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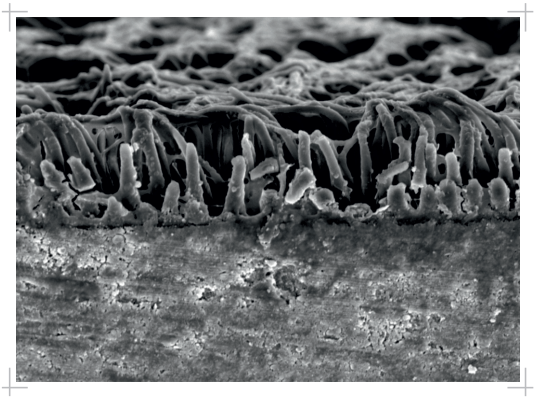

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1. Introduction

With 2.2 – 3.6 % annual failure rate [1], composite restorations durability is regarded as an important clinical issue [2]. In order to deliver successful adhesive restorations, the following conditions should ideally be met; a high quality hybrid layer should be formed between dentine and the restorative material, and hybrid layer should be long-term maintained [3–6]. Hydrolytic degradation and enzymatic activity in dentine are the main reasons for adhesive failure of composites [7,8]. As if water content in dentine and endogenous enzymes were not enough of a challenge for the restorations, bacteria from the oral cavity, can further threaten the restoration's viability, through secondary caries [9–11]. Since, extrinsic bacterial damage or intrinsic enzymatic degradation are localized at restoration margins, this thin adhesive interface is considered the Achille's ptern of adhesive restorations. Both phenomena are connected to each other forming a vicious circle – the greater the extent of marginal failure due to enzymatic collagenolysis, the larger the space created for water to flow in and as next for bacteria to gather. The ultimate goal would be the production of a dental material with antibacterial and at the same time anticollagenolytic characteristics which would inhibit both bacterial and enzymatic degradation.

Chlorhexidine is a known antiseptic which acts against *S. mutans* and is therefore widely used in oral hygiene products and in preventive dentistry [12–14]. The importance of chlorhexidine in restorative dentistry and its benefit in adhesive restorations has however recently been discussed [15–17]. Although dentine collagenolytic enzymes – matrix metalloproteinases and cysteine cathepsis – are responsible for the destruction of collagen matrix in the hybrid layer, chlorhexidine is shown to act against them and protect the collagen network of the hybrid layer [15–19]. Chlorhexidine can be delivered as dentine pre-treatment or admixed with the adhesives; however issues are raised regarding its potential interference with the mechanical properties and the bonding efficiency of the adhesives used as carriers [20,21].

Only one adhesive system with industrially incorporated 0.2% chlorhexidine is commercially available [22,23] and a direct comparison between the different ways of

chlorhexidine addition into the different steps of the adhesive procedure (primer or bonding agent) has never been made in a single study nor has it been directly compared with the use of chlorhexidine as a cavity pre-treatment agent, under the same circumstances. Whether chlorhexidine adhesives can protect the adhesive bond after 6- or 12-month storage, or whether they are able to protect restoration margins from secondary caries via antibacterial action, are questions which need to be answered.

2. Review of literature

2.1. Dentine and its Endopeptidases

Dentine is a collagen-based mineralized tissue with inorganic apatite crystals embedded in an extracellular organic matrix. This matrix consists mainly of type I collagen (~90% w/v), which is responsible for the tensile strength of dentine and for its biochemical properties. Non-collagen components (~10% w/v) such as proteoglycans and glycoproteins are present within the matrix and play fundamental roles during fibrillogenesis, crystal growth and mineralization [24]. Since dentinogenesis is an active phenomenon, it requires extracellular enzymatic control by different proteinases, mainly matrix metalloproteinsases (MMPs) [25] and cysteine cathepsins (CCs) [15,18]. Apart from the dentinal matrix, MMPs [26] and CCs [27] are detected in human saliva, while some MMPs are also found in dental plaque [28]. Therefore their role in dentistry and their significance in dental research is clear, since clinical issues like dental caries and adhesive restorations' failure are related to them [29,8,30,31,27,32,18].

