*	Consortium (3 species)	Bovine enamel and root dentine	4	UTEYEB static	10% sucrose, 8x, 1 min
(Fernandez et al., 2015)	Saliva filtered, 30 min, 37° *C*	Consortium (2 species)	Enamel bovine	4	UTEYEB static	10% sucrose, 8x, 3 min
(Vanni et al., 2015)	saliva, 4h, room temperature, shaking	Saliva + consortium (6 species)	HA	64.5h	Fresh processed Saliva	mFUM + 0.15% glucose + 0.15% sucrose, 45 min, 4x
(Ccahuana-Vásquez and Cury, 2010)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel	5	UTEYEB static	10% sucrose, 8x, 1 min
(Giacaman et al., 2012)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel and dentine	5	UTEYEB static	8% sucrose 8x, 1 min
(Ribeiro et al., 2012)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel	5	UTEYEB static	8% sucrose 8x, 2.5 min
(Muñoz-Sandoval et al., 2012)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel and root dentine	5	UTEYEB static	10% sucrose, 8x, 1 min
(Giacaman et al., 2013)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel	5	UTEYEB static	8% sucrose, fructose, aspartame, saccharine, stevia and sucralose, 3x, 5 min
(Giacaman et al., 2015)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel	5	UTEYEB static	10% sucrose, 3x, 5 min
(Fernandez et al., 2016)	Saliva filtered, 30 min, 37° *C*	*S. mutans*	Bovine enamel and root dentine	4	UTEYEB static	10% sucrose, 8x, 3 min

Appendix Table 2. Continued.

Papers and model	Surface coating	Type of inoculum	Type of substrate	Days	Medium and flow	Carbohydrate and flow**
CHEMOSTAT[1]						
(Bradshaw et al., 1989)	Not mentioned	Consortium (9 species)	-	10	BMM	0.5% glucose, 1x
(Bradshaw and Marsh, 1994)	Not mentioned	Consortium (9 species)	-	10	BMM	0.5% glucose, 1x
(li and Bowden, 1994)	Not clear	Consortium (3 species)	-	32h	ADM mod	10 ml, 5% glucose ,4x
(Bradshaw et al., 1996)	Not clear	Consortium (10 species)	HA	21	75 ml h^{-1} BMM	7.5 ml, 0.5% glucose, 1x
(Bradshaw and Marsh, 1998)	Not mentioned	Consortium (10 species)	-	10	75 ml h^{-1} BMM	7.5 ml 0.5% glucose, 1x
(Bradshaw et al., 2002)	Not mentioned	Consortium (9 species)	-	10	60 ml h^{-1} BMM	5ml, 0.5 % glucose, 4x
CDC REACTOR[1]						
(Rudney et al., 2012)	Sterile Saliva	Saliva + dental plaque	HA, Silicone based composite	3	17 ml min^{-1} BMM	20 ml min^{-1}, 5% sucrose, 5x
(Li et al., 2014)	30 µl of sterilized human saliva	Saliva + plaque	Root dentin	3	17 ml min^{-1} BMM	20ml min^{-1}, 20% sucrose, 5x
PETRI DISHES[2]						
(Totiam et al., 2007)	Not clear	*S. mutans*	Human molars	8	MW static	TSBS (sucrose 5%) 1x, 4h
(Nassar and González-Cabezas, 2011)	Not clear	*S. mutans*	Human molars	8	MW static	TSBS (sucrose 5%) 1x, 4h

Note: Studies are cited from oldest to newest within each type of inoculum: saliva, consortium and monoculture. *Different Artificial Saliva (AS1- AS7): each one is described in Appendix table 4. ** Frequency described daily. *** Include all models mentioned as flow cell/chamber and when was not possible to find a specific denomination for dynamic model. † Indirect information: from cited reference in that study.

Abbreviations: **CDM:** chemically defined medium **TSB-**Trypticase soy broth. **TSBS-** trypticase soy broth supplemented with 5% sucrose. **BMM:** basal medium mucin. **DMM:** defined medium mucin. **MS:** mineral solution. **MW:** mineral wash. **UTEYEB:** Tryptione-yeast extract broth. **BHI:** Brain heart infusion **THB:** Todd Hewitt broth. **HA-** Hydroxyapatite **MS-MTU-** isolation medium for mutans streptococci. **PYE**: Peptone Yeast-extract. **ADM mod**: modified defined actinomyces medium (ADM) **mFUM:** Tryptone-yeast-based broth medium modified **MRS:** De Man Rogosa Sharpe.

Appendix Table 3. Batch and dynamic models: positive and negative points

	Batch models	**Dynamic models**
Positive points	· Shorter experiments · Simple equipment · Easy manipulation · Low-cost · Multiple conditions can be tested simultaneously	· Longer experiments · Salivary flow, shear-stress and Stephan curves can be easily reproduced. · Environment closely reproducing *in vivo* conditions
Negative points	· Lack of flow-rate and shear-stress simulation	· High-cost and time-consuming models · Complex equipment and manipulation · Generally only one condition can be tested in each experimental run

Appendix Table 4. Artificial saliva solution composition of the reported studies

Saliva Type	Artificial saliva composition
AS1	Lab-lemco 1 g l-1, yeast extract 2 g l -1, proteose peptone 5 g l-1, type III hog gastric mucin 2.5 g l-1, sodium chloride 0.2 g l-1, potassium chloride 0.2 g l-1, calcium chloride 0.3 g l-1; 1,25 ml l-1 of a 0.2 mm filter sterilized solution of 40% urea.
AS2	(g l-1): Mucin (type II; porcine; gastric), 2.5; bacteriological peptone, 2.0; tryptone, 2.0; yeast extract, 1.0; NaCl, 0.35; KCl, 0.2; CaCl, 0.2; cysteine hydrochloride, 0.1; hemin, 0.001; and vitamin K1, 0.0002.
AS3	(mg l-1) 233 ammonium chloride, 210 calcium chloride dihydrate, 43 magnesium chloride, hexahydrate, 1162 potassium chloride, 354 potassium di-hydrogen orthophosphate, 222 potassium thiocyanate, 13 sodium citrate, 535 sodium hydrogen carbonate, 375 di-sodium hydrogen orthophosphate, 2500 glycoprotein, 25 albumin, 173 urea, 10.5 uric acid, 0.1 creatinine, 13 choline, 41 mixture of amino acids, 0.8 mixture of vitamins, 3.105 alpha amylase 3.105 somogyi units/ litre (pH 6.8).
AS4	2.5 g l-1hog gastric mucin Type III, 2 g l-1proteose peptone, 1 g l-1 trypticase peptone, 1 g l-1 yeast extract, 0.5 g l-1 glucose, 2.5 g l-1 KCl, 0.1 g l-1 cysteine–HCl and 1mg l-1 haemin (pH 7.4).
AS5	0.33 g l-1 KH2PO4, 0.34 g l-1 Na2HPO4, 1.27 g l-1 KCl, 0.16 g l-1 NaSCN, 0.58 g l-1 NaCl, 0.17 g l-1 CaCl2.2.H2O, 0.16 g l-1 NH4Cl, 0.2 g l-1 urea, 0.03 g l-1 glucose, 0.002 g l-1 vitamin C and 2.7 g l-1 mucin.
AS6	(in mmol l-1): 10.2 NaCl, 10.7 KCl, 0.29 KH2PO4, 4.59 K2HPO4, 2.38 NAHC03, 0.25g Bio-Trypticase, 0.25g yeast extract.
AS7	Na2S= 0.0008g; Mg2P2O7= 0.0008g; Mucin= 2.0000g; CO(NH2)2= 0.5000g; Na2HPO4= 0 .3000g; CaCl2= 0.3000g; KCI= 0.2000g; NaCl= 0.2000g and 500 ml of distilled H2O.
CDM	(in g l-1): (NH4)2SO4 (0.8); NaCl (0.6); ascorbic acid (0.5); MgCl2.6H2O (0.16); CaCl2.2H2O (0.01); cysteine hydrochloride (0.3). 20 ml l-1 of a vitamin mix (g l-1): biotin (0.002); folic acid (0.002); pyridoxine hydrochloride (0.01); riboflavin (0.005); thiamine (0.005); nicotinic acid (0.005); pantothenic acid (0.005); vitamin B-12 (0.0001); p-aminobenzoic acid (0.005); thioctic acid (0.005). 13C-labeled glucose and 13C-L-lactate. Glucose (2.5 g l-1) and lactate (1.5 g l-1).
DMM	Salts (mmol l^{-1}): $CaCl_2$ 1.0, $MgCl_2$ 0.2, KH_2PO_4 3.5, K_2HPO_4 1.5, NaCl 10.0, KCl 15.0, NH_4Cl 2.0, Urea 1.0, Mucin (g l^{-1}) 2.5, 5 g l^{-1} casein. Basal salivary amino acids (µmol l^{-1}): Alanine 50, Arginine 50, Asparagine 25, Aspartic acid 25, Cysteine 50, Glutamic acid 25, Glutamine 25, Glycine 100, Histidine 10, Isoleucine 25,Leucine 25, Lysine 50, Methionine 10, Phenylalanine 25, Proline 100, Serine 25, Taurine 75, Threonine 25, Tryptophan 10, Tyrosine 15, Valine 25. Vitamins and growth factors (µmol l^{-1}): Choline chloride 100, Citrate 50, Uric acid 50, Haemin 10, Inositol 10, Ascorbic acid 5, Menadione 5, Niacin 5 0.24, Pyridoxine 4, Creatinine 1, p-Aminobenzoic acid 1, Pantothenic acid 1, Thiamine 1, Riboflavin 0.3, Biotin 0.1, Cyanocobalamin 0.05, Folic acid 0.025.
MW	(mg l^{-1}): KCl 624.6, NaCl 866.6, K_2HPO_4 33.8, $MgCl_2$ 59.6, $CaCl_2.2H_2O$ 166.6.
MS	0.33g KH_2PO_4, 0.34g Na_2HPO_4, 1.27g KCl, 0.16g NaSCN, 0.58g NaCl, 0.17g $CaCl_2.2H_2O$, 0.16 g NH_4Cl, 0.2 g urea, 0.03 g glucose, 0.002 g vitamin C and 2.7 g mucin and 1000 ml of distilled H_2O (pH 6.8).

Note: Artificial saliva described according to paper citation. For references see appendix table 1.

Biofilm models and anticariogenic/ antimicrobial substances – Part II

Descriptive information about the 49 selected papers showing outcomes and bias risk is shown in Table 2. A discussion of the evaluation methods and response variables as well as the risk of bias assessment are presented in the sub-topics below.

Dose-response and reproducibility

Thirty of the 49 studies reported artificial caries lesion formation and most of them used dynamic biofilm models (76.7%) to investigate the effect of anticariogenic/ antimicrobial substances. A dose-response evaluation was only reported in 10 studies. MAM was evaluated for fluoride concentration (0, 0.5, 1, 2, 5, 100, 3,000, and 5,000 ppm), demonstrating a dose-response effect on both pH and de and re-mineralization (Cutress et al. 1995; Angker et al. 2011) and ethanol was evaluated at different concentrations (10, 20, 30, 40%), altering the pH and the microbiota formed under those different conditions (Sissons et al. 1996). Angker et al. (2011) used the model to produce human enamel caries-like lesions from microcosms cultivated for 28 days with DMM and with pH oscillations performed by the application of 10% sucrose five times daily. Cutress et al. (1995) produced bovine enamel artificial caries lesions using microcosms cultivated by BMM and with sucrose application four times daily (10 or 5%). Sissons et al. (1996) described the biofilm dose-response to ethanol using a protocol that also used BMM but with 5% sucrose applied only three times per day.

For the flow cell/chamber approaches, Lynch and Ten Cate (2006b) demonstrated dose-response effects on the pH, mineral, and microbial contents of calcium glycerophosphate at different concentrations (CaGP 0.10, 0.25, 0.50%). In this study, human enamel and dentin caries-like lesions were produced by a biofilm consortium cultivated in a mix of MV and TSB+5% sucrose applied 3× daily. A dose-response validation was also described by Maske et al. (2016) for chlorhexidine (0, 0.03, 0.06, and 0.12%). The protocol used in this chamber approach showed that bovine enamel artificial caries was responsive to a CHX solution when microcosm biofilms were grown for seven days by delivery of 0.06 ml min–1 of DMM associated exposure to 5% sucrose (three times per day).

Chlorhexidine (0.012, 0.024, 0.03, 0.06, and 0.12%) was also used to validate microplate models considering pH, mineral (van de Sande et al. 2011), and microbiological variables as outcomes (Ccahuana-Vásquez and Cury 2010). A microplate microcosm model was developed by van de Sande et al. (2011), and

the dose-response to CHX was determined by biofilm growth in bovine enamel. In this study, DMM was used in association with 1% sucrose and applied 1× per day. A monoculture microplate model was CHX-dose-validated by Ccahuana-Vásquez and Cury (2010). The authors used UTEYEB associated with 10% sucrose applied 8× daily to produce a bioflm by S. mutans in the bovine enamel substrate. Using a similar monoculture model approach, Ribeiro et al. (2012) showed a dose-response to iron (1.0, 10.0, and 100.0 μg Fe m–1) on mineral loss. In contrast to the previous model, these authors used 8% sucrose applied 8× per day for 2.5 min instead of 1 min. Fernández et al. (2016) and Giacaman et al. (2015) showed, respectively, a dose response to fluoride (0, 150, 450, and 1,350 ppmF, on bovine enamel) and fatty acid (0.1, 1, and 10 mM, on bovine enamel and dentin root). Both studies used a monoculture microplate bioflm model (S. mutans), however, with a different protocol for the 10% sucrose solution application. In a study conducted by Fernández et al. (2016), sucrose was applied eight times daily (3 min), whereas Giacaman and collaborators applied sucrose only three times per day. Biofilms were formatted in a bovine substrate (enamel and root dentin).

For validation without a dose-response effect, chlorhexidine (CHX) and fluoride were most frequently used (51.2%). Although not used at the same concentration, CHX generally showed a significant effect on the demineralization and microbiological outcomes in different models (dynamic and batch). Even with different methodological protocols to producing biofilms, the models were CHX-responsive and therefore closer to clinical reality. However, CHX was not responsive to the microbiological outcome when used as varnish at a 1% concentration. Thneibat et al. (2008) explained this phenomenon based on the experimental time (long) and the use of one application of the product. Fluoride was used in different application methods and also showed a response to outcomes evaluated mainly in demineralization. These findings can also be related to expected fluoride clinical use and therefore also represent a model that is more similar to clinical conditions.

Ideally, every proposed model should provide a (reproducible) dose-response pattern for both anti-caries and antimicrobial substances; this would be particularly important for studies lacking positive and negative controls (Sissons et al. 1991; Shu et al. 2000; McBain et al. 2003b; Fontana et al. 2004; Deng et al. 2005) because it would demonstrate the sensitivity of the model regardless of the effect of the investigated substance.

Of the 49 selected studies, only seven studies reported reproducibility. However, reproducibility to dose-response, with respect to CHX, was only shown by van

de Sande et al. (2011). Using MAM, Sissons et al. (1991, 1995) demonstrated reproducibility to plaque growth, PO4 levels, and pH readings. CDFF and other chamber/cell flow approaches also demonstrated reproducibility to an established protocol (Fontana et al. 2000; Deng et al. 2005; Seemann et al. 2006; Maske et al. 2016). For the remaining studies included in Part II, no reports were found to be reproducible.

However, in this review, reproducibility was only evaluated for the studies included in Part II, but the studies selected for Part I (without tests with standardized antimicrobial or anti-caries substances) also reported plaque growth reproducibility (Hodgson et al. 2001; McBain et al. 2003c; Deng and Ten Cate 2004; Rudney et al. 2012; Edlund et al. 2013), pH readings (Sissons et al. 1992), and mineral loss (Bradshaw et al. 1996; Fontana et al. 1996). These findings indicate that a biofilm model and its respective protocol, despite lacking a tested anti-caries pattern, is able to develop similar caries lesions and cariogenic biofilms in independent experiments under the same protocol of sucrose exposure and nutrient medium.

Evaluation methods and response variables

A wide variety of evaluation methods were used in the included studies. Some of these focused on the microbiological composition of the biofilm (microbiological counts, quantitative polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis (DDGE)), while others were designed to evaluate the chemical and physical biofilm structure (biofilm weight, insoluble and soluble extracellular polysaccharide analysis, confocal laser scanning microscopy, polarized light microscopy, Fourier transform infra-red spectroscopy, fluorescent microscopy, backscattered electron imaging-scanning electron microscopy, and chemical analysis of F, Ca, and P ions) and its acidogenic capacity (pH, organic acid production). Additionally, when artificial caries lesions are induced, response variables included mineral loss assessment (transverse microradiography, percentage of surface hardness change, scanning electron microscopy, and polarized light microscopy).

In a biofilm model, it is important to quantitatively express the prevalence of specific groups of microorganisms to better understand their relationship to the tested experimental conditions. To achieve this goal, most of the reported studies used classical bacterial identification methods based on the cultivation of strains on agar plates and the determination of CFU counts (Shu et al. 2000; Giertsen et al. 2011; Smullen et al. 2012; Schwendicke, Horb, et al. 2014; Vanni et al. 2015). Although this method has been widely used, the significance of the data is limited by the

total counts of bacteria (irrespective of the genus) or the counts of specific strains. This method seems to be less representative for microcosm biofilms since it may underestimate the counts of some important strains (Rudney et al. 2012). This lack of information impairs understanding of the ecological role of microorganisms and of the metabolic profile of biofilms during caries lesion development. In this context, only four of the reported studies in Part II (based on the microcosm inoculum) used molecular biology methods for the identification of the biofilm microbial content (McBain et al. 2003a, 2003b; Zaura et al. 2011; Cavalcanti et al. 2014).

New approaches, including metagenomic, metatranscriptomic, metaproteomic, and metabolome analysis of the oral biofilm, may improve understanding of the microbial community and the ecology of caries (Nyvad et al. 2013; Takahashi 2015); thus, future studies examining in vitro biofilm models should also focus on the ecological and metabolic evaluations of a biofilm.

Risk of bias assessment

This review found that most studies showed a high (16) or medium (28) risk of bias based on the method used for the risk of bias assessment, as described in Table 2. Dynamic models showed a higher risk of bias than the batch model. This finding is not related to the complexity of the models but to how the experiments were performed and reported. It is worth mentioning that studies should be performed with sufficient methodological detail to allow a risk of bias assessment.

A low bias risk was only found in the models reported by Cavalcanti et al. (2014), Fernández et al. (2015), Giacaman et al. (2015), Fernández et al. (2016), and Maske et al. (2016); however, these studies did not mention the sample size calculation. The absence of information in the evaluated studies may not imply the authors did not carry out those procedures, but it precludes verification by the reader. In this sense, there is a need for reporting guidelines for in vitro research to improve the quality and transparency of studies (Sarkis-Onofre et al. 2015).

Final considerations

The development of laboratory biofilm models is essential for the advancement of pre-clinical caries research. Several biofilm models have been proposed, and it is difficult to determine which is superior for a specific research question. This review was designed to summarize the current evidence regarding the more comprehensive biofilm models described in the literature. This summarized information may help researchers when choosing which parameters to adopt in

future studies. It is important to note, however, that most of these models are distinct from the complexity of the oral environment and only reproduce a small number of the possible events that may occur in vivo.

A general recommendation regarding the choice of model parameters for future research is to select models that are simpler for initial screening tests or for 'proof of principle' questions and more complex simulation conditions for final pre-clinical testing of the hypothesis before in situ studies or clinical trials.

Table 2. List of selected papers showing (or not) a dose-response effect, description of the studied conditions, and bias risk (Part II).