2.1.1. Matrix Metalloproteinases (MMPs)

MMPs are a family of 23 different multi-domain calcium- and zinc-dependent proteolytic enzymes (endopeptidases) that take part in physiological and pathological tissue development and remodeling, by cleaving collagen fibrils.The first member of this enzyme group was discovered by *Gross and Lapiere*, *1962* [33], and from then, up to 28 types of MMPs have been described. MMP-2 (gelatinase),-3 (stromelysin), -8 (collagenase), -9 (gelatinase) and -20 (other type) have been identified in dentine tissue [34,25,35–37] with MMP-2 and MMP-9 being the most common forms [35,38,39]. Dentine MMPs remain trapped in the tissue matrix during development. Specifically, MMP-2 is deposited in dentine during tooth development and is still present in mature age up to 40 years old [40].

All forms share a common structure, including (i) a signal peptide, which directs MMPs to the appropriate pathway, (ii) the pro-peptide domain with a cysteine residue, which occupies the active zinc site making the catalytic enzyme inaccessible to substrates until an activation signal is given, referred to as the "cysteine switch", (iii) the catalytic domain

with a zinc ion, and (iv) the C-terminal hemopexin-like domain, which mediates interactions with substrates and defines specificity [41]. Their function relies on two ions of Zn^{2+} and at least one ion of Ca^{2+} bound on various amino acid residues. Their function is to control metabolic processes which take place in the cell microcosm, by activation and deactivation of their activity. In pathological conditions, the balance is often shifted towards over-activation, leading to excessive degradation of the extracellular matrix.

Their activation, and thus their enzymatic activity, is regulated on several levels, including the regulation of transcription, secretions, activation and inhibition. For most of them (except MMP-2), the most important step in regulation is the transcription from DNA. Thrombin upregulates MMP-2 and -3 mRNA [42] and hypoxia longer than 24 hours causes an increase in MMP-2 mRNA expression [43]. MMPs are secreted from the odontoblasts as proenzymes and their activation is a critical step that leads to collagenolysis [41]. Although they are activated in acidic pH, they function best at neutral pH [34]. A wide range of systemic diseases (like arthritis, oncogenesis, multiple sclerosis, osteogenesis imperfecta, Alzheimer's disease, bronchial asthma etc) is associated with the over-activation of MMPs or with lack of their natural inhibitors [41].

2.1.2. Cysteine Cathepsins (CCs)

CCs (B, L and K) [15,18,44] consist another group of dentine endopeptidases, capable of degrading extracellular matrix proteins such as collage type I and III. Since they were detected only a few years ago, there is not much information available. Unlike MMPs which cleave collagen fibrils at a single site, generating two collagen fragments, CC-K can cleave collagen at multiple sites generating fragments of various lengths [45]. Their expression by human odontoblasts and their activity in dentine was recently investigated [44] and they are also associated with caries progression and failure of restorations [31,27,18]. Their collagenolytic activity varies according to the depth of their localization and in contrast to MMPs, their optimum acidity to function is pH 5, since they are rather unstable at neutral pH [27].

2.2 Adhesive bonding in dentine

Resin composites have gradually turned out to be the most indicated restorative material, also for posterior teeth [46,47,1]. Composite materials however require an intermediate bonding agent or dental adhesive, which penetrates enamel and dentine, primarily establishing the micromechanical bonding [48]. Bonding with any commercially available bonding system requires the same three phases: etching, priming, bonding [4,49,6]. Dental adhesives are however divided into two categories, regarding their bonding mechanism; total-etch (or etch-and-rinse) adhesives and self-etch adhesives [3,6]. Total-etch adhesives require a distinct etching phase performed by 35 – 37% phosphoric acid gel, while self-etch adhesives demineralise by means of acidic primers or acidic components.

Enamel and dentine bonding is a form of tissue bioengineering, where minerals are replaced by resin monomers to form a hybrid biostructure. During etching of dentine, inorganic content is removed with phosphoric acid or by means of an acidic primerin a 5 μm – 10 μm depth [3,6], to achieve a superficial demineralisation of dentine in order to create micro-retentive porosities [50]. Demineralised collagen network should then be primed before proceeding to the last step of infiltration of the collagen with the adhesive resin, in order to polymerize and form two basic structures [3,6]; the hybrid layer [51] and the resin tags [52]. The hybrid layer is a structure which connects the hydrophobic adhesive with the hydrophilic dentine and is comprised of collagen fibrils, and proteoglycans enveloped by infiltrated polymer chains of the adhesive [53]. This polymer-collagen layer is basically responsible for the bonding effectiveness of composites, which does not rely on its thickness or on the number and length of the tags but rather on its quality [54]. Resin tags represent the micromechanical anchores of the hybrid layer inside the etched dentine, and more specifically the open dentinal tubules.

2.3 Why do adhesive restorations fail?

The key to success in adhesive dentistry relies on the durability of the adhesive interface overtime, however this is practically hard to achieve. Almost 20 years ago it was exhibited that the adhesive bond to dentine fails [55] and since then, it still remains as a concern [56,2,15,4,31,18,57]. It is widely accepted that resin-dentine bonds deteriorate over time, as exhibited *in vitro* after 1 year [16], after 4 years [58], after 5 years [59], after 6 years [60],

in animal studies [61] and *in vivo* in human teeth [62–64]. Bond failure occurs mainly due to hydrolytic and enzymatic degradation, which are better described as a vicious circle, rather than two indepenent challenges. Several other factors, which result in either hydrolytic or enzymatic degradation, are associated in the literature with reduced longevity of the adhesive bond to dentine, those being: application of simplified adhesives [65,66], phase separation between hydrophobic and hydrophilic monomers [7], sub-optimal polymerization and monomer conversion [7], degradation of resin components [65,2], moisture control during bonding – or the absence of it [3,6,7] – and last but not least, the operator effect [67,68]. Apart from the intrinsic factors mentioned, bacteria from the oral cavity are able to adhere on restorative materials [69] and in case of favorable conditions, they can further extrinsically degrade the restoration margins [70], contributing this way to final restoration failure.

2.3.1. The role of water

Preservation of collagen network integrity in a well-formed hybrid layer is vital to preserving dentine bond overtime [18]. Since diffusion of resin monomers in the collagen network of demineralised dentine shows a decreasing concentration gradient [71], collagen fibrils at the bottom of the hybrid layer may remain uncovered and thus structurally unstable [53,15]. Moreover, depth of demineralisation can be greater than the infiltrating potential of resin monomers and the monomer size (~ 2 mm diameter for adhesive monomers such as TEGDMA) is not small enough to penetrate the nanometric voids between collagenmolecules, ranging from 1.26 – 1.33 nm [72]. In absence of hermetic encapsulation, these empty interfibrillar spaces are subsequently filled with water and are prone to hydrolytic and enzymatic degradation [56,30,32,73]. Moreover, ionic and hydrophilic resin monomers contained in dental adhesives [65], so as to enable bonding with wet dentine substrates, or to etch and bond dental tissues simultaneously, may have additional undesirable effects. Permeable, unstable resin matrices may be produced by them, allowing water sorption, resin leaching, plasticization of the polymer network and hydrolysis to occur over time [56,74,75]. In the long run, adhesion with etch-and-rinse adhesives is compromised by themselves [36], since those adhesives include a hydrophobic

resin which cannot sufficiently infiltrate dentine matrix and are therefore more prone hydrolytic degradation [58,2].