Biofilm models	Papers	Evaluation Methods	Development of caries lesion	Cariogenic biofilm	Dose-response	Anticariogenic / antimicrobial substance	pH / acid production	Demineralization/ remineralization	microbiologic	Reproducibility reported	**Sample size calculation/ Randomization of treatment allocation/ control group/ blinding/ sample standardization/ Sterilization**	Low- L Medium – M High -H
AMCC	(Mei, Chu, et al. 2013)	CFU, SEM, CLSM, FTIR	Y	Y	-	0.12% CHX	na	+	+	N	N / Y / Y / N / Y / Y	M
	(Mei, Li, et al. 2013)	CFU, SEM, CLSM, EDS, FTIR	Y	Y	-	38% SDF	na	+	+	N	N / N / Y / N / Y/ Y	M
CDFF	Pratten and Wilson (1999)	CFU, pH, Wet weight	N	Y		0.2% CHX	+	na	+	N	N / N / N / N / Y / Y	H
	(McBain et al., 2003b)	DGGE, CFU, MIC, MBC	N	Y	-	0.6 g ml^{-1} Triclosan	na	na	+	N	N / N / N / N / Y / Y	H
	(McBain et al., 2003a)	MIC, DGGE, CFU, MBC	N	Y	-	0.06% CHXM	na	na	+	N	N / Y / Y / N / Y / Y	M
	(Deng et al., 2004)	CFU, pH, Acid prod.	N	Y	-	0.2% CHX	+	na	+	N	N / N / N / N / Y / N	H
	(Zanin et al., 2005)	pH, CLSM	N	Y	-	HeNe laser or LED light	+	na	+	N	N / N / Y / N / Y / N	H
	(Deng et al., 2005)	TMR, Acid prod, CFU	Y	Y	-	135 ppm F, 135 ppm F+ 0.2% CHX	+	+	+	Y	N / N / N / N / Y / N	H
	(Cenci et al., 2009)	TMR	Y	Y	-	Fluoride released from GIC	na	+	na	N	N / Y / Y / N / Y / Y	M
	(Zaura et al., 2011)	TMR, qPCR protein and acid anions	Y	Y	-	0.12% CHX, SF4, SF5	+	+	+	N	N / N / Y / N / Y / N	H
MAM	(Sissons et al., 1991)	CFU, pH, EM, Chemical analysis (F, Ca, P)	N	Y	-	CPMU	+	+	+	Y	N / N / N / N / Y / Y	H
	(Sissons et al., 1995)	wet and dry weight, total protein content	N	Y	-	MHB 0.2%	na	na	+	Y	N / N / Y / N / Y / N	H
	(Cutress et al., 1995)	pH, Microrradiography	Y	Y	0, 0.5, 1, 2, 5 ppm F	F	+	+	na	N	N / Y / Y / N / Y / Y	M
	(Sissons et al., 1996)	pH, MIC, Wet weight	N	Y	10, 20, 30,40% ethanol	Ethanol	+	na	+	N	N / N / Y / N / Y / N	H
	(Shu et al., 2000)	TMR, pH, CFU	Y	Y	-	1 mmol l^{-1} F	+	+	-	N	N / N / N / N / Y / Y	H
	(Yip et al., 2007)	SEM-EDS, FTIR	Y	Y	-	GIC	na	+	na	N	N / Y / Y / N / Y / Y	M
	(Angker et al., 2011)	BSE-SEM, Inorganic analysis (Ca, F, P), pH	Y	Y	0, 100, 3000, 5000 ppm F	F, Casein 2%, CPMU	+	+	na	N	N / N / Y / N / Y / Y	M

Table 2. Continued

Biofilm models	Papers	Evaluation Methods	Development of caries lesion	Cariogenic biofilm	Dose-response	Anticariogenic / antimicrobial substance	pH / acid production	Demineralization/ remineralization	microbiologic	Reproducibility reported	**Sample size calculation/ Randomization of treatment allocation/ control group/ blinding/ sample standardization/ Sterilization**	Low- L Medium – M High -H
MSCBM	(Schwendicke, Doerfer, et al. 2014)	TMR, CFU, pH	Y	Y	-	*Lactobacillus rhamnosus* GG	+	+	-	N	N / Y / Y / N / Y / Y	M
	(Schwendicke, Horb, et al. 2014)	CFU, pH, TMR	Y	Y	-	viable or heat-inactivated *Bifidobacterium animalis*	+	+	+	N	N / Y / Y / N / Y / Y	M
FLOW CELL / CHAMBER	(Klimek et al., 1982)	[ppm F], [protein],	N	Y	-	200 ppm F	na	na	+	N	N / N / Y / N / Y / Y	M
	(Sorvari et al., 1994)	Microhardness and pH	Y	Y	-	0.2% CHX+ F varnish, 0.2% CHX, and F varnish	+	+	na	N	N / N / Y / N / Y / N	H
	(Zampatti et al., 1994)	SEM	N	Y	-	0.004% CHX in toothpaste	na	na	+	N	N / N / Y / N / N / Y	H
	(Fontana et al., 2000)	CFU, CLSM	Y	Y	-	CsAb and 59Ab	na	+	-	Y	N / Y / Y / N / Y / Y	M
	(Fontana et al., 2002)	pH, CLSM, CFU	Y	Y	-	Fluoride varnish (5% NaF)	-	+	-	N	N/ Y / Y / N / Y / Y	M
	(Fontana et al., 2004)	pH, CLSM, CFU	Y	Y	-	0.25 ppm F	-	+	+	N	N / Y / N / N / Y / Y	M
	(Seemann, Bizhang, et al. 2005)	CLSM	Y	Y	-	Sealants	na	+	na	N	N / Y / Y / N / Y / Y	M
	(Seemann, Klück, et al. 2005)	CLSM	Y	Y	-	Sealants	na	+	na	N	N / Y / Y / N / Y / Y	M
	(Seemann et al., 2006)	CLSM	Y	Y	-	10 ppm F, Glycan	na	+	na	Y	N / N / Y / N / Y / Y	M
	(Lynch and ten Cate 2006b)	TMR, %TVC, pH	Y	Y	0.10, 0.25 and 0.50% CaGP	CaGP	+	+	+	N	N / N / Y / N / Y / Y	M
	(Xie et al., 2008)	PLM, pH, FM	Y	Y	-	228 ppm F, *Galla ginensis* extract	+	+	+	N	N / N / Y / N / Y / Y	M
	(Thneibat et al., 2008)	CLSM, pH, CFU	Y	Y	-	1% CHX and copper varnish	+	+	-	N	N / Y / Y / N / Y / Y	M

Table 2. Continued

Biofilm models	Papers	Evaluation Methods	Development of caries lesion	Cariogenic biofilm	Dose-response	Anticariogenic / antimicrobial substance	pH / acid production	Demineralization/ remineralization	microbiologic	Reproducibility reported	**Sample size calculation/ Randomization of treatment allocation/ control group/ blinding/ sample standardization/ Sterilization**	Low- L Medium – M High -H
FLOW CELL / CHAMBER	(Lee et al., 2010)	TMR, bacterial viability	Y	Y	-	0.12% CHX, CCPN	na	+	+	N	N / Y / Y / N / Y / Y	M
	(Smullen et al., 2012)	CFU, pH, MIC	N	Y	-	Plants extracts*	+	na	+	N	N / N / Y / N / N / Y	H
	(Maske et al., 2016)	%SHC and CFU	Y	Y	0, 0.03, 0.06, and 0.12% CHX	CHX	na	+	+	Y	N / Y / Y / Y / Y/ Y	L
CHEMO-STATS	(Bradshaw and Marsh, 1994)	pH, CFU	N	Y	-	0.4% Xylitol, Sorbitol	+	na	+	N	N / N / Y / N / Y / N	H
	(li and Bowden, 1994)	pH, [F], CFU, SEM	N	Y	-	F	+	na	+	N	N / N / Y / N / Y / Y	M
	(Bradshaw et al., 2002)	CFU, pH	N	Y	-	10 ppm F	+	na	+	N	N / N / Y / N / Y / N	H
MICROPLATE	(Ccahuana-Vásquez and Cury, 2010)	%SHL, CFU, pH, dry weight, IEPS, SEPS	Y	Y	0.012,0.024, 0.12% CHX	CHX, 0.05% NaF	+	+	+	N	N / Y / Y / N / Y / Y	M
	(van de Sande et al., 2011)	% SHL, pH	Y	Y	0,03, 0.06, and 0.12% CHX	CHX	+	+	na	Y	N / N / Y / N / Y / Y	M
	(Giertsen et al., 2011)	CFU	N	Y	-	7.5% Xylitol	na	na	+	N	N / N / Y / N /Y / Y	M
	(Muñoz-Sandoval et al., 2012)	%SHL, CFU, pH, dry weight, IEPS, SEPS	Y	Y	-	Bovine milk	+	+	+	N	N / Y / Y / N / Y / Y	M
	(Giacaman et al., 2012)	%SHL, CFU, pH, dry weight, IEPS, SEPS	Y	Y	-	Milk- Fluoridated (5 ppm) and 0.05% NaF	+	+	-	N	N / N / Y / Y / Y/ Y	M
	(Ribeiro et al., 2012)	%SHL, CFU, pH, dry weight, IEPS, SEPS	Y	Y	1.0, 10.0, and 100.0 µg Fe ml^{-1}	Iron	-	+	-	N	N / Y /Y /N / Y / Y	M

Table 2. Continued

Biofilm models	Papers	Evaluation Methods	Development of caries lesion	Cariogenic biofilm	Dose-response	Anticariogenic / antimicrobial substance	pH / acid production	Demineralization/ remineralization	microbiologic	Reproducibility reported	**Sample size calculation/ Randomization of treatment allocation/ control group/ blinding/ sample standardization/ Sterilization**	Low- L Medium – M High -H
MICROPLATE	(Cavalcanti et al., 2014)	pH, %SHC, CLSM, qPCR, SEM	Y	Y	-	0.05% NaF	+	+	+	N	N / Y / Y / Y / Y/ Y	L
	(Vanni et al., 2015)	CFU	N	Y	-	Toothpaste or mouthwash with or without Propolis, or CHX	na	na	+	N	N / N / Y / N / Y/ Y	H
	(Kuper et al., 2015b)	TMR	Y	Y	-	Protect Bond adhesive	na	+	na	N	N / N / Y /N / Y / Y	M
	(Giacaman et al., 2015)	%SHL, CFU, pH, dry weight, IEPS, SEPS	Y	Y	0.1, 1 and 10 mM fatty acid	Oleic, Linoleic, Stearic acid	+	+	+	N	N / Y / Y / Y / Y/ Y	L
	(Fernandez et al., 2015)	%SHL, pH, CFU	Y	Y	-	Lactobacillus rhamnosus	+	+	+		N / Y / Y / Y / Y / Y	L
	(Fernandez et al., 2016)	%SHL, [µg F g^{-1} substrate], pH, [Ca, F]	Y	Y	0, 150, 450, or 1350 ppm F	F	+	+	na	N	N / Y / Y / Y / Y/ Y	L

Note: Acid Prod- Acid Production, **Ca-** calcium, **CaGP-** calcium glycerophosphate, **BSE-SEM-** Backscattered electron imaging-scanning electron microscopy, **CCPN-** cetylpyridinium chloride- containing nanoemulsion (25 vol.% soybean oil, 65 vol.% deionized water, 10 vol.%, Triton X-100, 1 wt.% cetylpyridinium chloride.), **CFU-** colony forming unit, **CLSM-** confocal laser scanning microscopy, **CHX-** chlorhexidine, **CHXM-** Chlorhexidine gluconate-containing mouthwash **CPMU-** calcium phosphate monofluorophosphate urea, **CsAb -**antibodies to *Streptococcus mutans* cell surface proteins, **DGGE-** denaturing gradient gel electrophoresis, **EDS-** energy dispersive spectroscopy**, EM-** Electron microscopy **F-** fluoride, **FM-** fluorescence microscope, **FTIR-** Fourier transform infrared spectroscopy, **GIC-** glass ionomeric cement, **IEPS-** insoluble extrapolysaccharide, **MHB-** Methyl paraben **MIC**-minimum inhibitory concentration, **MBC-** Minimum Bactericidal Concentration**, NaF-** Sodium Fluoride, **P-** phosphate, **PLM-** polarized light microscopy, **qPCR-** quantitative polymerase chain reaction, **SEM-** scanning electron microscopy, **SDF-** Silver diamine fluoride**, SF4 and SF5-** subfraction of shiitake extract, **SEPS-** soluble extrapolysaccharide, **SM-** *Streptococcus mutans,* **TMR-** Transverse microradiography, **%TVC-** percentage of the total viable count**, 59Ab-** cell surface 59-kDa protein (59Ab); **%SHL-**percentage surface hardness change**. +:** effect, **–:** no effect **na:** not applicable; **Y-** yes; **N**-no; * Extracts of R. officianalis L. and S. officianalis L (0.025%), unfermented cocoa (0.4%), red grape seed (0.05%), and green tea (0.2%).

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SECTION

1

Small gap size

CHAPTER

3

Minimal gap size and dentin wall lesion development next to resin composite in a microcosm biofilm model

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Abstract

This in vitro study investigated the development of dentin wall lesions next to resin composite containing very small gap sizes using an in vitro biofilm model, and evaluated whether a relevant threshold for the gap size could be established. Microcosm biofilms were grown for 14 days within small interfacial gaps between dentin-resin composite discs under intermittent cariogenic challenge. The factor under study was gap size: samples were either restored with composite resin without adhesive procedure (no intentional gap; no bonding [NB] group) or with intentional gaps of 30, 60, or 90 μm, or with complete adhesive procedure (no gap; bonding [B] group). Secondary caries wall lesion progression was measured in lesion depth (LD) and mineral loss (ML) using transversal wavelength independent microradiography at 3 locations: outer surface lesion and wall lesions at 200 and 500 μm distance from gap entrance. Results from linear regression analysis showed that the presence of an intentional gap (30, 60, and 90 μm) affected the secondary caries progression at 200 μm from the gap entrance ($p \leq 0.013$). The NB group did not show significant wall lesion development (ML and LD, $p \geq 0.529$). At 500 μm distance almost no wall caries development was observed. In conclusion, dentin wall lesions developed in minimal gap sizes, and the threshold for secondary wall lesion development was a gap of around 30 μm in this microcosm biofilm model.

3.1 Introduction

Secondary caries has been considered as one of the most common reasons for the failure and replacement of posterior restorations [Mjor et al., 2000; Demarco et al., 2012; van de Sande et al., 2013; Opdam et al., 2014]. It has been assumed that secondary caries lesions develop both as outer lesions on the tooth surface next to the restoration and as wall lesions within the tooth-restoration interface [Hals and Nernaes, 1971].

The current literature is still unable to determine the precise mechanism of secondary caries initiation and progression. However, one of the possible factors is the existence of a gap or defect at the cavity wall. For a long time, leakage between the tooth and restoration was considered as a risk factor for wall lesion initiation and progression [Gonzalez-Cabezas et al., 1995, 1999, 2002]. According to the microleakage theory, secondary caries could develop in any gap, but the wider the gap, the higher the risk that secondary caries could occur [Totiam et al., 2007].

Nevertheless, in clinical studies microleakage did not seem to be associated with secondary caries, and therefore the macroleakage theory started to be accepted. According to this theory, wall lesions will only develop when large gaps (225 μm or more) or cavities are present [Kidd et al., 1995; Thomas et al., 2007; Totiam et al., 2007].

Several recent studies showed that secondary caries lesions could develop in smaller gaps than originally thought [Kuper et al., 2014; Khvostenko et al., 2015; Turkistani et al., 2015]. Kuper et al. [2014] observed wall lesions in gaps with a width of around 70 μm, but a threshold for the minimal gap size in which wall lesions could develop was still not established. If such a threshold exists, it will most likely be found in the range of about 10–70 μm. Thus, this in vitro study aimed to evaluate dentin wall lesion development next to resin composite in very small gap sizes and to evaluate whether a relevant threshold for the gap size could be established. The hypothesis tested was that in minimal gaps secondary caries would develop and that the size of the gaps would influence the lesion depth (LD) and mineral loss (ML) of wall lesions.

3.2 Material and Methods

Ethical approval was granted by the local Ethics in Research Committee (School of Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil) under protocol No. 1.634.686/2016.

Sample Size Calculation

Sample size was calculated with data of wall lesion progression in gaps (0 and 100 µm) from a previously published study [Kuper et al., 2014], using the Power and Sample Size Program© (version 3.1.2, 2009). Power was set at 80% and type I error at 5%. This resulted in a sample size of 8 specimens per group. However, considering a 20% dropout rate due to the complex design of the sample preparation, a total of 10 samples per group was made.

Sample Preparation and Restorative Procedures

Sample preparation and restorative procedures are shown in Figure 1. Enamel-dentin discs were cut from the buccal surface of 25 freshly extracted sound bovine incisors using a water-cooled trephine drill. The enamel was removed by wet polishing with 80-grit SiC abrasive paper. To standardize the smear layer, samples were wet polished with 600-grit SiC paper for 1 min. The dentin discs were sectioned into 2 halves that were restored with resin composite (Filtek Z250 XT; 3M ESPE, St. Paul, MN, USA) according to the following conditions:

Complete Adhesive Procedure: Bonding (B). The bottom (pulpal) and interfacial sides of the dentin were etched with a 35% phosphoric acid gel (15 s, Ultra-Etch®; Ultradent Products Inc., South Jordan, UT, USA), a bonding agent was applied (AdpterTM Single Bond 2; 3M ESPE) and polymerized according to the manufacturer's instructions. Subsequently, the halves were inserted in an acrylic mold (2.5 mm thickness and 6 mm diameter), restored with composite resin and polymerized (20 s), resulting in a round specimen: half dentin and half composite at the surface, with a connecting composite layer of 1 mm at the bottom.

Without Adhesive Procedure: No Bonding (NB), 30, 60, and 90 µm. These samples were restored with composite in the same way as previously described, except that a central square window (3 mm^2) at the interface did not receive any adhesive procedure. At this square window either no matrix was used (NB group) or a metal

matrix with standard width (2.0 mm) and different thicknesses (30, 60, and 90 µm) was placed against the interfacial wall during composite placement, creating an intentional gap (Figure 1 b).

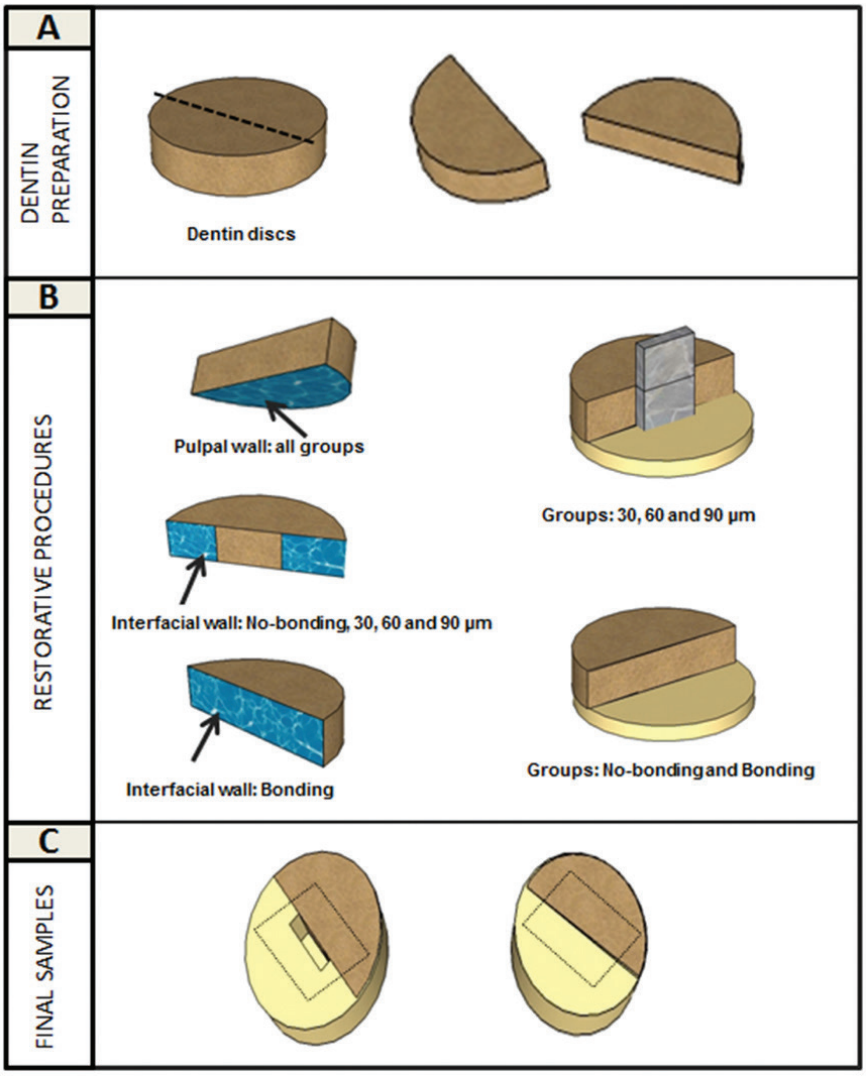

Figure 1. a Dentin preparation: dentin discs and halves prepared. **b** Restorative procedures. Left side: arrows indicate the areas which were etched with 35% phosphoric acid per group. Right side: placement of composite. Note the use of metal matrix for groups with gaps. **c** Final representation of the samples. Left side: samples with gaps. Right side: samples without intentional gaps. Note that the complete sample is covered by nail varnish except the square window around the interface between the dentin and resin composite.

After the restorative procedures, the final samples were covered with nail varnish except a central square window (2.0 × 2.0 mm) at the sample surface, incorporating the gap area (Figure 1 c). The gaps were measured with a WF10X lens (Future-Tech Corp., Tokyo, Japan) coupled to a microhardness tester (FM 700; Future-Tech Corp.) at 3 different locations: 200 µm from each edge and in the middle of the gap (1,000 µm from edge) as shown in Figure 2 a.

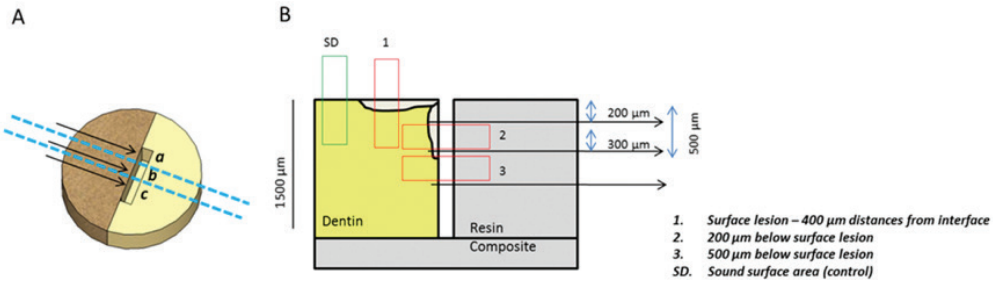

Figure 2. a Locations of gap measurement (a and c = 200 µm from each edge; b = 1,000 µm from edge – middle position). Dashed lines represent the section that was used for T-WIM (1 mm). **b** Section cut from the dentin-resin composite sample and the locations of T-WIM measurements: 1 (surface lesion: 400 µm from interface), 2 (200 µm from the surface / gap entrance), 3 (500 µm from the surface / gap entrance) and SD (sound surface area –control).

Sterilization of Samples

The samples were sterilized by gamma radiation in the Regional Center of Oncology/ Radiotherapy Service, Faculty of Medicine, Federal University of Pelotas, Pelotas, Brazil. All samples were kept moist in distilled water inside microtubes, placed at 2 cm from the radiation source, and subsequently submitted to sterilization with gamma radiation from a cobalt-60 source with particle energies of 1.25 MeV and 609.25 Gy/min, with a total dose of 4.08 KGy.