2.3.2. Hydrolytic and enzymatic degradation

As if bonding to dentine was not a challenge for the clinician by itself, evidence of collagenolytic activity in dentine was first reported both in carious and sound dentine in 1983 [76], and some years later, *Pashley et al. 2004* proved that MMPs are involved in dentine degradation in absence of bacteria [15]. Those degraded sites correspond to nanoleakage patterns [66] or increased MMP activity [15,77–79]. Collagen, which is suboptimally infiltrated by the adhesive during the formation of the hybrid layer [53], may be degraded overtime by dentine MMPs in presence of water [15,77–79,59]. Hydrolytic activity by host-derived proteases seems to start at the bottom of the hybrid layer, where the porous sub-optimally infiltrated areas are located. The major significance of water for the functionality of MMPs was demonstrated by a decrease of resin-dentine interface degradation after storage in mineral oil, proving that MMPs are in fact hydrolases [15]. Adding to that, the breakdown of collagen creates more available space to be filled with water, boosting the vicious circle of hydrolysis, enzymatic degradation and long-term bond deterioration [73].

Endogenous dentine peptidases (MMPs and CCs) are released and activated iatrogenically during adhesive procedures, exposing collagen network to their collagenolytic activity. There is compelling evidence that dentine treatment with either total-etch or self-etch adhesives activates precursor forms of proteases that would have otherwise remained inactive [78]. Routine application of acidic monomers (pH 1.5 – 2.7) on dentine promotes activation of MMPs without denaturing the enzymes, resulting in a 14- to 15-fold increase in their collagenolytic activity [15,79]. However, the extent of MMP activity seems to be pH-dependent [79]. Another study shows that self-etch adhesives with pH ~ 2.4 leave less exposed collagen, since demineralisation and infiltration occur simultaneously, exposing fewer proteases [74]. In contrast, the very low pH of the phosphoric acid (pH 0.1 – 0.4) during separate etching at etch-and-rinse systems, denatures the enzymes and decreases MMP activity [79,15,78]. Controversial results showing increased MMP activity after application of phosphoric acid, [80,36], are related to the influence of acid etching on

soluble vs matrix-bound proteases in dentine, since matrix-bound enzymes are unaffected by low pH and continue to act against collagen. A different explanation is given by the group of *Iwasa et al. 2011* which suggested the formation of a protective $CaHPO_7$-layer after separate etching, which temporarily masks the collagen fibrils from proteases activity [81]. Furthermore, while acid etching reveals endogenous MMPs, etching and rinsing can also cause loss of Ca^{2+} and Zn^{+} ions, which are necessary for their collagenolytic activity [82], leading to their inactivation.

Enzymatic degradation is detected by MMP-2 and MMP-9 during the adhesive procedure with three-step etch-and-rinse adhesives [36,59], two-step etch-and-rinse [83,84] with mild two-step self-etch adhesive [36,79] or with one-step self-etch adhesives [79,83,85] and MMP activation seems to be product-dependant [83].

Apart from their interference with the adhesive interface of bonded restorations, MMPs and CCs have also been related to autodegenerative processes in dentine, such as the inflammation of dental pulp [86] and progression of caries lesions [29,34,27,8,76]. Lactic acid produced by cariogenic bacteria during caries progression may also activate MMPs [29]. While salivary enzymes may access outer, caries-infected dentine, they do not contribute to degradation of caries-affected dentine, during which dentinal fluid rather than saliva could be the source of increased collagenolytic activity [87]. Likewise, dentine matrix-bound endopeptidases may not necessarily be readily activated after simple *in vitro* demineralisation. However endopeptidases decrease mechanical properties of caries-affected dentine and reduce its ability to remineralise [8].

If biodegradation of the adhesive interface is to be avoided, a complete infiltration of resin monomers into the collagen network is fundamental. Despite the fact that the goal is both clear and reasonable, it is not so easily attained. For that reason, strategies to counteract these actions in the adhesive interfaces have been explored, including inactivation or blockage of MMPs during application of the adhesive protocols and management of secondary caries which could further threaten the permeability of the composite restorations.