Microcosm Biofilm Model

Fresh whole saliva stimulated by paraffin film chewing (20 mL) was collected from a healthy volunteer (male, age 24 years) who had not been under antibiotic therapy for at least 6 months. The volunteer abstained from oral hygiene for 24 h and from food ingestion for 2 h prior to collection. A 0.4-mL volume of saliva was used to inoculate each dentin-restoration disc placed in a 24-microwell plate, and it remained at rest at 37°C. After 1 h, 1.8 mL of defined medium enriched with mucin (DMM) [Wong

and Sissions, 2001] containing 1% sucrose was added. The plates were incubated at 37°C under an anaerobic atmosphere (5–10% CO2, less than 1% O2). After 6 h, the samples were rinsed with 2 mL of sterile saline, inserted into a new plate containing DMM without sucrose, and incubated for 18 h under the same conditions. The biofilms were formed individually on the discs in each well for 14 days, during which the same daily routine of alternate exposure to DMM supplemented with sucrose (DMM+S) and without sucrose (DMM) was followed [van de Sande et al., 2011; Maske et al., 2015; Montagner et al., 2016]. The final pH of DMM+S and DMM medium was 4.6 ± 0.05 and 7.1 ± 0.07, respectively.

Transversal Wavelength Independent Microradiography

Secondary caries lesion development was evaluated using transversal wavelength independent microradiography (T-WIM). A 1-mm section was cut from each sample through the interface. Microradiographs were taken of the sections with an exposure time of 12 s and settings of 40 kV and 20 mA. A step wedge with the same absorption coefficient as the dentin (94% Al/6% Zn alloy) was used for proper quantitative measurement of LD (μm) and ML (μm × vol%). After X-ray exposure, the films were developed (10 min), fixed (7 min), rinsed (30 min), and dried. A digital image of each sample was captured using a light microscope (Leica Microsystems, Wetzlar, Germany) with a magnification of 10× and a CMOS camera (Canon EOS 50D, Tokyo, Japan). LD and ML for T-WIM were measured with a software program (T-WIM calculation program, version 5.25; J. de Vries, Groningen, The Netherlands) at 3 locations: surface lesion (400 μm distance from interface), and at 200 and 500 μm distance from the entrance of the gap.

As a control, LD and ML values were also determined from the sound surface (Figure 2 b).

Statistical Analysis

The correlation between ML and LD was determined with a Pearson correlation analysis. The effect of interface conditions on ML and LD were separately analyzed by linear regression models per location (surface lesion, and 200 and 500 μm distances from gap entrance) using the statistical software package SPSS (Statistical Package for Social Sciences, version 20.0, Chicago, IL, USA). The statistical significance was set at $p < 0.05$.

3.3 Results

The average gap size per group was as follows: NB = 13.6 ± 0.43 µm; 30 µm = 36.8 ± 1.33 µm; 60 µm = 64.2 ± 1.52 µm; and 90 µm = 94.0 ± 1.30 µm.

The average values for LD and ML considering each gap condition at the 3 locations are shown in Figure 3. LD and ML sound surface values were 49.1 ± 13.8 and 1,109.4 ± 246.0, respectively, and the range for sound values is indicated in Figure 3 by a rectangular box. Surface lesions showed highest ML and LD values. Dentin wall lesions were lower, with substantial lesion formation only in the intentional gaps at 200 µm from the surface.

The Pearson correlation analysis showed a high correlation between LD and ML data ($R > 0.906$).

Results from the linear regression analysis are shown in Table 1. The presence of intentional gap conditions significantly influenced the secondary caries lesion progression at 200 µm from the gap entrance ($p < 0.013$), whereas NB did not affect the lesion progression ($p \geq 0.529$). The effect on secondary caries progression was observed from the 30-µm gap size. At a distance of 500 µm from the gap entrance, only the gap size of 60 µm showed statistically significant lesion formation.

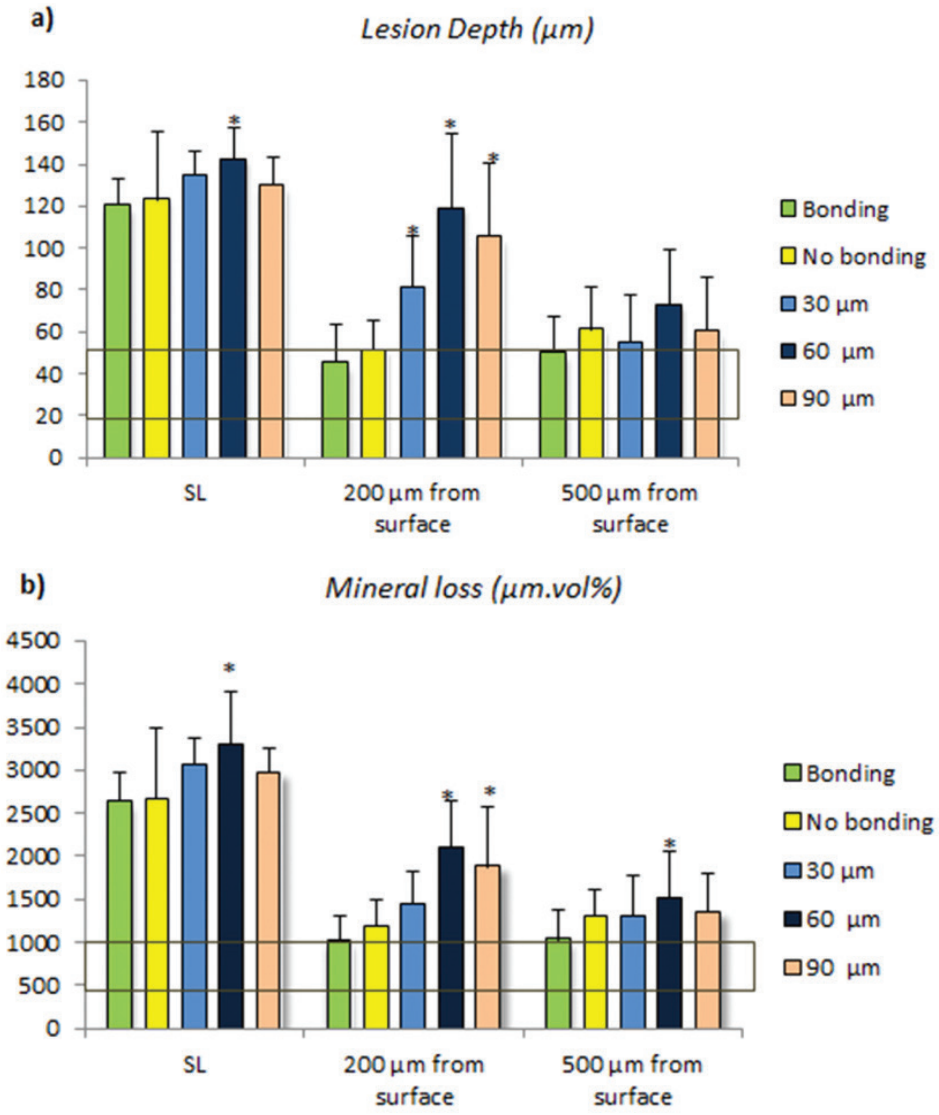

Figure 3. Bar charts showing the lesion depth (**a**) and mineral loss (**b**) values for each interface condition and area of analysis. Rectangular boxes represent the lesion depth and mineral loss sound surface values for reference: 49.1 ± 13.8 and 1,109.4 ± 246.0, respectively. Asterisks represent those interface conditions with a significant effect on secondary lesion development within each location evaluated (bonding vs. interface). SL, surface lesion.

Table 1. Linear regression of lesion depth and mineral values considering the gap condition in each area of analysis.

Area of analysis		Lesion Depth (µm)				Mineral Loss (µm.vol%)			
			p	95% CI of effect			*p*	95% CI of effect	
	Variable	Effect	*value*	Lower	Upper	Effect	*value*	Lower	Upper
Surface Lesion (SL)	**Intercept***	120.675	-	107.525	133.825	2638.50	-	2272.01	3004.99
	NB	2.263	.807	-16.334	20.859	24.00	.926	-494.29	542.29
	30 µm	13.925	.138	-4.671	32.521	429.89	.101	-88.42	948.17
	60 µm	21.625	.024	3.029	40.221	663.88	.013	145.58	1182.17
	90 µm	9.575	.279	-8.067	27.217	326.50	.187	-165.19	818.19
	Adj r2=.075					***Adj r2=***.121			
200µm from surface	**Intercept***	45.437	-	25.718	65.157	1026.63	-	675.78	1377.47
	NB	5.650	.684	-22.238	33.538	155.50	.529	-340.67	651.67
	30 µm	36.075	.013	8.187	63.963	425.38	.091	-70.80	921.55
	60 µm	73.250	.000	45.362	101.138	1075.63	.000	579.45	1571.80
	90 µm	60.253	.000	33.796	86.709	852.68	.001	381.97	1323.39
	Adj r2=.505					***Adj r2=***.376			
500µm from surface	**Intercept***	50.487	-	34.229	66.746	1038.38	-	729.10	1347.66
	NB	10.600	.356	-12.393	33.593	267.38	.223	-170.01	704.76
	30 µm	4.475	.696	-18.518	27.468	271.38	.217	-166.01	708.76
	60 µm	22.063	.060	-.930	45.055	470.75	.036	33.36	908.14
	90 µm	10.093	.355	-11.720	31.905	307.03	.142	-107.92	721.97
	Adj r2=.007					***Adj r2=***.022			

* Bonding group was used as reference group for analysis.

3.4 Discussion

This in vitro study showed that wall lesions can develop in dentin next to resin composite containing very small gaps and that the minimal gap size (threshold) for its development seems to be around 30 µm.

In this study, samples in the B group represent the best feasible interface condition where no or unavoidable small marginal defects are present and therefore the development of wall lesions was not expected. As interfacial gaps have been considered a crucial factor to secondary caries lesion formation [Totiam et al., 2007;

Diercke et al., 2009; Kuper et al., 2014], etching and bonding agents were omitted to induce a minimal gap in the NB group and enable wall lesion formation. Although a minimal gap was present in the NB group (13.6 ± 0.43 µm), secondary caries wall lesions were not observed there. As expected, samples in the B group also did not show any wall lesion development. LD and ML values presented in these groups were comparable to sound surface values (control) and are related to the T-WIM technique. In this method, an offset from zero and minimum unsharpness are unavoidable [Thomas et al., 2006]. The effect of gap size on secondary caries wall lesion formation was significant from 30 µm. Thus, the secondary caries progression seems to start from a gap size of between 13 and 30 µm. These results support the hypothesis that secondary caries can develop in the presence of very small gaps and that a threshold gap width may exist.

Recently, Khvostenko et al. [2015] showed that a viable biofilm could be formed inside small simulated interfaces varying in size from 15 to 30 µm. Our results are in agreement, with caries formation from a gap width of 30 µm.

The present findings do not support the clinical theory that large gaps (macroleakage) are needed for secondary wall lesion development [Mjor, 2005]. Two wall locations were analyzed in this study. The secondary wall lesions found in the first location (200 µm from gap entrance) showed clear lesion formation with intentional gap sizes while the deepest location (500 µm from gap entrance) showed hardly any lesion formation. This can be explained by the fact that access of bacteria deeper into the gap is more challenging and takes longer, especially when there is no loading involved. Cyclic loading helps bacterial penetration due to a hydraulic pumping effect [Khvostenko et al., 2015; Nedeljkovic et al., 2015]. If the gap closes and opens under the cyclic loading, it transports fresh media and bacterial cells into the gap, while possibly removing some of the saturated solution and bacterial waste from the gap [Khvostenko et al., 2015]. In addition, longer distances could decrease the rate of nutrients reaching the deeper parts of the gap as well as clearance of the demineralization products [Nassar and Gonzalez-Cabezas, 2011; Schwendicke et al., 2016]. Minerals dissolved from the tooth structure due to the acid attack would remain in the small gap, increasing the saturation level of the area, and therefore also limiting the progression of wall lesions [Totiam et al., 2007; Nassar and Gonzalez-Cabezas, 2011].

An increment of LD and ML was expected with the increase of gap size, but it was not observed in the present study. In a previous in vitro study, Kuper et al. [2015] found an association between gap size and wall lesion development using the same

biofilm model. However, in that study gap sizes varied roughly between 213 and 578 µm. In the present study we focused on finding a threshold and for that reason the gap sizes were very small and only ranged between 30 and 90 µm. It is much more unlikely to find an association over such a small range. The small range between gap sizes as well as the variability of biofilm formation on samples inherent to biological models could also explain the slightly higher secondary caries lesion formation at gap sizes of 60 µm than at those of 90 µm.

For this study a short-term microcosm biofilm model was used with a high cariogenic challenge regimen and saliva of only one person as inoculum. From previous studies we knew that this saliva was able to reproducibly induce caries lesions. However, we know that the disadvantage is that no interpersonal variation was simulated in this study. Considering these facts, it is interesting to point out that the secondary caries development shown here reflects a high cariogenic challenge and the internal capability to induce caries from that specific saliva donor. However, other studies have consistently shown that regardless of the type of inoculum, this biofilm model can produce similar caries-like lesions [Azevedo et al., 2011, 2014; Signori et al., 2016]. Further research should use in situ models to obtain intrapersonal variations on caries susceptibility. Another limitation of this study is that in this biofilm model, no fluoride was used in the artificial saliva (DMM). Fluoride delivered from the medium (representing fluoride from other sources inside the mouth) may have some preventive effect in the secondary caries development in these small gaps.

Within the limits of the present study, we conclude that the minimum gap size for secondary caries wall lesion development may be as small as 30 µm in this microcosm biofilm model.

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Disclosure Statement

The authors declare no conflicts of interest with respect to the authorship and/or publication of this article. Author Contributions M.S.C, T.T.M., N.K.K., and M.-C.D.J.N.M.H. conceived and designed the study. T.T.M. collected and analyzed the data. The paper was written by M.S.C., T.T.M., N.K.K., and M.-C.D.J.N.M.H.

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CHAPTER

4

A threshold gap size for in situ secondary caries lesion development

This chapter will be submitted in Journal of Dentistry as: Maske TT, Kuper NK, Hollanders A, Bronkhorst EM, Cenci MS, Huysmans, MCDNJM (2018). A threshold gap size for in situ secondary caries lesion development.

Abstract

Objectives: This in situ study aimed to investigate the influence of very small gaps in SC development and additionally to link the level of SC and a threshold gap size with the caries activity level of the volunteer.
Methods: For 21 days, 15 volunteers wore a modified occlusal splint loaded with dentin-composite samples restored according to different interface conditions: bonded (B = samples restored with complete adhesive procedure), no-bonded (NB = restored with composite resin without adhesive procedure), and 30, 60 and 90 µm (no adhesive procedure and with intentional gap). Eight times per day, the splint with samples was dipped in a 20% sucrose solution for 10 minutes. Before and after caries development, samples were imaged with T-WIM and lesion depth and mineral loss were calculated. Volunteers were grouped according to the average wall lesion depth and classified as high, mid and low caries activity levels.
Results: No wall lesion formation was observed in B and NB groups. In general, intentional gaps led to SC lesion depth progression independent of caries activity level of volunteers. No substantial wall lesions were found for two volunteers. A trend for deeper lesion in larger gaps was observed for the high activity group.
Conclusion: Very small gaps around or wider than 30 µm develop SC independent of the caries activity level of the patient and SC wall lesion progression seemed to be related to individual factors even in this standardized in situ model.

Significance: Independently of caries activity level of the patient, the threshold gap size for secondary caries wall lesion seems to be around 30 µm.

4.1 Introduction

Secondary caries is defined as a carious lesion developing adjacent to a restoration margin (Mjor, 2005). The presence of cariogenic biofilm in association with a discontinuity / gap at the restoration margin is considered a crucial factor for secondary caries lesion development (Carvalho and Manso, 2016; Ferracane, 2017).

Macroleakage is the most accepted theory to explain secondary caries development (Nedeljkovic et al., 2015). By this theory the width needed for biofilm development inside the gap should be large enough to harbour a cariogenic biofilm, sometimes estimated at larger than 225 µm (Kidd et al., 1995; Thomas et al., 2007). Recently, however, a group of in vitro and in situ studies have questioned this theory (Khvostenko et al., 2015; Kuper et al., 2014; Maske et al., 2017; Turkistani et al., 2015). These studies have shown that the width of gap required for secondary caries wall lesion development may well be smaller than this macroleakage theory proposes. Khvostenko et al. (2015) showed that gap sizes with ~15 µm were able to accumulate biofilm. In addition, Maske et al. (2017) showed that secondary wall lesions developed in vitro in very small gap sizes and suggested that a minimum gap size for lesion formation would be around 30 µm. Although these findings undermine the prevailing theory they still need complementary validations by clinical in situ studies.

In an in situ study focused on gap sizes and secondary caries development, it could be observed that although all subjects were exposed to a highly cariogenic protocol which should result in rapid caries progression, the level of wall lesion development varied greatly among the subjects. This suggested that gap width may be irrelevant where caries risk is low, but that when caries risk is high, a gap size of around 70 µm could suffice for secondary caries wall lesion formation (Kuper et al., 2014). This assumption could also imply that a minimum gap size (threshold) for secondary caries lesion development could be related to caries risk / activity caries level of the patient.

The present in situ study therefore aimed to investigate the influence of very small gaps between tooth and restoration in the secondary caries development and additionally to link the level of secondary caries and a threshold gap size with the caries activity level of the volunteer. The hypotheses raised in this study were that: i) secondary wall lesion would develop in very small gap sizes and ii) a high caries activity level would be related to secondary wall lesion development in the smallest gaps and a low caries activity related to lesion progression only in bigger gaps.

4.2 Material and Methods

Study participants

The protocol and design of this study were submitted to and approved by an Ethical Committee Board (CMO code NL 56622.091.16). All volunteers agreed and signed the written informed consent. Sixteen volunteers (five men and eleven women, aged 18–52 yr, mean age = 28.4 yr) with good general health were recruited within the Campus of Radboud University (Nijmegen, The Netherlands). Volunteers with active caries, periodontitis (DPSI > 2), ASA > 2, and wearing orthodontic or a removable prosthetic appliance in the mandibular jaw were excluded.

Sample preparation

Sound human molars were collected and ground flat using a 220-grit Sic paper on polishing wheel until complete enamel removal and dentin exposure of the coronal tooth surfaces (occlusal, proximal and smooth). A diamond saw was used to cut off the roots (Buehler diamond wafering blade nr.11-4244) and the remaining crowns were cut perpendicularly into two dentin bars with fixed width of 3.2 mm and varying lengths (Figure 1A-B). Subsequently the dentin bars were manually ground with 400-grit Sic paper to a height of 2.0 mm and gas-sterilized with ethylene oxide (Isotron Nederland B.V., Venlo, The Netherlands) (Kuper et al., 2014). Using a rectangular putty mould a long dentin-composite sample (15 mm) was created by attaching two dentin bars together on a thin layer of composite (0.5 mm) fixed with self-etching primer and bonding agent (SE Bond, Clearfil, Kuraray, Okayama, Japan) on the pulpal side. In each dentin-composite sample, five slots were made parallel to the dentin tubule with a 0.12 cylindrical bur with a depth of 1.9mm. Three slots were filled with resin composite (AP-X PLT, Clearfil, Kuraray, Okayama, Japan) without any adhesive procedures and a small gap was created by placing a metal matrix of 30, 60, or 90 μm thickness between the dentin and the composite. One slot was filled completely with composite, but no adhesive was used (no-bonded). The remaining fifth slot was restored with composite and adhesive procedure according to manufacturer's instructions (bonded, control group) (Figure 1C-E).

A modified occlusal splint for mandibular jaw (Figure 1F) with four metal slots (20 mm x 3.2 mm x 2.5 mm) containing dentin-composite samples was delivered to each volunteer. The samples described in this study were always located in the lower slots, but the left and right side were alternated per volunteer. Also the positions of different composite-dentin interfaces (B, NB, 30, 60 and 90 μm) were modified

between the volunteers (mesial to distal). The rotation of samples positions (more mesial or distal) and the side of the device (right or left) were manually generated using computer software (Microsoft Office Professional Plus 2010 Excel Program). The upper slots and their samples were used for one parallel study aiming to evaluate effect of a metalloproteinase inhibitor (2 % CHX solution) on secondary caries lesion development (Maske et al. 2018). The antimicrobial effect of that inhibitor was inactivated using a neutralizing solution (D/E Neutralizing Broth, Acumedia, Michigan, USA) to avoid residual effect on the present study.

Experimental protocol

For 21 days, volunteers wore the occlusal splints (24 h per day), removing them only for eating, drinking, sports or oral hygiene and keeping them in physiological salt solution during those periods. The splints were dipped in 20% sucrose-solution eight times per day for 10 min and with 1h intervals at least. The exact moments of sucrose exposure was recorded by each volunteer in a diary. After sucrose dipping, the splints were rinsed with tap water and immediately replaced in the mouth. All volunteers used fluoride dentifrice (1450 ppm; Colgate Caries Protection, Colgate-Palmolive-Company, The Netherlands) and once a day fluoride dentifrice slurry was applied on the samples for 2 min when they brushed their teeth. The slots containing the samples were not cleaned or brushed to promote biofilm formation and caries lesion development. Instructions were given both orally and in writing by a researcher involved in the study.

Transversal wavelength independent microradiography (T-WIM)

T-WIM pictures were carried out at baseline (T0) and after 21 days (T21). The settings for the microradiographs were 45 kV, 40mA and 8 s of x-ray exposure. A stepwedge (94% Al/6% Zn alloy) was used for quantitative measurement of LD and ML.

After x-ray exposure, films were developed (10 min), fixed (7 min), rinsed and dried. Digital images of each sample were recorded with a light microscope (Leica Microsystems, Germany) with a magnification of ×10 and a CMOS camera (Canon EOS 50D, Japan). The T-WIM pictures were edited using Adobe Photoshop CS6 (v. 13.0, Adobe Systems, San Jose, CA, USA) to distinguish between gaps and caries lesions. The contour of the interfaces on the baseline (T0) images was selected and copied to second T-WIM image (T21) to calibrate the pictures. Subsequently, the selected contour was coloured black to allow caries lesion development to be

identified and distinguished from gap width (Kuper et al. 2014). Wall lesions in the dentin around the gaps were measured using a software program developed by our laboratory at a fixed area 400 µm under the surface. Baseline measurements (T0) were subtracted from measurements taken from pictures after 21 days (T21) to estimate the true LD and ML values. The same software program was used to measure the actual gap sizes (30, 60 and 90 µm) from baseline T-WIM images as described elsewhere (Kuper et al. 2014).

Statistical analysis

Descriptive statistics were used to analyse mineral loss (ML) and lesion depth (LD) data for all interface conditions and volunteers. In order to evaluate a possible relationship between caries activity levels and threshold gap size for wall lesion development, the volunteers were grouped into 3 equal groups (n=5) according to the average wall lesion depth in intentional gap sizes (30, 60 and 90 µm) and classified as high, mid and low caries activity. The relationship among caries activity level, gap size and wall lesion depth was also analysed descriptively.

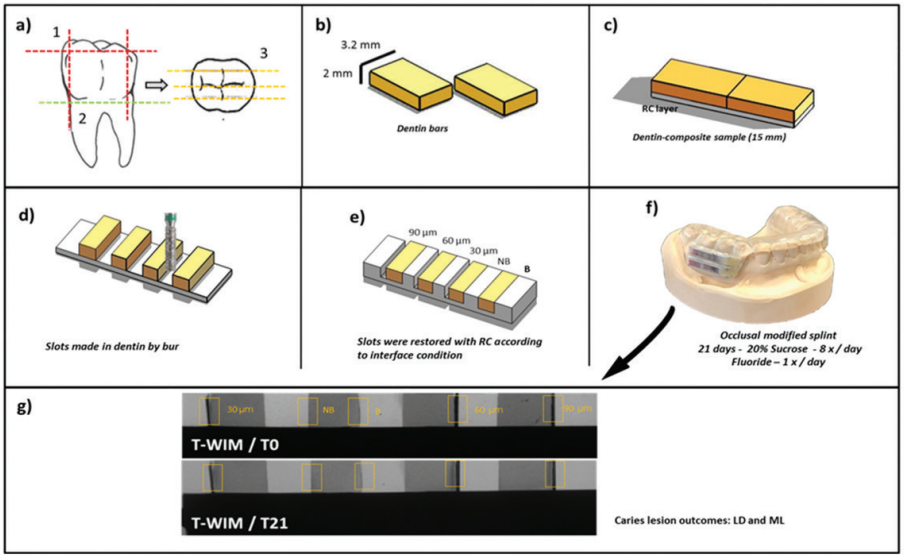

Figure 1. Preparation of samples and overview of study: A) 1- Complete enamel removal and dentin exposure. 2- Roots are cut off. 3- Dentin bar are made by cutting. B) Dentin bars (2 mm thickness and 3.2 mm width). C) Two dentin bars were attached together with a thin layer of composite to obtain a dentin-composite sample. D and E) Five slots are made in the dentin-composite sample and restored with resin composite according to interface condition (Bonded-B, No-bonded-NB, 30, 60 and 90µm). F) Splint was loaded with dentin-composite-samples. G) After experimental running, sample were analysed by TWIM.

4.3 Results

Fifteen volunteers completed the study in 21 days. One volunteer did not comply with the study protocol and was therefore excluded from data-analysis. In addition, 3 volunteers had one sample excluded from B or NB group because the enamel layer had not been completely removed during sample preparation.

Actual gap sizes are presented in Table 1. Average values of LD and ML considering all volunteers included in the study are also shown in this table. In general, dentin wall lesion development was seen when intentional gap sizes were present. No considerable wall lesion formation was observed in B and NB condition.

Table 1. Mineral loss (ML) and Lesion Depth (LD) values considering all volunteer *vs.* interface condition per lesion location evaluated.

Interface condition	Actual gap size (µm) Mean ± SD	Wall lesion Mean ± SD		Surface lesion Mean ± SD	
		LD (µm)	ML (µm.vol%)	LD (µm)	ML (µm.vol%)
B	-	3.7 ± 7.4	16.6 ± 451.3	69.1 ± 57.8	841.5 ± 938.2
NB	-	2.6 ± 7.8	196.5 ± 327.3	52.8 ± 56.8	721.3 ± 915.4
30 µm	45.5 ±10.5	57.2 ± 44.9	904.1 ± 658.0	45.7 ± 53.4	857.4 ± 1050.7
60 µm	72.2 ± 9.4	83.8 ±57.6	1289.3 ± 1113.5	40.6 ± 43.0	689.2 ± 725.5
90 µm	103.5 ±4.6	82.3 ± 69.4	1483.9 ±1399.5	43.7 ± 32.3	1014.8 ± 666.7

Note: B [n= 14] / NB [n=13] / 30, 60 and 90 µm [n= 15].

Figure 2 shows the relationship between secondary caries wall lesion depth and threshold gap sizes according to caries activity levels. In general, the presence of an intentional gap, even the smallest one, led to secondary caries wall lesion depth progression. A trend for deeper lesions in larger gaps could only be observed for the high caries activity group. In two volunteers there were no substantial wall lesions in any interface condition evaluated.

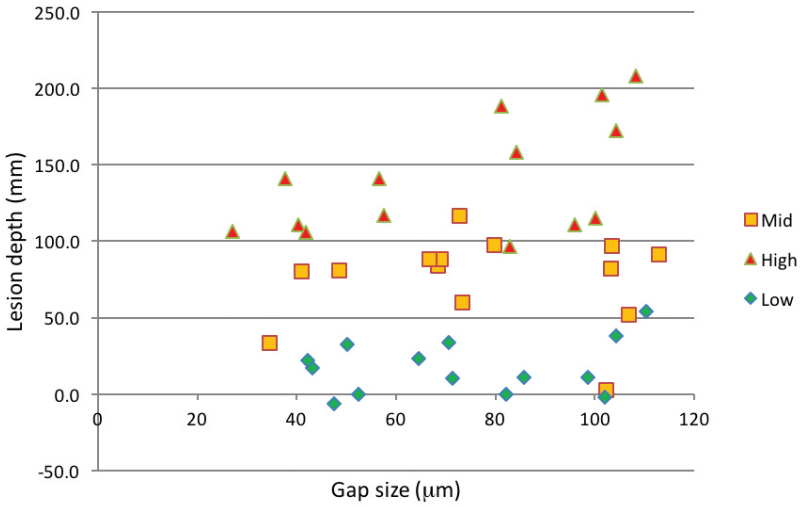

Figure 2. Secondary caries wall lesion depth results for all (measured) gap sizes regarding the caries activity level of volunteers.

4.4 Discussion

This is the first in situ study trying to find a relationship between threshold gap size for secondary caries wall lesion development and caries activity level of the patient. The present study showed that secondary caries wall lesions occurred independently of caries activity level when a gap size around or wider than 30 µm was present. The second hypothesis raised in this study was therefore not supported.

In this study, the best feasible interface condition, ideal bonding, was represented by samples from group B. To introduce the smallest possible gap size, etching and bonding agents were intentionally omitted in samples assigned to NB group. As expected, samples in the B group did not show any wall lesion development. Similarly, no considerable wall lesions were observed in the NB group. Although the method used to measure the gap width in this study was unable to determine the very small space between tooth and restoration in the NB condition, other studies have reported that this condition represents around 7 to 13 µm gaps (Cenci et al., 2008; Maske et al., 2017). Considering the existence of such minimal space in NB group, the ML and LD results confirm that a gap size, larger than 7-13 µm, is needed to secondary caries wall lesion development.

This study confirmed that a gap size of about 30 µm was sufficient for the development of secondary caries wall lesion and thus supports the hypothesis that wall lesions may develop in very small gaps and that a threshold gap width around 30 µm may exist (Maske et al. 2017). When data were individually split by volunteers it was noticed that, independently of caries activity level, wall lesions developed in very small spaces (> 27.4 µm). This implies that the presence of a minimal gap size is the critical factor to develop secondary caries wall lesion.

Obviously subjects classified as low, high or mid secondary caries activity level demonstrated different patterns of lesion depth progression. Figure 2 illustrates the differences in lesion development across subjects. If all subjects were exposed to the same cariogenic protocol (20% sucrose, 8 x per day), what may explain this large variation? It must be assumed that there are individual factors (either protective or risk factors) that modulate the lesion progression at least in gap sizes around and from 30 µm. It may be hypothesized that substantial gaps may be a risk factor for secondary caries development only in individuals with an elevated caries risk profile. This hypothesis could also explain why 2 volunteers did not show any wall lesion depth progression, and it is supported by observations by Kuper et al. 2014, where the pattern of lesion progression was also variable among subjects enrolled in that in situ study.

A trend for larger wall lesion with wider gaps was observed in high caries activity patients in this in situ study. It is worth to be mentioned it could be related to caries activity level criteria used in this study, but it could be also related to more biofilm accumulation in the space between tooth and restoration (Kuper et al., 2015; Totiam et al., 2007) and related to an individual capability to modulate the development of secondary caries lesion.

Although this study was limited by use of a simplified method to classify caries activity level of each volunteer, the above-mentioned findings acknowledge that dentists should clinically consider the patient's individual risk profile. The marginal defect seems to be a crucial factor for secondary lesion development (Diercke et al., 2009; Kuper et al., 2014; Totiam et al., 2007), but only focusing on gap size may be an oversimplification.

Within the limitations of the present study, we concluded that: i) secondary caries lesions develop in very small gaps, ii) the threshold gap size for secondary caries wall lesion seems to be around 30 µm independent of caries activity level of the patient and iii) secondary caries lesion progression seems to be related to individual factors even in a standardized in situ model.

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SECTION

2

MMP inhibitor

CHAPTER

5

Matrix metalloproteinase inhibitor and secondary caries wall lesion development in a microcosm biofilm model

This chapter was submitted in Caries Research as: Maske TT, Kuper NK, Cenci MS, Huysmans, MCDNJM (2018). Matrix metalloproteinase inhibitor and secondary caries wall lesion development in a microcosm biofilm model.

Abstract

This study investigated the role of MMP inhibitor (2% CHX) in secondary caries wall lesion development in different interface conditions with small (run 1) and wider gaps (run 2). Dentin discs were restored and pre-treated with or without chlorhexidine 2%. In run 1, interfaces were made with gaps of 30, 60 or 90 µm. Interfaces with composite placed directly onto the dentin were either bonded (Adpter Single Bond 2) or not bonded. In run 2, interfaces were made with gaps of 100 µm, with or without adhesive on the composite side (Clearfil SE Bond). Interfaces were either bonded or not bonded as in run 1. Microcosm biofilms were grown on dentin-composite samples (14 days). Caries lesion outcomes were analysed by transversal wavelength independent microradiography (T-WIM) at three locations: outer surface and interface wall (200 and 500µm distance from gap entrance). Linear regression analyses showed that pre-treatment with MMP inhibitor did not influence the wall lesion progression at either location ($p \geq 0.218$). Interfaces with intentional gaps showed positive and significant effect on the wall lesion progression at 200 µm distance from the gap entrance ($p \leq 0.005$). A small trend of increase in wall lesion development was observed at the 200 µm location when bonding was present on the composite side. In conclusion, the dentin pre-treatment with MMP inhibitor (CHX 2%) was not able to slow down the secondary caries wall lesion development in small and wide gaps in this biofilm model.

5.1 Introduction

Dentin is a collagen-based mineralized tissue consisting of inorganic apatite crystallites embedded in an extracellular matrix [Linde, 1989]. Approximately 10% of this matrix is composed by noncollagenous proteins that include proteoglycans, phospholipids, and enzymes. Among these enzymes, the matrix metalloproteinases (MMPs) have recently received more attention. MMPs are a family of Zn^{+2} - and Ca^{+2} -dependent endopeptidases and are capable of degrading collagenous dentin proteins [Visse and Nagase, 2003; Mazzoni et al., 2015].

MMPs are involved in dentin caries progression and collagen degradation in the adhesive hybrid layer under composite restorations [Tjaderhane et al., 1998; Tjaderhane et al., 2013; Mazzoni et al., 2015]. In a mineralized state (sound substrate), the MMPs remain inactive, but they can become activated by acids delivered from cariogenic bacteria and/or acidic materials used in restorative procedures such as phosphoric acid and acid monomers [Pashley et al., 2004]. Self-etching and etch-and-rinse adhesives remove mineral crystals around collagen fibrils (exposing the collagen) to provide space for bonding infiltration, but simultaneously trigger MMP activation [Tjaderhane et al., 2013; Mazzoni et al., 2015]. In dentin caries progression, the acidic environment created by cariogenic biofilm can have a similar result [Tjaderhane et al., 1998; Mazzoni et al., 2015; Takahashi and Nyvad, 2016]. MMPs activated by bacterial acid may initiate a partial degradation of the exposed tooth organic materials. Once the organic degradation starts, complete remineralisation may no longer be possible [Kuboki et al., 1977; Takahashi and Nyvad, 2016].

In dentin matrices, MMP activity can be reduced by endogenous and exogenous inhibitors. Endogenous inhibitors originate from different human cells, while exogenous inhibitors are synthesized as therapeutic agents. Most of these inhibitors chelate calcium or replace the zinc ions at the active site and/or interact with the MMP propeptide fragment, while others may prevent MMP access and inhibit activity by coating the substrate [Tjaderhane et al., 2013; Mazzoni et al., 2015].

One potent exogenous inhibitor is chlorhexidine (CHX) which reduces the activity of MMPs by chelating mechanism [Gendron et al., 1999]. Through this mechanism, ions such as Ca^{+2} and Zn^{+2} are sequestered and the catalytic activity of MMPs doesn't evolve [Sorsa et al., 2004]. CHX has been shown to preserve bond stability even at concentrations as low as 0.2% [Carrilho et al., 2007a; Komori et al., 2009; Breschi et al., 2010; Montagner et al., 2014]. In addition, in situ studies have also demonstrated

that gels or solutions delivering CHX are able to prevent dental demineralization caused by erosion [Magalhaes et al., 2009; Kato et al., 2010]. These observations suggest that approaches to avoid organic matrix degradation might also be useful in caries prevention [Tjaderhane et al., 2015; Takahashi and Nyvad, 2016].

As MMPs are involved in dentin caries progression and collagen degradation of the hybrid layer, it may be hypothesized that the use of CHX in the interface location of composite resin restorations will slow down the secondary caries process. Therefore, the aim of this study was to evaluate the role of MMP inhibitor (2% CHX) in the secondary caries wall lesion development and progression in different interface conditions, including both bonded and non-bonded conditions with several gap sizes.

5.2 Material and Methods

Ethical approval was granted by the local Ethics in Research Committee (School of Dentistry, Federal University of Pelotas- Pelotas, RS, Brazil) under protocol number 1.634.686.

Study design

This study, using a microcosm biofilm model, was carried out in two runs. Run 1 investigated the effect of MMP-inhibitor (chlorhexidine 2%, CHX) in secondary caries development at the tooth-composite interface in small gaps. Run 2 investigated the effect of MMP-inhibitor in wider gaps, while also considering the possible influence of an adhesive present at the interface. Dentin discs were restored with different composite-interfaces as shown in figure 1. The dentin of the interfaces was either pre-treated with chlorhexidine 2% (=CHX +) or not (=CHX-). In the small gap samples, interfaces were made with gaps of 30, 60 or 90 μm. Interfaces with composite placed directly onto the dentin, either bonded (Adpter Single Bond 2; 3M ESPE, St Paul, USA; AdSB) or not bonded. In the wide gap samples, interfaces were made with gaps of 100 μm, with or without the presence of an adhesive on the composite side (ClearFil SE Bond, Kuraray, Japan; CSE). Interfaces with composite placed directly to the dentin were also either bonded (CSE) or not bonded.

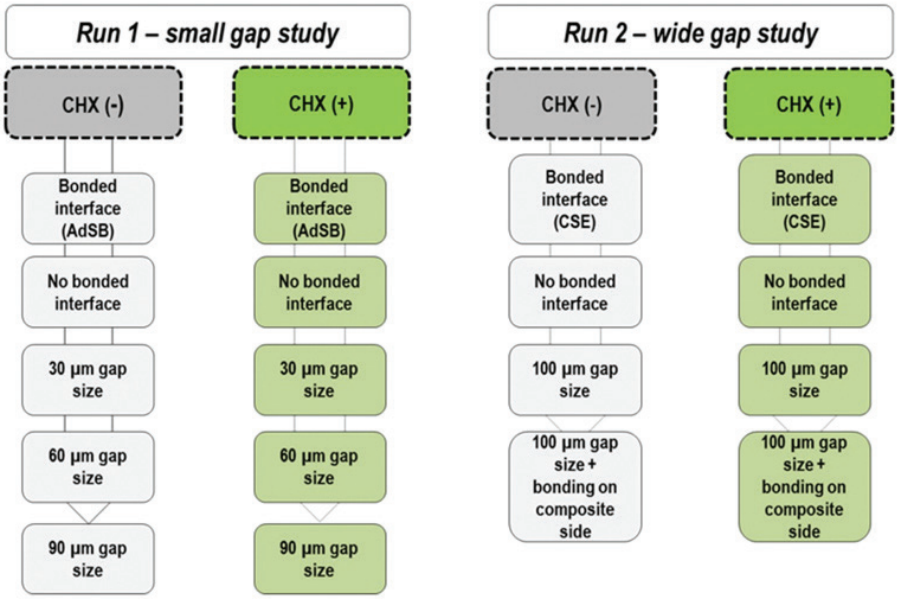

Figure 1. Study design / interface conditions per run. All groups n=10. Run 1 – small gap study using Adpter Single Bond (AdSB) for bonded interface. Run 2 – wide gap study using ClearFil SE Bond (CSE) for bonded interface and 100 µm+B group.

Sample preparation and restorative procedures

Sample preparation and restorative procedures are shown in figure 2. Enamel-dentin discs were cut from bovine incisors using a water-cooled trephine drill. The enamel was removed by wet polishing with 80-grit SiC abrasive paper. To standardize the smear layer of the dentin, samples were wet polished with 600-grit SiC paper for 1 min. The dentin discs were sectioned which resulted in two halves that were restored with resin composite (Filtek Z250 XT, 3M, ESPE, St. Paul, USA).

The bottom (pulpal) and interfacial side of the dentin hemi-sections assigned to small gap study ***(run 1)*** were etched with 35% phosphoric acid gel (15 s, Ultra-Etch®, Ultradent Products. Inc., South Jordan, USA) and rinsed with water. When samples were allocated to pre-treatment with CHX, chlorhexidine 2% was applied after the etching on the interfacial wall. After 60 s, the excess of CHX-solution was removed with paper towels. Samples assigned to the bonding group (B), received an adhesive procedure (AdSB). Subsequently all halves (n=10 per group) were inserted in an acrylic mold (2.5 mm thickness and 6 mm diameter), restored with composite resin and polymerized (20 s), resulting in a round specimen: half dentin and half

composite with an overall layer of 1 mm layer of composite. At the interfacial wall either no matrix was used (NB group) or a metal matrix with standard width (2.0 mm) and different thicknesses (30, 60, 90 μm) was placed against this wall during composite placement, creating an intentional gap.

Samples assigned to wide gaps study **(run 2)** were pre-treated with 2% CHX-solution (60 s) after acid primer application (CSE) on pulpal and interfacial side of dentin hemi-sections. B and NB groups were restored as in run 1, only now using CSE bonding agent. During restorative procedure, a metal matrix was placed against the dentin wall to create an intentional gap of 100 μm. Bonding agent was applied on the metal matrix to create the group with adhesive located at the side of resin composite (100 μm + B) .

After the restorative procedures, the samples were covered with nail varnish except a central square window (2.0 x 2.0 mm) on top of the sample, incorporating the gap area. The gaps of the samples were measured with a WF10X lens (Future-Tech Corp., Tokyo, Japan) coupled to a Microhardness tester (FM 700, Future-Tech Corp., Tokyo, Japan) at three different locations: 200 μm from each edge and in the middle of the gap (1000 μm from edge).

Inactivation of antimicrobial effect of 2% CHX

All samples treated with 2 % CHX solution were immersed in a neutralizing solution (D/E Neutralizing Broth, Acumedia, Michigan, USA) before sterilization and biofilm formation procedures, in order to avoid a direct effect of the antimicrobial properties from CHX solution on biofilm growth. Samples treated with CHX were placed in a 2ml of neutralizing solution and homogenized for 10s. This process was repeated twice and subsequently the samples were cleaned with 2 ml of saline solution by homogenization (10s). The group that was not treated with CHX was subjected to the same protocol using distilled water instead of neutralizing solution.

Sterilization of samples

All samples were sterilized by gamma radiation in the Regional Center of Oncology/ Radiotherapy Service (Faculty of Medicine, Pelotas-RS, Brazil). The samples were kept moist in distilled water and placed at 2 cm from the radiation source. They were sterilized with gamma radiation from a cobalt-60 source using particle energies of 1.25 MeV and submitted to 609.25 Gy/min. Total dose was 4.08 KGy.