2.3.3. Dental caries

Dental caries is an irreversible, infectious disease of the calcified tooth tissue, involving demineralisation of inorganic compounds, mainly hydroxyapatite, by acids produced by oral bacteria. Therefore, caries affects teeth through a series of episodic and cyclical bacterial and chemical events that result in carious lesions and, if remain untreated, finally cause tooth loss. Caries is produced when pathogenic bacteria are gathered on a susceptible host – the tooth surface – in an environment rich in fermentable carbohydrates, for a sufficient length of time for the cariogenic process to take place. Cariogenic properties of *S. mutans* [88] and their relationship with caries [89] and with sugar intake [90], have led to the perception that mutans streptococci are the main pathogen which causes dental caries. Apart from *S. mutans*, *Lactobacillus spp*. is also highly associated with caries [91]. Cariogenicity however depends more on diet than the prevailing bacterial species, and thus bacterial counts alone cannot alter caries activity or caries risk [91].

Caries initiation and progression involves a continuous balance between demineralisation and remineralisation, which take place in the oral cavity. Demineralisation occus when pH in dental plaque, and thus on the tooth surface, decreases below 5.5. In acidic pH, bacterial organic acids are able to diffuse into calcified dental tissues, leading to dissolution of apatite crystals [29], which can be clinically seen as a ‘’white spot’’. Active enamel lesions comprise of surface erosion and subsurface porosity [92]. Demineralisation may then continue deeper in dentine, leading to destruction of organic matrix by proteases, either of bacterial origin as initially described [93] or as more recently exhibited, by endogenous MMPs [29,34,27,8,76]. As next, saliva buffers neutralize bacterial acids and allows for remineralisation to take place, even after half of the mineral compound from caries-affected dentine is lost. This dynamic process is repeated numerous times daily and if balance is lost, demineralisation predominates and caries progression takes place [29]. However, regarding remineralisation, recent evidence demonstrates that dentine collagen matrix does not necessarily remain as intact as believed during caries demineralisation, since structural changes in collagen are noted [87] and a true subsurface remineralisation is rarely achieved [92,92].

2.3.4. Secondary caries

Replacement of failed restorations is a major problem contributing to the expensive circle of re-dentistry [10]. Although in the 1970s restorations were failing due to degradation and wear of materials, nowadays the main cause of restoration failure is secondary caries [1,10,94,95]. Specifically for Class V restorations, it has been demonstrated that 62.5% of them fail after 5 years due to reasons associated with their marginal integrity [96]. According to FDI (World Dental Federation), secondary or recurrent caries is defined as "positively diagnosed carious lesion which occurs at the margins of an existing restoration" [97] and has the same pathology with primary caries [10]. This lesion usually consists of two regions: an outer lesion formed on the tooth surface, having similar histological characteristics with primary caries, and a wall lesion, which is a narrower defect in the enamel or dentine along the cavity wall [9].

Secondary caries is caused because of microleakage of fluids, bacteria, toxins and ions through the material – tooth interface [11]. This space varies between 2 - 20 μm at the cavity floor and 1 – 10 μm at the lateral restoration walls [98] and bacterial biofilm gathered in up to 8 weeks, may be 2 – 15 μm [52]. The growth of bacteria is either due to invasion of oral bacteria through restoration margins or due to retained bacteria in the cavity, which gain access to their nutrients though microleakage [99]. Even though a threshold marginal gap size for clinical failure of the restorations has not been established [100], restorations with marginal defects fail more frequently [101]. There is a close relationship between marginal adaptation of restorations and bacterial growth in cavities, underlining the fact that bacterial growth results from the communication of the oral environment with the restored cavity [99]. The development of wall lesions could depend both on the amount of the accumulated plaque on the outer surface and the micro-gap between the restoration and the tooth tissue [10]. Since composite restorations accumulate more biofilm, they are subject to faster bacterial degradation [69], and the extent of degradation of the restorative composite or of the adhesive, are dependent on their chemical formulation [70]. Furthermore, issues which arise from, or are connected to adhesive bonding [7] (such as polymerization shrinkage, hydrolytic and enzymatic bond

degradation) or technical failures such as fractures, defective contours, overhanging margins [9], can result in gap formation between the composite and the tooth tissues.

2.4 How can restoration failure be avoided?

In order to elongate the duration of adhesive restorations and prevent their failure, action should be taken against hydrolytic degradation, enzymatic deterioration and bacterial attack during secondary caries process. Since hydrolysis is difficult – if not impossible – to prevent, in a tissue which consists of ~ 22% water and 33% organic compounds, research is focused on ways to reduce enzymatic activity intrinsically and on antibacterial strategies to reduce the risk of secondary caries extrinsically.

2.4.1 Chlorhexidine (CHX)

CHX is a cationic-bisguanide ($C_2H_7N_5$), broad-spectrum antimicrobial agent which is extensively used inoral hygiene products. Although clinical studies have shown that CHX varnishes are effective in reducing *S. mutans* counts for 3 months after a 10-day application period [13], there is not enough evidence that over-the-counter preparations can influence caries progression [12]. However when a CHX was tested in artificial mouth systems, it presented positive results against early caries as an emulsion [14] and reduced successfully bacterial counts inside cavities compared to ozone disinfection [102].

CHX possesses both bacteriostatic and bactericidal effects against Gram+ and Gram- [103,104] depending on its concentration and acts by disruption of the cell membrane [105] by binding on the lipopolysaccharides of the bacteria [104]. Its antibacterial action against *S. mutans* [106] was known long before the importance of CHX in restorative dentistry and its beneficial role in adhesive restorations was discussed. CHX was first used as a dentine disinfectant and re-wetting agent prior to adhesive bonding, since it did not influence the immediate bond strengths [107,108], before realizing its activity against collagenases and gelatinases (MMPs) [109,15,110] and more recently against CCs, and specifically against CC-B, -K and –L [19,44]. It is believed to act by cationic chelation, sequestrating Ca^{2+} and Zn^{2+} ions, which would otherwise activate the MMP catalytic domains [110, 82].

CHX is able to bind to acid etched dentine and be slowly released overtime [111] without promoting deleterious changes on collagen structure [112]. It has strong positive ionic

charges, which electrostatically bind the protonated NH_3^+ in the CHX molecule to the negatively charged phosphate groups in mineralized dental tissues or to carboxylic acids in demineralised dentine [111,113]. This binding is increased by acid etching through a potential increase in surface free energy [111,114]. CHX binding mechanism to dental tissues and to enzymes is dependent on CHX saturation and when applied in higher concentrations, CHX may oversaturate the enzyme binding sites and remain bound to collagen fibrils for later release. CHX is then released at therapeutic levels, a phenomenon known as substantivity [115]. This characteristic allows enzyme-bound CHX to remain active after initial application. The association between CHX concentration and its protective effect on bond strength is related but the correlation is not linear [17,111]. It is also exhibited that CHX molecular charge and not its concentration is responsible for its localization in dentine [105] since substantivity levels were same when either 0.2% or 2% CHX was tested [115]. Extent of CHX debinding from the dentinal substrate is greater when rinsed water, than with HEMA, ethanol or NaCl solution [111].

2.4.2 Inhibition of enzymatic degradation

Inhibition of enzymatic degradation would be advantageous in preservation of hybrid layer overtime [18]. Several methods have been suggested in order to inhibit degradation and achieve long-term stability of the resin-dentine interface; among them, incorporation of MMP inhibitors in materials, use of hydrophobic adhesives which exhibit lower water sorption and solubility, application of multiple layers, increasing the curing time and solvent evaporation, or using collagen-cross-linking agents [56,4,32,65,74,31].