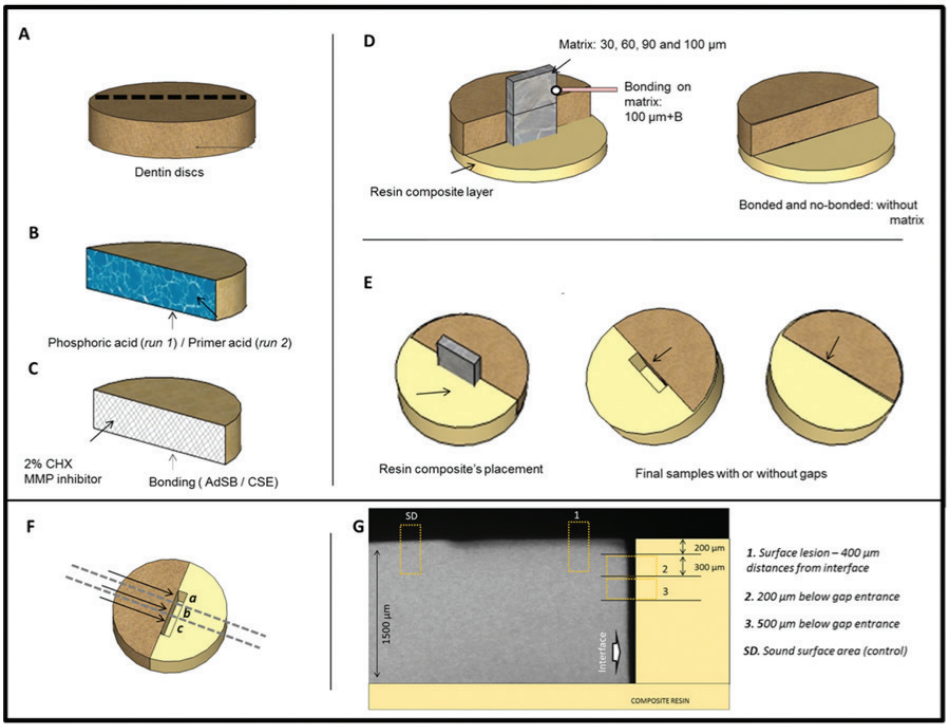

Figure 2. Sample preparation, gap measurement and T-WIM areas of analysis. A- Dentin discs were cut and two halves were obtained. B- Etching with phosphoric acid or primer acid was performed in the pulp and interfacial walls of all samples. C- MMP inhibitor was applied on the interfacial etched area in allocated groups. Bonding was applied on the pulpal wall of all samples and also on the interfacial wall of the samples assigned to bonding group. D- Halves with interfacial dentin wall pre-treated or not pre-treated with MMP inhibitor were placed in the resin composite layer. E- Final restorative procedures. Note the presence of metal matrix for 30, 60, 90, and 100 µm groups and no matrix for bonded and no-bonded groups. Bonding agent was applied on the metal matrix to create the group with adhesive on the composite side. F - Locations of gap measurement (a and c = 200 µm from each edge, and b = 1000 µm from edge – middle position). Dash lines represent the section that was used for T-WIM (1mm). G - Section cut from the dentin-resin composite sample and the locations of T-WIM measurements (1, 2, 3 and Sound Dentin: SD).

Microcosm biofilm model

Each run was carried out independently. Samples were submitted to cariogenic challenge using a microcosm biofilm model previously described elsewhere [Maske et al., 2015; Montagner et al., 2016]. Fresh whole saliva stimulated by paraffin film chewing was collected from a healthy volunteer (male, 24 years) who had not been

under antibiotic therapy for at least six months and abstained from oral hygiene for 24 h and from food ingestion for 2 h prior to collection. A 0.4 ml volume of saliva was inoculated onto each sample placed in a 24-microwell plate. The saliva remained at rest for 1 h at 37°C. Subsequently, 1.8 mL of defined medium enriched with mucin (DMM) [Wong and Sissions, 2001] containing 1% sucrose was added, and the plates were incubated at 37°C under an anaerobic atmosphere (5–10% CO2, less than 1% O2). After 6 h, the samples were rinsed with sterile saline (2 ml), inserted into a new plate containing DMM without sucrose, and incubated for more 18 h under the same conditions. The biofilms were formed individually on the resin-composite discs and in each well for 14 days. The same daily routine of alternate exposure to DMM supplemented with and without sucrose was followed (DMM+s and DMM, respectively).

Biofilm supernatant pH analysis

After the medium replacements, in both runs, biofilm supernatant pH readings were carried out in the DMM+S and DMM mediums used. The pH was individually and randomly recorded from each three wells per interface condition treated or not with MMP inhibitor (Quimis 50w - Quimis Aparelhos Científicos Ltda, Diadema, SP, Brazil; V621 electrode – Analion, Ribeirão Preto, SP, Brazil).

Microbiological control

Total microorganisms counts were performed considering the groups treated or not treated with 2% CHX to confirm that pre-treatment had not affected biofilm development. On the 14^{th} day of each independent run, the dentin-composite discs (n = 3 / interface condition) were removed from the wells and washed with sterile saline. The biofilm was then collected from the surface of each disc (including the gap surface) with a sterile microbrush and disposed in pre-weighed sterile tubes. The biofilms were dispersed by vortexing, sonicated for 30s (20 w), serially diluted (10^{-1}-10^{-7}) in saline solution, and inoculated in duplicate in the Brain Heart Infusion agar (Acumedia, Michigan, USA). The plates were incubated under anaerobic conditions for 96 h. The number of CFUs was determined by a blinded and trained researcher, and the results were expressed as CFUs/mg of biofilm (wet weight).

Transversal Wavelength Independent Microradiography (T-WIM)

Secondary caries lesion development was evaluated using T-WIM. A 1 mm section was cut from each sample through the interface. Microradiographs of the sections

were taken with an exposure time of 12s and settings of 40kV and 20 mA. A step wedge with the same absorption coefficient as the dentin (94% Al / 6% Zn alloy) was used for proper quantitative measurement of lesion depth (LD, µm) and mineral loss (ML, µm.vol%). After X-ray exposure, the films were developed (10 min), fixed (7 min), rinsed (30 min) and dried. A digital image of each sample was captured using a light microscope (Leica Microsystems, Wetzlar, Germany) with a magnification of 11 X and a CMOS camera (Canon EOS 50D, Tokyo, Japan). Lesion depth (LD) and mineral loss (ML) for T-WIM were measured with a software program (TWIM calculation program, version 5.25, J.de Vries, Groningen, NL) at three locations: surface lesion (400 µm distance from interface), and wall lesions at 200 and 500 µm distance from the entrance of the gap. As a control, LD and ML values were also determined from the sound surface at 400 µm distance from surface lesion (figure 2G).

Statistical Analysis

The effect of pre-treatment with MMP inhibitor and interface conditions on caries outcomes (mineral loss and lesion depth) at wall lesion locations were analyzed using linear regression models per run. Data from total microorganisms (log_{10}) counts and pH readings were analyzed using Two-Way ANOVA and Tukey *post hoc* test. All tests were conducted using the statistical software package SPSS (Statistical Package for Social Sciences, version 20.0, Chicago, IL, USA) and the statistical significance was set at $p < 0.05$.

5.3 Results

Run 1- Small gap size

Actual average gap sizes were: CHX+: NB = 13.4 µm (±0.39), 30 µm = 36.2 µm (±1.57), 60 µm = 63.3 µm (±0.74), 90 µm = 93.8 µm (±2.89); and CHX-: NB = 13.7 µm (±0.91), 30 µm = 33.3 µm (±1.39), 60 µm = 62.0 µm (±0.98), 90 µm = 93.1 µm (±0.85).

The average values for LD and ML for each treatment, gap condition and location are shown in Figure 3. The dash line in this figure is a reference for LD and ML average values measured in sound dentin. Surface lesions showed highest ML and LD values. Dentin wall lesions were lower, with substantial lesion formation only in the intentional gaps at 200 µm from the surface.

Results from the linear regression analyses are shown in Table 1. The pre-treatment with MMP inhibitor did not influence the wall lesion progression at

either location ($p \geq 0.218$). A gap of 60 and 90 µm significantly increased the lesion progression at 200 µm from the gap entrance ($p < 0.001$), but not at 500 µm distance.

Total microorganism and pH data are shown in Table 2. The final pH of DMM+S and DMM medium was 4.6 ± 0.04 and 7.1± 0.01, respectively. MMP inhibitor did not significantly affect pH readings and total microorganism counts ($p \geq 0.171$), nor did interface condition ($p \geq 0.152$). No significant interactions between the factors evaluated were observed.

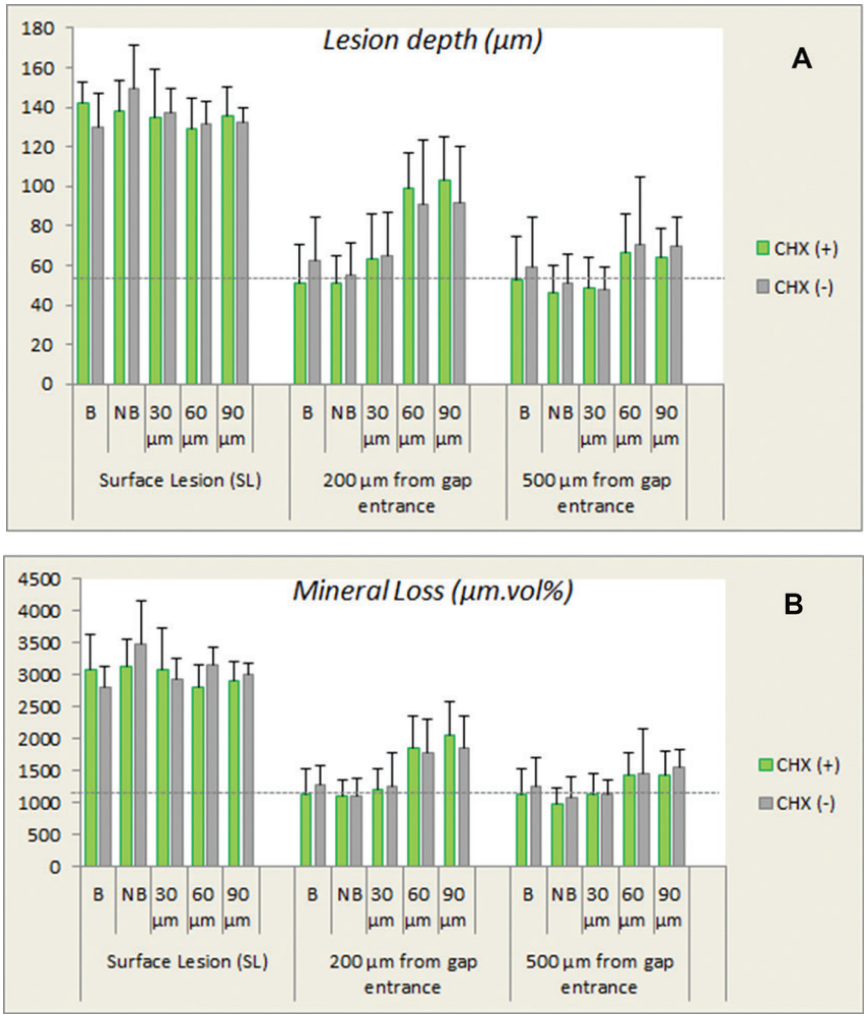

Figure 3. Run 1: bar charts of a) lesion depth (LD; µm) and b) mineral loss (ML; µm.vol%) for each interface condition, pre-treatment and location of analysis. All groups n=10. The average value for sound surfaces is presented in the charts as a dash line.

Table 1. Results from linear regression analyses for both experiments performed: run 1 (small gap size) and run 2 (wide gap size)

			Lesion Depth (µm)				Mineral Loss (µm.vol%)			
					95% CI of effect				95% CI of effect	
	Area of analysis	Variable	Effect	*p value*	Lower	Upper	Effect	*p value*	Lower	Upper
Run 1 Small gap size	200µm from SL	Intercept*	56.45	-	44.92	67.97	1198.37	-	990.395	1406.34
		MMP inhibitor	1.39	0.777	-8.33	11.10	38.30	0.663	-135.92	212.51
		NB	-3.79	.636	-19.64	12.07	-108.64	0.453	-394.97	177.69
		30µm	6.96	.364	-8.20	22.11	-48.16	0.723	-317.89	221.58
		60 µm	37.65	<0.001	22.73	52.57	599.02	<0.001	329.61	868.43
		90 µm	40.81	<0.001	25.69	55.93	735.50	<0.001	462.53	1008.48
	500µm from SL	Intercept*	58.53	-	47.88	69.17	1259.82	-	1043.45	1476.21
		MMP inhibitor	-5.60	0.218	-14.57	3.38	-109.98	0.234	-292.40	72.44
		NB	-7.31	0.324	-21.95	7.33	-1.12	0.267	-464.75	130.42
		30µm	-3.49	0.616	-17.28	10.30	18.48	0.896	-261.87	298.83
		60 µm	12.69	0.071	-1.09	26.46	235.43	0.098	-44.57	515.43
		90 µm	10.98	0.127	-3.19	25.14	281.70	0.055	-6.22	569.61
Run 2 Wide gap size	200µm from SL	Intercept*	45.42	-	32.59	58.24	1004.49	-	745.47	1263.51
		MMP inhibitor	5.05	0.376	-6.27	16.37	119.08	0.302	-109.47	347.62
		NB	5.29	0.496	-10.14	20.72	36.28	0.817	-275.30	347.85
		100 µm+B	49.59	<0.001	33.54	65.64	735.59	<0.001	411.43	1059.76
		100 µm	38.93	<0.001	22.30	55.56	484.48	0.005	148.67	820.29
	500µm from SL	Intercept*	50.67	-	37.07	64.27	1120.73	-	874.76	1366.70
		MMP inhibitor	2.75	0.634	-8.73	14.23	-6.63	0.949	-214.19	200.92
		NB	4.00	0.621	-12.07	20.08	-19.42	0.894	-310.16	271.33
		100 µm+B	12.89	0.129	-3.84	29.59	191.9	0.209	-110.26	494.21
		100 µm	7.92	0.348	-8.80	24.63	157.86	0.301	-144.38	460.08

*Bonding group was reference group for analysis. n=10 per group

Table 2. pH and total microorganisms average (SD) considering pre-treatment with MMP inhibitor or not, interface condition, and runs performed (small or wide gap study).

		CHX (+)			CHX (-)		
	Interface condition	**DMM**	**DMM+S**	**Total Microorganisms CFU (log)/mg**	**DMM**	**DMM+S**	**Total Microorganisms CFU (log)/mg**
small gap study ***run 1***	B	7.06 (0.08)	4.54 (0.05)	7.24 (0.73)	7.09 (0.05)	4.60 (0.05)	7.05 (0.29)
	NB	7.07 (0.06)	4.61 (0.05)	7.19 (0.23)	7.07 (0.04)	4.52 (0.18)	7.28 (0.17)
	30 µm	7.00 (0.09)	4.55 (0.02)	7.53 (0.24)	7.10 (0.01)	4.50 (0.04)	7.28 (0.24)
	60 µm	7.07 (0.04)	4.57 (0.04)	6.90 (0.21)	7.06 (0.11)	4.59 (0.03)	7.31 (0.21)
	90 µm	7.03 (0.09)	4.63 (0.01)	7.04 (0.44)	7.08 (0.07)	4.63 (0.08)	7.21 (0.33)
wide gap study ***run 2***	B	7.02 (0.06)	4.64 (0.04)	7.43 (0.14)	7.06 (0.01)	4.67 (0.05)	7.33 (0.30)
	NB	7.03 (0.09)	4.65 (0.02)	7.15 (0.18)	6.96 (0.02)	4.71 (0.04)	7.40 (0.43)
	100 µm	6.99 (0.04)	4.70 (0.03)	7.38 (0.35)	7.05 (0.03)	4.64 (0.03)	6.97 (0.10)
	100 µm +B	7.07 (0.08)	4.69 (0.03)	7.16 (0.44)	7.00 (0.02)	4.70 (0.02)	6.84 (0.31)

Note: DMM = pH readings performed in defined medium enriched with mucin without 1% sucrose after 18h incubation. DMM+S = pH readings performed in DMM enriched with 1% sucrose after 6h incubation. Interface condition and pre-treatment applied did not affect the pH readings and microbiological counts. No interaction factors were showed. (Two-Way Anova, $p < 0.05$).

Run 2- wide gap size

Actual average gap sizes were: CHX+: NB = 12.3 µm (±0.47), 100 µm = 104.2 µm (±0.53), 100 µm+B = 102.4 µm (±1.47); and CHX-: NB = 13.7 µm (±0.66), 100 µm = 102.8 µm (±0.85), and 100 µm+B = 107.0 µm (±1.81).

LD and ML results for all groups are shown in Figure 4, showing a similar trend to that seen in Figure 3.

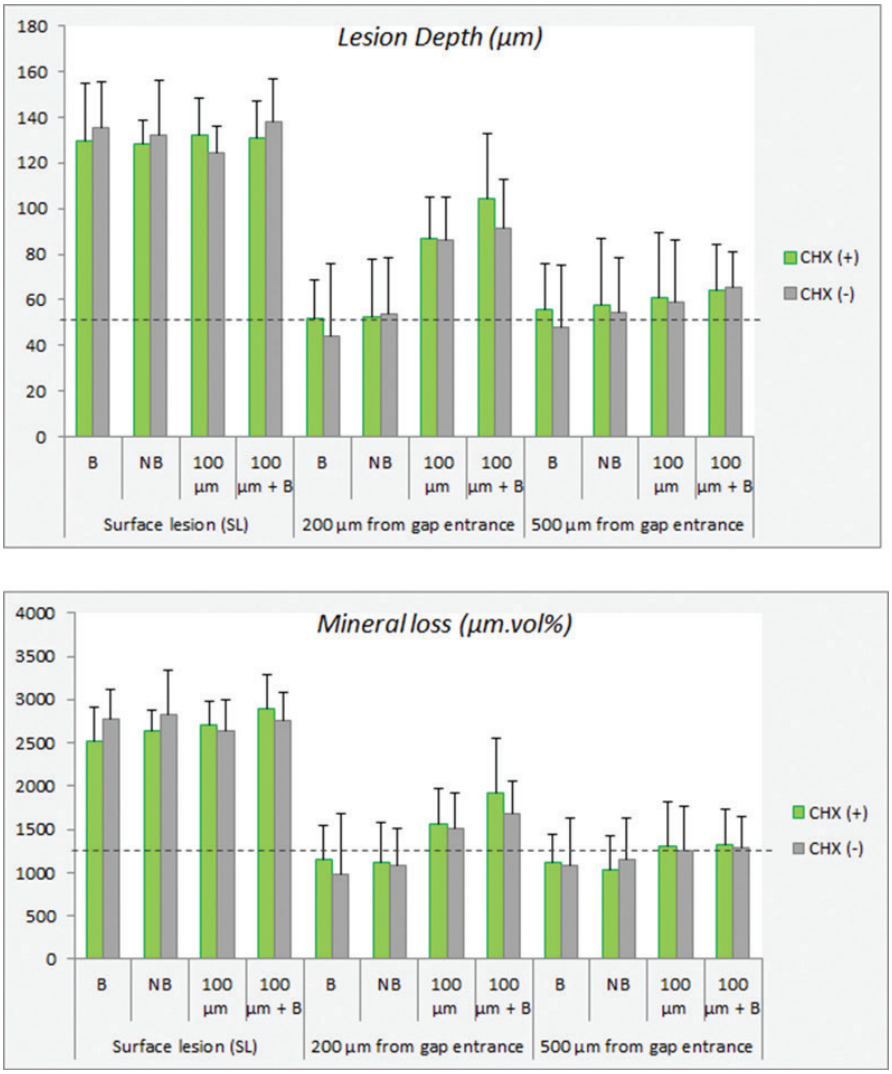

Figure 4. Run 2: bar charts of a) lesion depth (LD; µm) and b) mineral loss (ML; µm.vol%) considering each interface condition, pre-treatment performed, and areas of analysis. All groups n=10. The average value for sound surfaces is presented in the chart as a dash line.

The linear regression results are also shown in Table 1. The pre-treatment with MMP inhibitor did not affect the wall lesion progression at either location ($p \geq 0.302$). Interfaces with gaps showed positive and significant effect on the wall lesion progression at 200 µm distance from the gap entrance ($p \leq 0.005$). A small trend for the presence of bonding on the composite to increase wall lesion development at the 200 µm location could be observed, but overlapping 95% CIs show that this was not significant.

Pre-treatment and interface condition did not affect the pH readings (DMM and DMM+S) and the microbiological counts ($p > 0.05$). The factors evaluated did not show significant interactions (Table 2).

5.4 Discussion

In this study it was shown that the application of MMP inhibitor (2% CHX) did not reduce secondary caries development in dentin in small or wider gaps. As MMPs are thought to be involved in dentin caries progression and collagen degradation [Tjaderhane et al., 2013; Mazzoni et al., 2015] we expected that the application of MMPs inhibitor (2% CHX solution) on the dentin would reduce secondary caries lesion progression. However, this hypothesis could not be confirmed.

It has been demonstrated that CHX used as MMP inhibitor in demineralized dentin after etching and prior adhesive procedures improves the hybrid layer integrity and bond strength between tooth and composite resin [Carrilho et al., 2007b; Montagner et al., 2015], but these results have not been confirmed in recent clinical trials [Gostemeyer and Schwendicke, 2016; Favetti et al., 2017]. It is speculated that MMPs remain inhibited for as long as CHX remains bonded to collagenous matrix, but there is still no consensus about how long a CHX solution will retain its MMP inhibitory activity in a dentin substrate [Carrilho et al., 2007b]. The mechanism by which CHX binds to demineralized dentin seems to be by electrostatic process [Kim et al., 2010]. Thus, as a covalent and strong bonding doesn't occur between CHX and demineralized substrate, it is likely that the CHX leaches from the hybrid layer, reducing the inhibition of collagen degradation [Pashley et al., 2011].

When a good restoration is made (i.e., without marginal defects), it is likely that CHX-saturated demineralized matrix becomes sequestered by adhesive resin coating collagen fibrils, and covered by an overlying adhesive layer, which may produce prolonged retention of CHX and inhibition of MMPs [Carrilho et al., 2007b].

When marginal defects are present along the interface of the restoration, leaching of CHX could be facilitated, and its inhibiting effect compromised. In all simulated interfaces (small and wide gaps), except for the bonded groups, no adhesive barrier covering the dentin substrate was reproduced in this study. This lack of barrier may have led to an accelerated leaching of CHX from the demineralized dentin, which would explain the lack of caries-reducing effect. . However, CHX retention was not evaluated on dentin interface condition and future analyses are still needed to confirm the findings.

Unlike other studies evaluating the effect of MMPs inhibitors as treatment in dentin substrate [Nishitani et al., 2006; Magalhaes et al., 2009; Almahdy et al., 2015], all samples in this study were sterilized by gamma radiation (4.08 KGy) after restorative procedures. This sterilization method was used to avoid external contamination in the biofilm model used. This might raise some concerns about the presence of MMP activity in our samples once a high dose-radiation could inactivate the proteases in the substrate. However, gamma radiation is reported to be a suitable sterilization method of biological/ bioactive products or tissues, maintaining ~ 90% of proteases activity when doses between 0-10 KGy where applied. Bioactivity of collagen type I is preserved after sterilization with total dose up to 20 KGy [Furuta et al., 2002; Wiegand et al., 2009; Dyankova et al., 2014]. Also the integrity of MMP-2 and -9 and their pro-and active forms were maintained in bovine dentin substrate even after using a high dose of gamma radiation (25 KGy) [Kellen Gasque [2011]. In addition, we recently observed that gamma sterilized dentin substrate showed collagen type I degradation after cariogenic challenge produced by the same biofilm model used in the present study [Maske et al., 2015]. This means that MMP activity was still present even after radiation with 4.08 KGy. Considering these findings, the authors do not believe that the sterilization method used could interfere in MMPs activity and therefore influence the results found in the present study. However, we recognize that MMP activity on sound substrate was not evaluated in dentin substrate used and more studies are still needed to confirm the findings presented here.

The results of this study showed that secondary wall lesions can develop in gaps larger than 30 µm (34. 6 ±4.0µm) and as small as 60 µm (62.6 ±0.84). Although LD and ML values were measured for gaps lower than 30 µm (NB= 13.58±0.75 µm), it is important to point out that these are not to be interpreted as secondary caries wall lesion, as they are similar to values measured in sound surfaces. This means that the gap threshold for wall lesion development in the current biofilm model

seems to be around 30 µm. In a recent study with gaps not pre-treated with CHX the results were essentially the same [Maske et al., 2017].

The secondary caries wall lesion progression showed a slight trend to increment when bonding material was present on the resin composite at first wall location evaluated (Run 2). The presence of bonding material may act as a retention factor for biofilm growth and this could explain the increase of secondary caries progression in this condition. Results from a recent in situ study performed by our group support the present finding [Montagner et al., 2015].

This was the first study trying to show the role of MMP inhibitor on secondary lesion development and limitations need to be pointed out. 1) Sound instead of caries-affected dentin substrate was used and maybe the effect of CHX would have been clearer when some MMPs had already been activated by the dental caries process. 2) Bonded and non-bonded conditions with several gap sizes were simulated; however other conditions possibly including aged interfaces should be tested. 3) MMPs may also be present in the oral environment from other origins such as gingival crevicular fluid and from salivary glands [Chaussain-Miller et al., 2006] and these could have a role in secondary caries development. The present model could not include these MMP sources, and in situ and/or clinical studies may be needed to assess their role.

5.5 Conclusion

The dentin pre-treatment with MMP inhibitor (CHX 2%) was not able to slow down the secondary caries wall lesion development in small and wide gaps in this biofilm model.

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Authors contributions

Conceived and designed the study: M.S.C, T.T.M, N.K.K, and M.C.D.J.N.M.H. Collected and analysed the data: T.T.M. Wrote the paper: M.S.C, T.T.M, N.K.K, and M.C.D.J.N.M.H.

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CHAPTER

6

Secondary caries development and the role of a matrix metalloproteinase inhibitor: a clinical in situ study

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Abstract

Objectives: This in situ study aimed to investigate whether the dentin treatment with MMPs inhibitor (CHX 2%) could influence the development of secondary caries wall lesions in different dentin-composite interfaces.
Material and Methods: For 21 days, 15 volunteers wore a modified-occlusal splint loaded with dentin-composite samples treated or not with CHX and restored according 4 different interface conditions: Bonding (B = samples restored with complete adhesive procedure), no bonding (NB = restored with composite resin without adhesive procedure), 100 µm (no adhesive procedure and with intentional gap) and 100 µm + B (adhesive material on composite side and intentional gap). Eight times per day, the splint with samples was dipped in a 20% sucrose solution for 10 minutes. Before and after caries development, samples were imaged with T-WIM and lesion depth (LD) and mineral loss (ML) were calculated.
Results: Linear mixed effect analysis showed that dentin treatment with CHX did not significantly affect the caries lesion progression (LD and ML; $p \geq 0.261$). Dentin wall lesions were observed in the 100 µm and 100 µm+B groups independently of MMP inhibitor treatment.
Conclusion: The treatment of dentin with MMP inhibitor was not able to slow down the secondary caries wall lesion development in this in situ study.

Significance: The dentin treatment with 2% CHX did not prevent secondary caries wall lesion initiation.

6.1 Introduction

Dental work in general practice consists of a significant proportion of placing and replacing restorations. Secondary caries has been shown to be the most common reason for posterior restoration failure (Demarco et al., 2012; Mjor et al., 2000; Opdam et al., 2014).

Over time, dynamics in the oral cavity, e.g. masticatory forces, enzymatic activity of dentin (proteinases), and biofilm activity may negatively affect the quality of the restoration interface leading to marginal gaps or defects. In the presence of cariogenic plaque and fermentation products this can result in secondary caries development (Carvalho and Manso, 2016). Therefore, the good sealing between dentin and restorative material is the main focus to prevent secondary caries and to prolong the lifetime of composite restorations (Nedeljkovic et al., 2015).

Several studies have shown that enzymatic activity in dentin contributes significantly to adhesive interface degradation (Carvalho and Manso, 2016; Tjaderhane et al., 2013a; Tjaderhane et al., 2013b). This phenomenon can be attributed to host-derived enzymes such as Matrix Metalloproteinases (MMPs) which are Zn+2 - and Ca+2 -dependent endopeptidases and are considered to be mainly responsible for degrading collagenous dentin proteins(Mazzoni et al., 2015; Visse and Nagase, 2003) . The MMPs are also claimed to be involved during progression of dentin caries lesions, where they are responsible for breakdown of the collagenous organic matrix of dentin after demineralization occasioned by acid from bacteria metabolism (Mazzoni et al., 2015; Takahashi and Nyvad, 2016).

The activity of MMPs on dentin substrate can be retarded by use of inhibitors. Several studies have demonstrated increase of bonding strength and reduced interfacial degradation over time when MMP inhibitors are used during adhesive procedure (e.g., chlorhexidine – CHX, galardin, hesperidin, etc.) (Breschi et al., 2010; Montagner et al., 2014). Recently, exogenous MMPs inhibitors were reported to reduce the degradation of human dentin matrix (acid-demineralized dentin) in situ (van Strijp et al., 2015) and retard the caries process in rats (Sulkala et al., 2001). Similarly, other in situ studies have been showing that gels or solutions delivering MMPs inhibitions including CHX are able to prevent dental demineralization caused by erosion (Kato et al., 2010; Magalhaes et al., 2009). These observations suggest that approaches against organic matrix degradation might also be useful in caries prevention (Takahashi and Nyvad, 2016; Tjaderhane et al., 2015).

A number of studies have investigated the effect of CHX as MMP inhibitor on bonding stability when there is a good marginal seal (Breschi et al., 2009; Carrilho et al., 2007a; Komori et al., 2009). To the authors' knowledge there are no previous studies evaluating the effect of this MMP inhibitor on secondary caries development at interfaces with a compromised marginal seal due to defects at the interface (e.g. gaps). Therefore, the aim of this in situ study was to investigate whether the dentin treatment with MMP inhibitor (CHX 2%) could influence the development of secondary caries wall lesions in different interfaces, including bonded and non-bonded conditions and interfacial gaps. The hypothesis of this study was that MMP inhibitor would reduce secondary caries wall lesion progression.

6.2 Material and Methods

Study design

This was a mono-centre in situ study with a split mouth design. The protocol and design of this study were submitted and approved by an Ethical Committee Board (CMO code NL 56622.091.16). Independent variables were dentin treatment applied or not (2% CHX, MMP inhibitor) and interface conditions whereas the outcome variables were mineral loss (ML) and lesion depth (LD).

Sample size calculation

Since a split mouth design would be used, the equation for sample size calculation was applied: $n= f(\alpha,\beta)*\sigma^2/(\mu1-\mu2)^2$ (Pandis, 2012). Using a power of 90%, significance level of 5% and considering the outcomes from a previously published study (Kuper et al., 2014), the following parameters were used: the average between the SD from no gap and smallest gap size (σ =26.5); average of lesion progression in dentin samples restored with composite and with wall lesion development ($\mu1$= 48.5 μm); and difference on lesion progression lower than 50%, which would not be clinically meaningful ($\mu2$= 24.3 μm). The sample size needed was 13 volunteers. Considering a drop-out rate of 20%, the final sample required was 16 volunteers.

Volunteers

Sixteen volunteers with good general health (5 men and 11 women, mean age = 28.4 years) were recruited within the Campus of Radboud University (Nijmegen, The Netherlands). All the volunteers agreed and signed the written informed consent.

Exclusion criteria were active caries, periodontitis (DPSI > 2), ASA > 2, and the wearing of orthodontic or a removable prosthetic appliance in the mandibular jaw.

Sample preparation

Thirty-two sound human molars were collected and ground flat with 220-grit Sic paper until complete enamel removal and dentin exposure. The roots were cut off with a diamond blade (Buehler diamond wafering blade nr.11-4244) and the remaining crowns were perpendicularly cut in 64 dentin bars with fixed width of 3.2 mm and various lengths. Subsequently the dentin bars were manually ground with 400-grit Sic paper to a height of 2.0 mm (figure 1A-B) and sterilized with ethylene oxide (Isotron Nederland B.V., Venlo, The Netherlands) (Kuper et al., 2014). One dentin-composite sample was created by two dentin bars that were attached to each other with a thin layer of composite (0.5mm) fixed with self-etching primer and bonding agent on the pulpal side (ClearFil SE Bond, Kuraray, Okayama, Japan; CSE) (figure 1C). In each dentin-composite sample, four slots were made parallel to the dentin tubule with a 0.12 cylindrical bur with a depth of 1.9mm.Self-etching primer was applied on the dentin wall of each produced slot. Subsequently, 2% CHX solution was applied with a disposable syringe for 60 s on dentin samples assigned to pre-treatment (figure 1D-F).

Two slots were filled with resin composite (AP-X PLT, color A2, Clearfil, Kuraray, Okayama, Japan) and a gap was created by placing a matrix of 100 μm of thickness between the dentin and the composite. One of these slots received a layer of bonding material on side of metal matrix creating an interface where bonding was located on resin composite side of the gap (100 μm+B). From the remaining two slots, one was filled completely with composite, but no adhesive was used (no bonding; NB) and the other slot was restored with composite and adhesive procedure (Bonding; B - control group; CSE) (figure 1G). Resin composite and bonding agents were activated according to manufactures' instructions using a Bluephase® 20i light curing (Ivoclar Vivadent Ltda). Those dentin-composite samples treated with MMP inhibitor were immersed in 2 ml of neutralizing solution (2 x; D/E Neutralizing Broth, Acumedia, Michigan, USA) for 10s and followed by immersion in distilled water (10 s) to inactivate the antibacterial effect of MMP inhibition solution used and therefore to avoid crossed effects in the study. The same protocol was used to dentin-composite samples not treated with MMP inhibitor, but distilled water was used instead of neutralizing solution.

Each volunteer received a modified-occlusal splint for mandibular jaw (figure 1H) with buccal flanges holding four embedded metal slots of 20 mm x 3.2 mm x 2.5 mm. Only the two upper slots were used for this study. Thirty-two dentin-composite samples were placed at left or right side alternately per volunteer considering the treatment applied (n=16 / treatment). Positions of different composite-dentin interface conditions (B, NB, 100 µm and 100 µm+B) were changed per volunteer (mesial to distal). The sequence was manually generated using computer software (Excel Program).

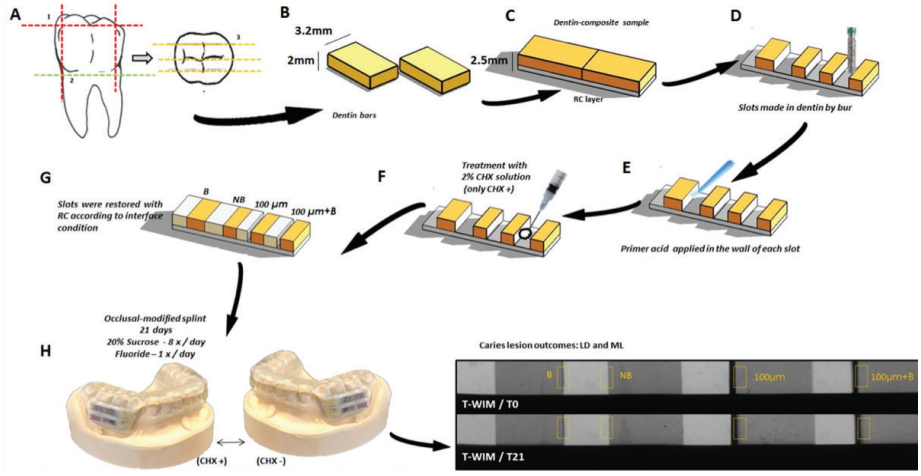

Figure 1. Sample preparation and experimental design: A) 1- Complete enamel removal and dentin exposure. 2- Roots were cut off. 3- Dentin bars were made by cutting. B) Dentin bars (2mm thickness and 3.2 mm width). C) Dentin-resin composite bars: two dentin bars were attached to each other by a resin composite (RC) layer. D) Four slots were made in the dentin-composite bars. E) Primer acid was applied at all dentin walls. F) CHX solution was applied at the dentin wall of samples assigned to pre-treatment. G) Each slot was restored with resin composite according to interface condition. Those samples treated with CHX were immersed in neutralizing solution. H) Modified-occlusal splint loaded with dentin-composite samples: bonding (B), no bonding (NB), 100 µm+B, and 100µm. Samples were placed at left and right side alternately per volunteer considering the treatment applied. T-WIM images were made before and after experimental run (21 days) and true lesion depth (LD) and mineral loss (ML) were calculated.

Experimental protocol

The occlusal splints were worn 24 h per day for 21 days (3 weeks), and were only removed during eating, drinking or oral hygiene. During these periods the device remained in physiological salt solution. Volunteers were instructed to dip the splint

in 20% sucrose solution eight times per day (10 min). The intervals between sucrose dippings were at least 1h. They received a diary to record the exact moments of sucrose exposure. After the dipping in sucrose, the splint was rinsed with tap water and replaced in the mouth. All volunteers used fluoride toothpaste (1450 ppm; Colgate Caries Protection, Colgate-Palmolive-Company, The Netherlands) and were asked to apply the fluoride toothpaste slurry on the samples once a day (2 min) when they brushed their teeth. The volunteers were instructed not to clean or brush the samples. Instructions were given both orally and in writing by a researcher involved in the study.

Transversal wavelength independent microradiography (T-WIM)

T-WIM images were made at baseline (T0) and after 21 days (T21). The microradiographs were taken using 45 kV, 40mA and 8s of x-ray exposure. A step wedge with the same absorption coefficient as tooth material (94% Al/6% Zn alloy) was used for quantitative measurement of LD and ML. After x-ray exposure, films were developed (10 min), fixed (7 min), rinsed and dried. Digital images of each sample were recorded with a light microscope (Leica Microsystems, Germany) with a magnification of ×10 and a CMOS camera (Canon EOS 50D, Japan). The T-WIM images were edited using the method of Kuper et al. (2014). From each sample the wall lesions in the dentin facing the gaps were measured using a software program developed in our laboratory at a fixed area 400 µm under the surface. Baseline measurements (T0) were subtracted from measurements taken after 21 days (T21), in order to estimate true LD and ML. The subtracted values were used in the statistical analysis. Actual gap sizes were measured on gaps from baseline T-WIM images using the same software program as described elsewhere (Kuper et al., 2014).

Statistical analysis

The effect of MMP inhibitor on LD and ML was analysed using linear mixed-effects models. Absolute differences between averages of LD and ML considering the effect of MMP inhibitor were entered into the model as fixed effect. More complex mixed-effects models were tested to verify the effect of added factors such as location of the gap (more distal or mesial) and interface conditions. The created models were compared among them by ANOVA. As there was not an improvement in the models by addition of factors ($p > 0.05$), a simple mixed-effect model was used. All tests were conducted using R statistical program with the significant level set as 5%.

6.3 Results

Fifteen volunteers completed the study successfully in 21 days. One volunteer did not comply with the study protocol and wore the device for only 6 consecutive days during the trial running and was therefore excluded of data-analysis. Unfortunately, the enamel of four samples wasn't completely removed during sample preparation. In total, four volunteers had samples excluded in three different interface conditions. Two volunteers had samples excluded from 100 µm group without CHX (-), another volunteer had one sample from 100 µm+B group with CHX (+) excluded, and one volunteer had one sample from 100 µm +B group with CHX (-) excluded.

Of the samples with an intentional gap of about 100 µm, the actual gap size was 125.9 ± 24.24 µm.

Descriptive values for LD (µm) and ML (µm.vol%) are shown in Figure 2. Dentin wall lesions were observed in intentional gaps (100 µm and 100 µm+B) independently of MMP inhibitor treatment applied. B and NB did not showed substantial secondary caries wall lesions.

The linear mixed effect analysis showed that the effect of treatment with MMP inhibitor on LD was -1.89 µm (SE=7.31 and CI 95%= - 16.67 / 12.86), which is not significant (p=0.797). The effect of pretreatment with MMP inhibitor on ML was -138.0 µm.vol% (SE=121.4 and CI 95% = -373.3 / 107.2), which is also not significant (p=0.26). More complex mixed-effects models showed that factors such as gap location (more distal or mesial) and interface conditions did not influence the effect of treatment with MMP inhibitor ($p \geq 0.09$).

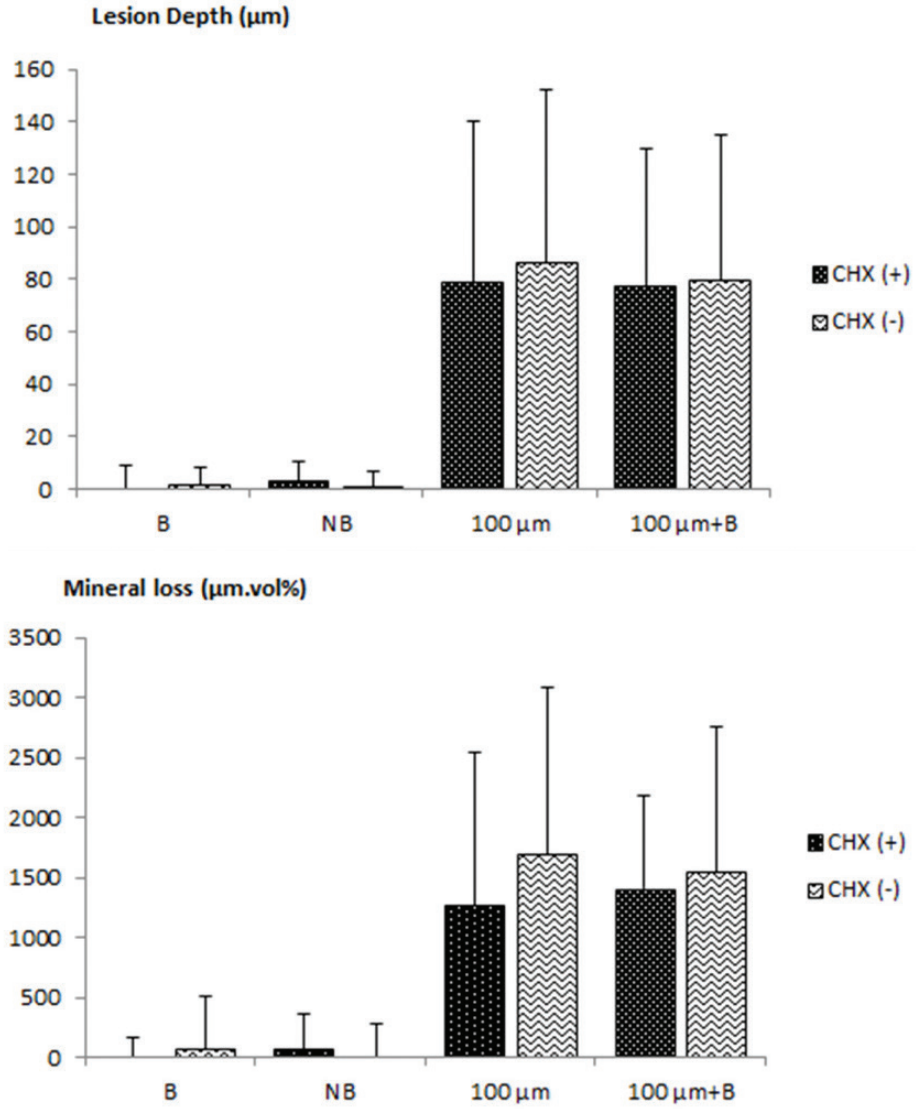

Figure 2. Bar charts showing the LD and ML average values for each group considering dentin treatment. Bars on the left side represent the application of pre-treatment (CHX+), and those on the right side represent not treatment (CHX-). The linear mixed effect analysis showed that the effect of treatment with MMP inhibitor on LD and ML was not significant ($p > 0.26$).

6.4 Discussion

This study showed that dentin pre-treatment with MMP inhibitor (CHX 2%) did not influence secondary caries wall lesion development in any interface condition. Therefore, the hypothesis stated was rejected in this in situ study.