Tissue inhibitors of MMPs (TIMPs) are substances which balance the activity of MMPs in dentine and can either be natural or synthetic. The natural compounds include long-chain fatty acids, epigallocatechin and other polyphenols, flavonoids and others and generally exceed the concentration of MMPs in extracellular fluids. So far four proteins have been identified as natural inhibitors (TIMP-1, TIMP-2, TIMP-3, TIMP-4) and they do not demonstrate specificity [41]. Synthetic inhibitors on the other hand, such as CHX, mimic the structure of the natural ones and show selectivity, as at lower concentrations they preferentially target some MMPs rather than others [116]. Although the exact mechanism of inhibition of proteolytic activity is not understood, the inhibitory mechanism is thought

to occur via chelation. Chelation is a particular way that ions and molecules bind metal ions, involving a chelator (ligand) and a single central atom. Inhibitors must therefore contain a functional group capable of chelating the catalytic domain of MMPs, thus preventing binding of the substrate [43,86].

CHX in restorative dentistry is either applied extrinsically as a dentine pre-treatment agent, or added in restorative materials and adhesives, which would act as a carrier or CHX-reservoir.

CHX as dentine pre-treatment agent

Application of CHX in a prepared cavity as dentine pre-treatment is shown to suppress collagenolytic activity in dentine *in vitro* [77,15,80,16,110,117,84,109,17,118–123,16,125] and *in vivo* [63,64,126,127,120,128], even in low concentrations [110] and short-time applications (15 – 30 seconds), in presence of caries-affected [129,130,122] or artificially eroded dentine [121]. Although CHX shows no specificity against certain MMP types, there is a distinct difference between CHX concentrations which are able to inhibit MMP activity when CHX is applied on dentine; 0.002% for MMP-9, 0.0001% for MMP-2 and 0.02% for MMP-8 [110], but *Colares et al. 2013* [17] showed that this correlation is not clear. CHX concentration seemed to have no effect on bond strength degradation after 12 months [16]. In most studies CHX solution as dentine pre-treatment is used at 0.2% - 2% concentrations and is rubbed against or applied and let to act on the etched dentine surface for 15 – 60 seconds [109]. Two studies also demonstrated that application method (with or without rinsing, before or after etching) had no impact on immediate bonding efficiency [131,132]. CHX solutions are produced either with water, with ethanol [133–135] or as CHX-methacrylate [49]. Despite the fact that both solutions (in water or in ethanol) are equally saturated by dentine substrate [135], studies show that ethanol based CHX solutions exhibit a worse behavior in terms of bond strength, when evaluated immediately or after storage [134,133]. CHX as dentine pre-treatment does not generally affect the immediate bond strength with dentine negatively with either total-etch or self-etch adhesives [132], when used in 0.2% [136] in 2% [109,7,131,137,138,121,129,122,132,139,140], in 4% [108], or in 5% concentration [122], since wettability of dentine surface by water or ethanol, which are components of the respective dental adhesive, is shown not to be

compromised by CHX [141] and moreover, formation of hybrid layer is enhanced [125]. A single study even demonstrated that use of 2% CHX led to higher immediate bond strength [84].

Despite the fact that CHX could be tracked in the hybrid layer after 5 years [142] and although it has become the most popular specific MMP inhibitor, it is not known how long the inhibition effect after its application on dentine can last [56]. Studies provide contradictory results, which looks as if they are related to the concentration of CHX and / or to the chemical composition of the adhesive [109]. Some show that CHX pre-treatment exhibits a positive effect after 6-months [134,77,129,143,144], 9 months [138], 1 year [16,49], 2 years even at lower 0.2% concentration [80] or even 5 years [142]. Moreover, there are some studies exhibiting that CHX application does not produce better bonding values immediately [145,109,137,139,140], after 15 days [146] or after 6- [123,133,136,140,147], 15-month storage [133] or 2 years [122]. No difference was shown when instead of a CHX solution, dentine was pre-treated with a CHX-containing air-abrasion powder [148]. Last but not least, other studies indicate that its positive effect on bonding preservation at 6 [109] or 9 months [149], is lost after long-term storage and despite its substantivity CHX eventually leaches out of the hybrid layer due to its electrostatic nature of binding with water, which in turn acts as the desorption medium [113]. Even when CHX is tracked inside the hybrid layer after 8 weeks [16,115] or 5 years [142], the concentration may still be very low to exhibit a therapeutic result.