As it is ethically not justified to induce caries in humans intentionally and as it is technically not possible to assess the true outcome of the caries disease (e.g., mineral loss) in a clinical study (Askar et al., 2017), in situ studies seem to be ideal to investigate factors related to caries lesion development. These models optimize the balance between clinical relevance and control of key variables (Ferracane, 2017). Recently our group investigated the effect of MMP inhibitor (CHX 2%) using an in vitro microcosm biofilm model (data not published yet), but interpersonal variations and oral cavity environment could not be considered in those results. The present study reflects therefore a more clinically relevant scenario to evaluate the role of dentin treatment with MMP inhibitor on secondary wall lesion progression in interfaces containing gaps.

Four interface conditions were evaluated in this study. Secondary wall lesions only developed when there was an intentional gap at the restoration interface (100 µm and 100µm+B). Samples from B group (control) didn't show any wall lesion. In NB group, a minimal gap size was simulated by bonding material omission, but ML and LD values showed the same results as in the control group (B). This confirms that a critical gap size (threshold) is needed for secondary wall lesion development (Kuper et al., 2014; Maske et al., 2017).

The presence of bonding material on the composite side may act as a predisposing factor for biofilm retention at interface and an increase of wall lesion progression was recently observed by Montagner et al. (2015), but a similar behaviour was not observed in the present study. The differences in gap sizes used in each study could explain the conflicting results. Wider gaps (~200µm) with bonding material on composite side may favour the retention of more biofilm at interface than smaller gaps (~100 µm).

The dentin treatment with MMP inhibitor had a minimal and not significant estimated effect of slowing down wall lesion progression (-1.89 µm to LD and -138.0 µm.vol% to ML). A possible hypothesis to the lack of effect of CHX treatment in this study may be related to the presence of defects at the interface (gaps). The theory behind this is that CHX might be leached from the interface. When an optimally bonded restoration is made (i.e., without marginal defects), it is likely that CHX-

saturated demineralized matrix becomes sequestered by adhesive resin coating collagen fibrils which may produce prolonged CHX retention and inhibition of MMPs (Carrilho et al., 2007b). However, when marginal defects are present along the interface of the restoration, leaching of CHX could be facilitated, and its inhibiting effect compromised.

In vitro studies developed by Kim et al. (2011; 2012) have shown that demineralized dentin treated with 0.2 or 2% CHX and stored in a body fluid (6 weeks and without successive acid challenges) displayed collagen integrity and deposition of granular minerals along collagen fibrils when compared to the no CHX-treated. The present in situ study showed that demineralized dentin treated with 2% CHX was ineffective to slow down demineralization on tooth-resin-interfaces containing gaps. The former studies seem to be limited to mimic the oral cavity dynamics over 6 weeks storage time. The lack of pH-cycling episodes during the storage time (activating other latent MMPs in the substrate) could explain the conflicting results.

Other in situ studies evaluating the use of MMP inhibitors in prevention of dentin demineralization have demonstrated a positive effect of this therapy. Sulkala et al. (2001) using non-antimicrobial chemically modified tetracyclines applied 5 times per day (during 7 weeks) showed a reduction of fissure caries progression. Magalhaes et al. (2009) and Hannas et al. (2016) demonstrated that green tea and CHX applied either 2x (by toothpaste) or 4x (by solution) per day during 5 days were also able to prevent demineralization by erosion. These positive effects on slowing down dentin demineralization could be related to frequent availability of the MMP inhibitor (used more than once per day) in the area prone to demineralization. The use of other vehicles as gels to deliver MMP inhibitor compounds could also increase the contact time with dentin and enhance substantivity of the product (Kato et al., 2010). In this in situ study 2% CHX was used as solution and only applied once as pre-restoration treatment protocol and therefore it could explain the lack of effect in slowing down caries lesion.

To knowledge of the authors, this study is the first to show the role of MMP inhibitor on secondary lesion development in a more clinically relevant scenario and limitations need to be highlighted. A sound substrate was used instead of caries-affected dentin and maybe the effect of CHX would have been clearer when collagen matrix had already been exposed by the dental caries process and some MMPs had already been activated. Moreover, in a clinical situation adhesive interfaces may degrade creating failed bonded interface conditions (Montagner et al., 2015; Montagner et al., 2016). The role of MMP inhibitor on secondary caries

lesion considering such failed-interfaces could bring interesting findings. These suppositions should still be confirmed in further studies.

6.5 Conclusion

Within the limits of this in situ study the pre-treatment of dentin with MMP inhibitor was not able to reduce secondary caries wall lesion development.

Declaration of interest
The authors declare no conflict of interest with respect to the authorship and/or publication of this article.

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CHAPTER

7

General discussion and conclusions

7.1 General discussion

As previously discussed we know that certain factors have already been related to secondary caries lesion development and other factors such as threshold gap size, caries risk and dentin enzymatic degradation still needed to be researched. These factors and other relevant findings will be discussed in the following sections considering the results found in the present PhD research.

7.1.1 In vitro and in situ biofilm models - study design

Through the systematic review presented in the **Chapter 2** it was concluded that few biofilm models showed dose-response validation to anti-caries or antimicrobial substances and reproducibility. Validation of in vitro biofilm models is important to confirm that they reflect clinical reality with similar sensitivity and reproducibility of response found in the oral cavity.

Microcosm-based biofilm models seem to be closer to natural dental biofilm (McBain, 2009). Using an inoculum source based on saliva or dental plaque thousands of different strains may compete or collaborate with each other to establish a microbial community. As dental caries is a result of metabolic interactions of a diverse microbial community, this source seems to be the best to simulate the complexity of biofilm-caries process (Sissons, 1997). In the present thesis two kinds of biofilm models were used (in vitro and in situ). **Chapter 3 and 5** used a microcosm laboratorial model dose-responsive to CHX (0 - 0.12%) and with a pattern of reproducibility against this antimicrobial solution (van de Sande et al., 2011). **Chapter 4 and 6** showed studies in a standardized in situ model to investigate secondary caries lesions (Kuper et al., 2014; Kuper et al., 2015a; Montagner et al., 2015). In situ studies have been considered the bridge between the natural uncontrolled clinical situation and the highly controlled laboratory situation. These models aim to mimic what occurs in the natural caries process and provide clinically relevant information in a relatively short period of time without damage to the natural dentition (Zero, 1995).

According to Maltz and Beighton (2012) studies focusing on caries lesion progression should be tested first in the best available laboratorial model and subsequently tested in situ or in a clinical situation to build the evidence base in a progressive scale of complexity. Studies presented in **Section 1** (small gap size) and **Section 2** (MMP inhibitor) tried to construct the evidence in a progressive complexity of biofilm models.

It is worth mentioning that independent of in vitro or in situ biofilm models used **(Chapters 3-6)** the studies showed similar and consistent results. For example, wall lesions developed in gaps of around 30 µm in both in vitro and in situ studies, even though in situ studies showed wide interpersonal variation in the extent of the lesions. In addition, secondary caries lesion development after dentin pre-treatment with 2% CHX solution or without such pre-treatment was also essentially the same in both models.

The above-mentioned findings highlight that the use of a validated in vitro model with high complexity (microcosms) could bring relevant evidence before in situ tests. It is particularly important to economize scientific work and to not expose volunteers to test situation without necessity.

7.1.2 Marginal defect and gap sizes

The cause of tooth demineralization around restorations has been attributed to leakage of bacteria or their acid products into the gap formed in the tooth-material interface (Carvalho and Manso, 2016; Ferracane, 2017).

Even using acid etching and bonding agent during restorative procedure to produce the best feasible interface condition, an unavoidable gap of 6-10 µm may be found at the tooth-restoration interface (Irie et al., 2002). Results from in vitro and in situ studies carried out in **Chapters 3 – 6** showed that no secondary caries wall lesions developed in this condition, implying that gaps wider than 10 µm are needed to allow for secondary caries wall lesions development. **Chapters 3 and 4** also showed that samples restored with omission of bonding material, resulting in a gap of about 13 µm, did not develop secondary caries wall lesions either. Wall lesion development was only observed when gap size of around 30 µm was present. We therefore concluded that the threshold gap size for wall lesion development lies somewhere between 13 and 30 µm. These findings are in agreement with Khvostenko et al. (2015) who showed biofilm colonization in similar gaps (~15 and ~30 µm). In addition, Derand et al. (1991) using a monoculture biofilm model observed that secondary caries lesions around amalgam restorations occurred when a gap size of 30 µm or more (40, 60 and 80 µm) and a thick biofilm covering the restoration margin were present. In **Chapter 6** (in situ study), secondary caries progression occurred in gaps wider than 27.5 µm in most of the volunteers. However, we also observed that in two volunteers no secondary caries lesions developed in any gaps, including the widest gaps of about 100 µm. This may point towards an

important role for individual protective or risk factors and this will be addressed later on.

Totiam et al. (2007) compared secondary lesion development in several gaps (25, 250 and 1000 µm) in an vitro study and concluded that the gap size affected lesion progression along the cavity wall, with a trend for deeper lesions associated with bigger gap sizes. Kuper et al. (2015) also showed increased wall lesion progression with wider gaps. Findings from our in vitro study **(Chapter 3)**, however, did not demonstrate the same trend. The gap sizes we used have a relatively small variation in size between tested groups and this could be the reason we did not find a trend. In **Chapter 5**, 60 and 90 µm gap sizes also showed essentially the same lesion progression (independent of MMP treatment applied).

Section 1 showed that secondary caries wall and outer lesions had a different progression depending on the study design used. In an in situ condition, wall lesions were bigger than surface lesions and in the in vitro condition it was other way around (see table 1).

Table 1. Secondary caries lesions in in vitro (Chapter 3) and in situ (Chapter 4) studies from Section 1

Study and condition (examples)	*Lesion Depth (µm) surface lesion*	*Lesion Depth (µm) wall lesion*
Chapter 3 – 90 µm*	130.3 (13.8)	105.7 (34.9)
Chapter 4 – 90 µm*	43.7 ± 32.3	82.3 ± 69.4

*average of group

This may be explained by different condition for biofilm development. In **Chapter 3** the biofilm was statically formed on dentin samples / gap entrance without the complex dynamic of oral cavity that occurs in the in situ model such as presence of salivary flow and tongue movements. The biofilm accumulation on top of the in vitro samples without external disturbances is likely to result in deeper surface lesions.

Moreover, in both in vitro studies considering small gap sizes **(Chapter 3 and 5)** we observed that wall lesions in intentional gaps (30, 60 and 90 µm) were formed in the location closest to the surface (200 µm from the gap entrance). It could be also explained by stagnation of biofilm on top of samples without external disturbances mainly related to occlusal loading. As there is no loading to improve biofilm colonization inside these very small gaps, the lesions formed were more superficial (Khvostenko et al., 2015).

7.1.3 Caries risk

Dental caries is a complex multifactorial disease. It is known that caries is a biofilm-sugar-dependent pathology with other factors, such as salivary function and composition acting in its progression (Hicks et al., 2003; Marsh, 2006; Selwitz et al., 2007).

In our in situ studies **(Chapter 4 and 6)** we controlled two oral factors related to caries risk progression: oral hygiene and cariogenic diet. Volunteers were asked to not brush the samples inside the device since this is one of the most important factors to develop caries lesion. Moreover, all volunteers dipped their samples 8 x per day in sucrose solution to mimic a highly cariogenic diet. Although all volunteers were exposed to the same cariogenic condition, widely different patterns of secondary caries lesion development were observed (from no lesion development at all to significant lesion development). These findings indicate that patients' caries activity is not only influenced by oral hygiene and diet, but that other individual intra-oral factors play a significant role (either protective or promoting). A possible explanation for why patients experiencing the same cariogenic challenge have such different caries progression may lie in genetic predisposition acting on factors such as biofilm composition and saliva composition (Bretz et al., 2003; Opal et al., 2015).

In **Chapter 4**, we tried to link the level of secondary caries and a threshold gap size to the caries activity level (caries risk) of the volunteer. Thus, volunteers and their pattern of wall lesion depth progression in intentional gaps were grouped into three secondary caries activity levels (low, mid and high). It was observed that independent of caries activity level of the volunteers, the secondary caries wall lesions developed already in the smallest gaps present (> 27.5 μm). Possibly due to the limited number of volunteers we were not able to show a relation of gap threshold with caries risk. We did notice a trend for larger wall lesions in wider gaps for the high caries activity volunteers. It is worth pointing out that it could be related to caries activity level criteria used, but it could also be related to more biofilm accumulation in the space between tooth and restoration (Kuper et al., 2015; Totiam et al., 2007).

Clinically, the findings presented in **Chapter 4** show that a dentist should consider their patients' individual caries risk profiles when making treatment decisions regarding restorations with imperfect margins. Although marginal defects have been shown to be a factor in secondary lesion development (Diercke et al., 2009; Kuper et al., 2014; Totiam et al., 2007), focussing only on gaps may be an oversimplification.

7.1.4 Tooth-material interface

In the general introduction it was discussed how dental materials located at the interface could influence secondary caries wall lesion in several ways, with a possible role for the type of dental material, failure mode and properties of the bonding (Kuper et al., 2015a; Kuper et al., 2015b; Montagner et al., 2015; Montagner et al., 2016; van de Sande et al., 2014). In the present thesis we evaluated three conditions of tooth-material interface: i) adhesive material present on interface to bond tooth and composite, ii) adhesive material omitted from interface and iii) adhesive located at the side of the composite on interface with gap.

Interfaces conditions i (named bonding / bonded) and ii (no bonding / no-bonded) showed consistently the same results both in in vitro and in situ studies. Although ML and LD values were measured for these conditions in the studies, they did not reflect actual caries lesions and but should be considered an artefact of the T-WIM method (Thomas et al., 2006). As we never found caries wall lesion development in the restorations without intentional gaps, whether they were perfectly bonded or not bonded at all (independent of small gaps formed due shrinkage or incomplete polymerization), we may conclude that a tooth-restoration interface containing very small gaps up to about 13 μm are likely not susceptible to wall lesion development.

Interface condition iii (named 100μm+B) was evaluated in **Chapter 5 and 6**. This condition was already studied in situ by Montagner et al. (2015) using a gap size of around 200 μm. In that situation, the presence of bonding material on composite side increased secondary caries wall lesion progression. Both studies presented in this thesis (in situ and in vitro) showed a small trend to increase the lesion progression. The difference in the effect may be related to the difference in gap sizes. Wider gaps (~200 μm) could allow more biofilm accumulation than the smaller gaps (~100 μm) used in this thesis. The main conclusion that can be draw for this condition is that when, after failure, the adhesive is located at side of composite there may be a higher risk for wall lesion progression.

7.1.5 Dentin enzymatic degradation and Matrix metalloproteinases inhibitor

It is well established that matrix metalloproteinases (MMPs) play a central role in several biological and pathological process. MMPs have been claimed to play an important role in the dentin organic degradation following demineralization by bacterial acids and therefore in the control of the dentin caries process (Takahashi and Nyvad, 2016).

During self-etching and etch-and-rinse adhesive procedures, after application of acid primer etching or phosphoric acid, the exposed collagen matrix is also vulnerable to degradation by MMPs (Mazzoni et al., 2006; Mazzoni et al., 2015). The enzymatic degradation of collagen by MMPs is reported as an important factor for destruction of the bonded interface (Montagner et al., 2014). The application of MMP inhibitor on dentin after acid etching improved the integrity and stability of tooth restoration over time (De Munck et al., 2010; Loguercio et al., 2009). The most used MMP inhibitor in adhesive procedures is chlorhexidine (CHX) applied in concentrations between 0.2% to 2% (Montagner et al., 2014; Tjaderhane et al., 2015).

Considering the pre-treatment of dentin with CHX during adhesive procedures in dental restorations and that this solution (MMP inhibitor) could act in the dentin caries control, the main goal of the **Chapter 5 and 6** was evaluate the role of MMP inhibitor used during the adhesive procedure on prevention of secondary caries wall lesion progression.

Both study designs (in vitro and in situ) used in the present thesis showed that 2% CHX solution used as a dentin pre-treatment on tooth-restoration with gaps did not slow down the wall lesion progression. By these findings we can formulate some hypotheses: i) presence of a gap in the interface may help the CHX leaching from this space and therefore terming its effect as MMPs inhibitor, ii) to be effective the MMP inhibitor should be consistently present on interface to allow inactivation of new MMPs activated by pH cycling episodes.

It is worth mentioning that a sound substrate was used instead of caries-affected dentin in both studies performed. Maybe the effect of CHX-MMP inhibitor would have been clearer when collagen matrix had already been exposed by the dental caries process and some MMPs had already been activated by this process. On the other hand, we tried to mimic the MMP activation using acid etching and acid primer before restorative procedures to therefore maximize the MMP inhibitor effect. Moreover, only bonded and no-bonded interfaces with gap sizes were simulated in our MMP inhibitor studies (Section 2). Other clinical conditions where adhesive could be degraded by ageing and creating failed bonded interfaces should also be tested.

Although a theory behind MMP inhibitors and their effect on caries progression is well discussed in the literature (Buzalaf et al., 2015; Mazzoni et al., 2015; Takahashi and Nyvad, 2016; Tjaderhane et al., 2015) there are only few experimental studies focused on this topic. Sulkala et al. (2001) using a non-antimicrobial chemically

modified tetracyclines showed a reduction of dentin fissure caries progression when applied five times per day (during 7 weeks). Van Strijp et al. (2015) showed that hesperidin applied 2 x per day was able to decrease collagen degradation by 24% compared to control samples. It is worth mentioning that in that study hesperidin did not have its antimicrobial properties inactivated and therefore the effect found by authors could be in part related to an antimicrobial effect. On the other hand, Islam et al. (2012) using a pH cycling model (no microbial model) showed that hesperidin solution was able to slow down primary dentin caries progression and decreased collagen degradation.

In addition, Islam et al. (2012) also tested 0.2 % CHX solution as MMP inhibitor. TMR measurements showed that incubation in CHX (2h per day during 8 days) did not contribute to suppression of mineral loss. Moreover, findings from Hiraishi et al. (2011) also confirm this assumption showing that CHX did not show significant difference in root dentin lesion depth compared to positive control (no CHX pre-treatment). Even using a simplified model to test caries progression, former studies confirm the results found in this thesis and reported in Chapter 5 and 6.

The experimental literature about the role of MMP inhibitor on caries lesion progression thus does not present a consistent picture, with some positive findings and some negative (no effect) findings. More studies are still needed to clarify this condition.

7.2 Conclusions

Based on the main findings of this PhD research, it is concluded:

Chapter 2

- There are several in vitro biofilm models to study dental caries available in literature, however, only few studies showed dose-response and reproducibility validation.
- Multiplaque artificial mouth (MAM), microplate and flow chamber/ cell biofilm model approaches showed dose-response to anticaries or antimicrobial substances.
- Reproducibility of the dose-response pattern only was found for microcosm microplate model.

Section 1 (Chapter 3 and 4)

- The minimum gap size for secondary caries wall lesion development may be as small as 30 μm.
- In gaps narrower than about 13 μm no secondary caries wall lesion formation could be observed.
- No relationship between gap size threshold and caries activity level could be observed in situ.

Section 2 (Chapter 5 and 6)

- 2% CHX used as MMP inhibitor on dentin pre-treatment during adhesive procedures did not slow down secondary caries wall lesion progression in the interface conditions tested in complex in vitro and in situ biofilm models.

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CHAPTER

8

Summary /Resumo / Samenvatting

Thanks

Personal aspects

8.1 Summary

This PhD thesis is based on five studies that aimed to investigate the role of local factors in relation to the development of secondary caries lesions

Chapter 1 describes the etiology and histology of secondary caries reviewing the several factors that could influence the secondary caries process.

Chapter 2 presents a systematic review of in vitro biofilm models to study dental caries. This review addresses key methodological aspects of *in vitro* biofilm models for caries-related research, verifying their reproducibility and dose-response to anti-caries and/or antimicrobial substances. The review included models that produce a cariogenic biofilm and/or caries-like lesions with pH fluctuations and models showing an effect of anti-caries and/or antimicrobial substances. Basically the models consisted of dynamic or batch biofilm models varying in inoculum source and in carbohydrate exposure protocols. Dose-response validation was reported in 20.4% and reproducibility assessment in 14.3% of the studies. Almost a third (32.7%) of the models with dose-response validation were classified as high risk of bias. It was concluded that several *in vitro* biofilm caries models are available, but most of them lack validation by dose-response and reproducibility experiments.

Section 1 (Chapter 3 and 4) focuses on small gap sizes as a local factor in secondary caries lesion development.

In **Chapter 3** an in vitro study evaluated the development of dentin wall lesions in very small gaps next to resin composite using a biofilm model, and evaluated whether a relevant threshold for the gap size could be established. Samples were either restored with composite resin without adhesive procedure (no intentional gap; no bonding [NB] group) or with intentional gaps of 30, 60, or 90 μm, or with complete adhesive procedure (no gap; bonding [B] group). Microcosm biofilms were grown for 14 days within the small interfacial gaps under intermittent cariogenic challenge and secondary caries wall lesion progression was measured using Transversal Wavelength Independent Microradiography (T-WIM) at 3 locations: outer surface lesion and wall lesions at 200 and 500 μm distance from gap entrance. Results showed that the presence of an intentional gap (30, 60, and 90 μm) affected the secondary caries progression at 200 μm from the gap entrance ($p \leq 0.013$). The NB group did not show significant wall lesion development ($p \geq 0.529$). At 500 μm distance almost no wall caries development was observed. In conclusion, dentin wall lesions developed in very small gap sizes, and the threshold for secondary wall lesion development was a gap of less than 30 μm in this microcosm biofilm model.

Chapter 4 investigated the influence of very small gaps between tooth and restoration in secondary caries (SC) development and additionally to link the level of SC and a threshold gap size with the caries activity level of the volunteer. For 21 days, 15 volunteers wore a modified occlusal splint loaded with dentin-composite samples restored according to different interface conditions: bonded (B = samples restored with complete adhesive procedure), no-bonded (NB = restored with composite resin without adhesive procedure), and 30, 60 and 90 µm (no adhesive procedure and with intentional gap). Eight times per day, the splint with samples was dipped in a 20% sucrose solution for 10 minutes. Before and after caries development, samples were imaged with T-WIM and lesion depth and mineral loss were calculated. Volunteers were grouped according to the average wall lesion depth and classified as high, mid and low caries activity levels. No wall lesion formation was observed in B and NB groups. In general, intentional gaps led to SC lesion depth progression independent of caries activity level of volunteers. No substantial wall lesions were found for two volunteers. A trend for deeper lesion in larger gaps was observed for the high activity group. In conclusion, very small gaps around or wider than 30 µm develop SC independent of the caries activity level of the patient and SC wall lesion progression seemed to be related to individual factors even in this standardized in situ model.

Section 2 (Chapter 5 and 6) is also composed by an in vitro and in situ studies and aimed to investigate the role of CHX, an MMP inhibitor, acting as local factor to prevent secondary caries lesion development.

Chapter 5 using an in vitro study investigated the role of MMP inhibitor (2% CHX) in secondary caries wall lesion development in different interface conditions in two runs (run 1 / small gaps = Bonded, no-bonded, 30, 60 or 90 µm and run 2 / wider gaps= bonded, no-bonded 100 µm and 100 µm+B). Dentin discs were restored and pre-treated with or without chlorhexidine 2%. Microcosm biofilms were grown on dentin-composite samples (14 days) and caries lesion outcomes were analysed by transversal wavelength independent microradiography (T-WIM) at three locations: outer surface and interface wall (200 and 500 µm distance from gap entrance). Linear regression analyses showed that pre-treatment with MMP inhibitor did not influence the wall lesion progression at either location ($p \geq 0.218$). Interfaces with intentional gaps showed positive and significant effect on the wall lesion progression at 200 µm distance from the gap entrance ($p \leq 0.005$). A small trend of increase in wall lesion development was observed at the 200 µm location when bonding was present on the composite side. In conclusion, the dentin pre-

treatment with MMP inhibitor (CHX 2%) was not able to slow down the secondary caries wall lesion development in small and wide gaps in this biofilm model.

In Chapter 6, an in situ study aimed to investigate whether the dentin treatment with MMPs inhibitor (CHX 2%) could influence the development of secondary caries wall lesions in different dentin-composite interfaces. Similarly to Chapter 4, 15 volunteers wore a modified-occlusal splint loaded with dentin-composite samples treated or not with CHX and restored according 4 different interface conditions: Bonding (B = samples restored with complete adhesive procedure), no bonding (NB = restored with composite resin without adhesive procedure), 100 µm (no adhesive procedure and with intentional gap) and 100 µm + B (adhesive material on composite side and intentional gap). Eight times per day, the splint with samples was dipped in a 20% sucrose solution for 10 minutes. Before and after caries development, samples were imaged with T-WIM and lesion depth (LD) and mineral loss (ML) were calculated. Results showed that dentin treatment with CHX did not significantly affect the caries lesion progression (LD and ML; $p \geq 0.261$). Dentin wall lesions were observed in the 100 µm and 100 µm+B groups independently of MMP inhibitor treatment. In conclusion, the treatment of dentin with MMP inhibitor was not able to slow down the secondary caries wall lesion development in this in situ study.

Finally, **in Chapter 7** the available literature on secondary caries and study designs / gap size / caries risk / tooth-material interface / MMP inhibitor is discussed. The strengths and limitations of the different methodologies used in this thesis are also discussed.

8.2 Resumo

Esta tese de doutorado está baseada em cinco estudos que objetivaram investigar o papel de fatores locais associados ao desenvolvimento de lesões de cárie secundária (limiar para o gap interfacial / limiar para o gap interfacial e risco de cárie / inibidor de MMP) e adicionalmente mostrar uma visão geral sobre modelos de biofilmes in vitro para o estudo da cárie dentária.

O **capítulo 1** descreve a etiologia e a histologia das lesões de cárie secundária perfazendo uma visão geral sobre diversos fatores que poderiam influenciar no desenvolvimento da cárie secundária.

O **capítulo 2** apresenta uma revisão sistemática de literatura sobre modelos de biofilme in vitro para o estudo da cárie dentária. Essa revisão objetivou

caracterizar e discutir os aspectos metodológicos chaves de modelos de biofilme in vitro relacionados a pesquisa de cárie dentária e verificar a reprodutibilidade e dose-resposta desses modelos considerando a resposta para agentes anti-cárie e substâncias antimicrobianas. Nessa revisão foram incluídos modelos de biofilme que gerassem biofilme cariogênico e / ou lesões artificiais de cárie com flutuações de pH e que mostrassem o efeito de substancias anti-cárie e / ou antimicrobianas. Basicamente, os modelos consistiram de modelos dinâmicos e estáticos variando em relação a fonte de inóculo e a protocolos de exposição a carboidratos. Respectivamente, 20,4 e 14,3% dos estudos reportaram validações dose-responsta e reprodutibilidade. Quase um terço (32,7%) dos modelos com validações dose-resposta foram classificados como alto risco de viés. Apesar de diversos modelos de biofilme in vitro estarem disponíveis na literatura para pesquisas relacionadas a cárie dentária, a maioria deles ainda carecem de validações dose-resposta e experimentos de reprodutibilidade para cada protocolo proposto.

A **sessão 1 (capítulos 3 e 4)** tem como foco a avaliação da presença de pequenos tamanho de gaps como fatores locais no desenvolvimento da cárie secundária.

No **capítulo 3** um estudo in vitro usando um modelo de biofilme avaliou o desenvolvimento de lesões de parede próximas a resina composta contendo gaps muito pequenos. Além disso, avaliou se um limiar relevante de tamanho de gap poderia ser estabelecido. O fator em estudo foi o tamanho do gap: amostras foram ou restauradas com resina composta sem procedimento adesivo (sem gap intentional; grupo sem adesão [NB]) ou com gaps intentionais de 30, 60, or 90 μm, ou com procedimento adesivo completo (sem gap, adesão perfeita [B]). Biofilme de microcosmos foram crescidos por 14 dias dentro de gaps interfaciais entre discos de resina composta-dentina sobre um regime intermitente de desafio cariogênico. As lesões de cárie secundárias de parede foram mensuradas usando microrradiografia de onda tranversal independente em 3 localizações: na lesão superficial e na lesão de parede em 200 e 500 μm da entrada do gap. Os resultados mostraram que a presença de gaps intencionais (30, 60, and 90 μm) afetaram a progressão de cárie secundária em 200 μm a partir da entrada do gap ($p \leq 0.013$). O grupo NB não mostrou significantimente desenvolver lesões de parede ($p \geq 0.529$). Em 500 μm da entrada do gap lesões de parede não foram praticamente observadas. Em conclusão, lesões de parede em dentina se desenvolveram em tamanhos muito pequenos de gaps e o limiar para o desenvolvimento de cárie secundária de parede foi de um gap de tamanho de aproximadamente 30 μm, considerando o modelo de microcosmos.

O **capítulo 4** investigou in situ a influência de gaps muito pequenos entre dente e restauração no desenvolvimento de cárie secundária e adicionalmente correlacionou o nível de cárie secundária com o limiar de tamanho de gap com o nível de atividade de cárie do voluntário. Por 21 dias, 15 voluntários usaram um dispositivo oclusal modificado com amostras de dentina restauradas de acordo com diferentes condições de interfaces: aderida (B = amostras foram restauradas com resina composta e procedimento adesivo completo), sem adesão (NB = amostras foram restauradas com resina composta sem procedimento adesivo), e 30, 60 e 90 µm (sem procedimento adesivo e com gaps intencionais). Oito vezes por dia, o dispositivo oclusal foi imerso em solução de sacarose a 20% por 10 minutos. Antes e depois do desenvolvimento das lesões de cárie, imagens das amostras foram realizadas através de T-WIM e a profundidade de lesão e a perda mineral foram calculadas. Os voluntários foram agrupados de acordo com a média de profundidade das lesões de parede e classificados em níveis de atividade de cárie alta, média e baixa. Lesões de cárie de parede não foram formadas nos grupos B e NB. De forma geral, a presença de gaps intencionais levaram a progressão das lesões independente do nível de atividade de cárie do voluntário. Não foram observadas lesões consideráveis em dois voluntários. Uma tendência para lesões de parede mais profundas em gaps mais largos foi observada para os indivíduos classificados como alto nível de atividade de cárie. Em conclusão, gaps muito pequenos em torno ou maiores de 30 µm desenvolveram cárie secundária independentemente do nível de atividade de cárie do voluntário e a progressão das lesões de parede pareceu estar relacionada a fatores individuais mesmo em um modelo in situ padronizado.

A sessão 2 (capítulos 5 e 6) é também constituída por estudos in vitro e in situ, e objetivou investigar o papel da clorexidina (CHX), um inibidor de metaloproteinases da matrix (MMP), atuando como um fator local para o desenvolvimento de cárie secundária.

No capítulo 5 um estudo in vitro investigou o papel de um inibidor de MMP (2% CHX) no desenvolvimento das lesões de cárie secundária de parede frente a diferentes condições de interfaces em duas rodadas (rodada 1 / pequenos gaps = adesão, sem adesão, 30, 60, 90 µm e rodada 2 / gaps amplos = adesão, sem adesão, 100 µm e 100 µm+adesivo no lado da resina composta). Os discos de dentina foram restaurados e pré-tratados com e sem CHX 2%. Biofilme de microcosmos foram crescidos sobre as amostras de dentina-resina composta (14 dias) e os desfechos de cárie dentária foram analizados por microrradiografias de onda transversal independente (T-WIM) em 3 localizações: lesão superficial e na lesão de parede (em

200 e 500 µm da entrada do gap). Análises de regressões lineares mostram que o pré-tratamento com inibidor de MMP não influenciou a progressão das lesões de parede em qualquer localização ($p \geq 0.218$). As interfaces com gaps intencionais mostraram um efeito positivo e significante na progressão das lesões a partir de 200 µm da entrada do gap ($p \leq 0.005$). Uma pequena tendência para o aumento das lesões de parede foi observado em 200 µm da entrada do gap quando o adesivo estava presente no lado da resina composta. Conclui-se que o pré-tratamento da dentina com inibidor de MMP (CHX 2%) não foi capaz de diminuir o desenvolvimento das lesões de cárie secundária de parede em gaps pequenos e amplos utilizando esse modelo de biofilme.

O **capítulo 6,** objetivou investigar in situ se o tratamento da dentina com inibidor de MMP (CHX 2%) poderia influenciar o desenvolvimento de lesões de cárie secundária de parede em diferentes interfaces de dentina-resina composta. De forma similar ao capítulo 5, 15 voluntários usaram um dispositivo oclusal modificado contendo amostras de dentina-resina composta tratadas ou não com CHX e restauradas de acordo com quatro diferentes condições de interface: adesão (B = amostras restauradas com procedimento adesivo completo), sem adesão (NB = amostras restauradas com resina composta e sem procedimento adesivo), 100 µm (sem procedimenro adesivo e com gap intencional) e 100 µm + B (adesivo localizado ao lado da resina composta). Antes e depois do desenvolvimento das lesões, imagens das amostras foram realizadas com T-WIM e a profundidade de lesão (PL) e perda mineral (PM) foram calculadas. Os resultados mostraram que o tratamento da dentina com CHX não afetou significantemente a progressão das lesões de cárie (PL e PM; $p \geq 0.261$). Lesões na parede de dentina foram observadas nos grupos 100 µm and 100 µm+B independentemente do tratamento com inibidor de MMP. Conclui-se que o tratamento com MMP inhibitor não foi capaz de diminuir o desenvolvimento das lesões de parede no estudo in situ.

Finalmente, no **capítulo 7**, a literatura disponível sobre cárie secundária e design de estudos / tamanho de gaps / risco de cárie / interface dente-material / inibidor de MMP foi discutida. Além disso, os pontos fortes e as limitações das diferentes metodologias usadas nessa tese também foram comentadas.

8.3 Samenvatting

Dit proefschrift is samengesteld uit vijf studies die als doel hadden de rol van lokale factoren in het ontstaan van secundaire cariëslaesies te onderzoeken.

Hoofdstuk 1 beschrijft de etiologische factoren en histologische processen die ten grondslag liggen aan het ontstaan van secundaire cariëslaesies.

Hoofdstuk 2 beschrijft een systematisch literatuuroverzicht van biofilm modellen die worden gebruikt om secundaire cariës *in vitro* na te bootsen. De centrale methodologische aspecten van biofilm modellen die *in vitro* worden toegepast voor cariësgerelateerd onderzoek worden hierin vergeleken. De reproduceerbaarheid en dosis-respons validatie van anticariogene of antimicrobiële stoffen worden geverifieerd. Modellen die een cariogene biofilm en/of op cariës gelijkende laesies produceerden door middel van pH schommelingen werden geïncludeerd, evenals modellen die het effect van een cariostatisch of antimicrobieel middel onderzochten. In feite bestonden de modellen uit dynamische of constante biofilm simulaties met verschillende entmaterialen en protocollen voor blootstelling aan koolhydraten. Dosis-respons validatie werd in 20,4% van de gevallen gerapporteerd en een inschatting van de reproduceerbaarheid werd in 14,3% van de studies gemaakt. Bijna een derde (32,7%) van de studies die een dosis-respons validatie toepasten had een hoog risico op vertekening (bias). Concluderend: er zijn verschillende *in vitro* biofilm modellen beschikbaar voor cariësonderzoek, maar de meesten missen adequate validatie in de vorm van een test op dosis-respons of reproduceerbaarheid.

Sectie 1 (Hoofdstukken 3 en 4) richt zich op smalle randspleten als een lokale factor in het ontstaan van secundaire cariëslaesies.

In **Hoofdstuk 3** werd door middel van een *in vitro* studie het ontstaan van wandlaesies in dentine in zeer smalle randspleten naast composietrestauraties geëvalueerd, gebruikmakend van een biofilm model. Een belangrijke vraag hierbij was of er een relevante drempelwaarde voor de grootte van de randspleet kon worden vastgesteld. Proefstukjes werden gerestaureerd met composiet zonder adhesieve middelen (geen intentionele randspleet; geen bonding [NB] groep), met een intentionele randspleet van 30, 60 of 90 µm, of met een complete adhesieve procedure (geen randspleet; bonding [B] groep). Microkosmos biofilms werden gedurende 14 dagen gekweekt in de kleine randspleten terwijl deze werden blootgesteld aan periodieke cariogene omstandigheden. Secundaire cariësprogressie van de wandlaesies werd gemeten door gebruik te maken van T-WIM (transversale, golflengteonafhankelijke microradiografie) op drie locaties:

oppervlaktelaesies, wandlaesies op 200 µm van de ingang van de randspleet en wandlaesies op 500 µm van de ingang van de randspleet. De resultaten lieten zien dat de aanwezigheid van een intentionele randspleet (30, 60 of 90 µm) het ontstaan van secundaire cariëslaesies op 200 µm afstand van de ingang van de randspleet beïnvloedde ($p \leq 0{,}013$). De NB groep liet geen significante laesieontwikkeling zien ($p \geq 0{,}529$). Op 500 µm afstand van het oppervlak werd nauwelijks ontwikkeling van wandlaesies waargenomen. Dit leidt tot de conclusie dat wandlaesies zich in dentine naast erg smalle randspleten kunnen ontwikkelen, en dat de drempelwaarde van de randspleet voor het ontstaan van secundaire wandlaesies in dit microkosmos biofilm model niet meer dan 30 µm bedroeg.

Hoofdstuk 4 bestudeerde de invloed van zeer smalle randspleten op secundaire cariësontwikkeling in een *in situ* model, en probeerde verder de drempelwaarde voor de ontwikkeling van secundaire wandlaesies naast composietrestauraties te relateren aan de hoeveelheid cariësactiviteit van de proefpersonen. Gedurende 21 dagen droegen 15 vrijwilligers een gemodificeerde occlusale opbeetplaat die proefstukjes van dentine gerestaureerd met composiet bevatten. De proefstukjes waren gerestaureerd volgens verschillende condities: met composiet zonder adhesieve middelen (geen intentionele randspleet; geen bonding [NB] groep), met een intentionele randspleet van 30, 60 of 90 µm, of met een complete adhesieve procedure (geen randspleet; bonding [B] groep). De vrijwilligers doopten de opbeetplaat die de samples bevatte 8 keer per dag 10 minuten lang in een oplossing van 20% sucrose. Voor en na deze drie weken durende cariogene omstandigheden werden de proefstukjes gefotografeerd met behulp van de T-WIM methode. Laesiediepte en mineraalverlies werden berekend. De vrijwilligers werden in verschillende groepen ingedeeld afhankelijk van de gemiddelde laesiediepte van de wandlaesies. In de B en NB groepen werden bij geen enkele vrijwilliger wandlaesies waargenomen. Over het algemeen werden bij de intentionele randspleten wandlaesies waargenomen, onafhankelijk van het niveau van cariësactiviteit bij de vrijwilligers. Bij twee vrijwilligers werden helemaal geen substantiële wandlaesies gemeten. Er was een trend zichtbaar van diepere laesies in grotere randspleten in de groep met een hoog niveau van cariësactiviteit. Duidelijk is dat zich in erg smalle randspleten van ten minste 30 µm wandlaesies lijken te ontwikkelen, onafhankelijk van de hoeveelheid cariësactiviteit van de patiënt. Verder leek de progressie van wandlaesies ook in dit gestandaardiseerde *in situ* model gerelateerd te zijn aan individuele factoren.

Sectie 2 (Hoofdstukken 5 en 6) beschrijft ook een *in vitro* en een *in situ* studie. Deze richtten zich op het vermogen van chloorhexidine (CHX), een matrix metallo proteïnase (MMP) remmer, om lokaal de ontwikkeling van secundaire cariëslaesies te voorkomen.

In **Hoofdstuk 5** werd de invloed van een MMP remmer (2% CHX) op de ontwikkeling van secundaire wandlaesies bestudeerd in verschillende interface condities in twee rondes (Ronde 1 / smalle randspleten: bonding, geen bonding, 30, 60 en 90 µm randspleten. Ronde 2 / bredere randspleten: bonding, geen bonding, 100 µm randspleten en 100 µm randspleten met bonding). Dentine schijfjes werden al of niet behandeld met een 2% CHX oplossing en gerestaureerd met composiet. Microkosmos biofilms werden gekweekt op de proefstukjes (gedurende 14 dagen) en laesiediepte en mineraalverlies werden geanalyseerd met T-WIM op drie locaties: op het oppervlak, in de randspleet op 200 µm van de ingang van de randspleet en in de randspleet op 500 µm van de ingang van de randspleet. Lineaire regressie analyse liet geen invloed zien van de behandeling met een MMP remmer op de ontwikkeling van wandlaesies op een van beide wandlocaties ($p \geq 0{,}218$). De aanwezigheid van een randspleet in het proefstukje had een positief significant effect op het ontstaan van wandlaesies op 200 µm afstand van de ingang van de randspleet ($p \leq 0{,}005$). Een lichte neiging tot grotere wandlaesies werd waargenomen in de samples waarin bonding aanwezig was op het composiet. Behandeling van het dentine met een MMP remmer (2% CHX) leek de formatie van secundaire cariëslaesies in dit biofilm model in grote noch kleine randspleten te vertragen.

In **Hoofdstuk 6** werd een studie beschreven waarin door middel van een *in situ* model nogmaals het effect van behandeling van dentine met een MMP remmer (2% CHX) op het ontstaan van wandlaesies in dentine naast composiet is onderzocht. Vergelijkbaar met de studie in hoofdstuk 4 werd 15 vrijwilligers gevraagd om een gemodificeerde occlusale opbeetplaat te dragen waarin proefstukjes van dentine en composiet waren gemonteerd. De proefstukjes waren in vier groepen ingedeeld: bonding (B = proefstukjes gerestaureerd met een volledige adhesieve procedure), geen bonding (NB = gerestaureerd met composiet zonder adhesieve procedure), 100 µm (geen adhesieve procedure en met intentionele randspleet) en 100 µm + B (adhesief bonding materiaal aangebracht op composiet zijde, met intentionele randspleet van 100 µm). De vrijwilligers doopten de opbeetplaat met de proefstukjes acht keer per dag in een 20% sucrose oplossing gedurende tien minuten. De proefstukjes werden voor en na blootstelling aan het mondmilieu gefotografeerd met behulp van T-WIM. Laesiediepte (LD) en mineraalverlies (ML) werden berekend.

Uit de resultaten bleek dat behandeling van het dentine met CHX de progressie van wandlaesies niet significant beïnvloedde (uit zowel LD als ML; $p \geq 0.261$). In de groepen 100 µm en 100 µm + B werden, onafhankelijk van de behandeling met MMP remmer, wandlaesies in het dentine waargenomen. Concluderend liet deze studie geen effect zien van behandeling met een MMP remmer op de ontwikkeling van secundaire wandlaesies.

Tot slot bevat **Hoofdstuk 7** een geïntegreerde discussie van de resultaten.

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Personal Aspects

Tamires Timm Maske - MASKE TT

Personal aspects	Tamires is Brazilian; she was born in Pelotas –RS, a city at extreme south of Brazil. By chance, she is an only child. By choice, she has lovely friends as brothers and sisters.
Professional aspects	Tamires is graduated in Dentistry (2012) at Federal University of Pelotas, RS, Brazil. She is Master of Dental Science (2015) at Federal University of Pelotas. She is very honoured to be a Joint PhD student at Radboud University Medical Center and Federal University of Pelotas during period of 2016 up to the present moment.
Interests	She loves research and discovers new things! She is a bit hardworking person, but also likes enjoying the little things in life with friends and family!
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