Clinical trials provide controversial data, as some studies suggest that 2% CHX application on Class V dentine, provides after 36 months reduced retention – however not significantly [150] – and after 6 or 18 months delivered same retention rates [109,151,152] as without CHX. Marginal quality of restorations with and without CHX pre-treatment showed no difference after 12 months [128]. Clinical results in Class I cavities are more encouraging, since they exhibited increased μTBS after 6 months [127,63], 12 months [153,64], 14 months [126] or up to 20 months [120].

Addition of CHX in dental materials

Avoiding to add another step in the adhesive procedure while treating dentine, investigators have been studying the incorporation of CHX in dental materials, either being etchants, adhesives or restorative materials, since 1983 [154].

CHX exhibited positive results regarding bond strength preservation when admixed with the etchant [155,156,142], the adhesive on permanent teeth [157,144,158,142,36,159,160], the adhesive on primary teeth [161] or with restorative materials [103,20]. Hence, addition of an external component in restorative materials, may influence their water sorption, solubility, degree of conversion and mechanical properties [20,21]. However admixing CHX into resins up to 1% concentration exhibited no alteration in their degree of conversion and immediate bond strength [21,162,157]. On the contrary, addition of 1% CHX in restorative composites significantly decreased their compressive strength [154] and CHX release led afterwards to pore formation, diminished mechanical properties [163] and water induced swelling, which is enhanced by the hydrophilicity of the respective composites, resulting in undesirable situations [20]. Similarly, when mixed with glass-ionomer cements, it led to 30% decrease in their fluoride release, due to fluoride interaction with CHX [164] and the material demonstrated decreased compressive strength [165], just as composite resins.CHX is also believed to interfere with the bonding mechanism and maturation reaction of resin-modified glass-ionomer cements, when added in their composition [166].

Since literature shows that restorative materials may be compromised by CHX addition, adhesives were then studied, however studies are scarce. A 2-step total-etch adhesive with 0.2% built-in CHX is commercially available, but according to literature, it can only inhibit anaerobic bacteria [22] and CHX has no effect on the bond strength after 6-month storage [23]. Their degree of conversion was not influenced when CHX was mixed with the adhesives [157,167] but adhesives may become stiffer, as its elasticity decreased [167]. Solubility and water sorption remain unaffected after 28 days [158] or longer [168]. 2% CHX incorporated into the primer showed better inhibition of MMPs after 20-second application [169]. In another study, 2% CHX replaced liquid A of a two-bottle self-etch adhesive, and whatsoever did not alter its bonding efficiency even after 6-month storage

[170]. The authors attributed these results to the similar ionizing effect of CHX solution and liquid A, the later composed by water and HEMA. CHX added into experimental adhesives was proven not to jeopardize immediate bond strength to dentine [159,162,168,144,36] and moreover reduced bond degradation after 12-months [159,160,22,168,36]. CHX addition in adhesives has been investigated in concentrations 0.01% - 5% (Table 1, page 17-18). However, if a long-term release is feasible from this CHX-reservoir, lower concentration of CHX could be preferably admixed in terms of safety regarding mechanical properties. In that direction, it has recently been proposed, that CHX delivery could be accomplished through incorporated nanocapsules, a method which showed adequate CHX release up to 25 days [171]. Since clinical studies with CHX experimental materials are difficult to be performed due to ethical issues, only one *in vivo* study with self-etch CHX-adhesives is available and exhibits no difference in retention rates of 126 restorations after 2 years [172].

The biggest advantage of creating a CHX-reservoir inside the material instead of applying CHX on dentine, would be its elongated release and long-term action. Mechanism of CHX release has however been associated to water-induced swelling [20], which is enhanced by the unavoidable hydrophilicity within the adhesive interface structure. Despite the fact that the exact duration of CHX action is not known, CHX released from CHX-etchant and CHX-primer has been identified inside a 5-year hybrid layer [142], possibly due to its high substantivity [115]. Rate of CHX release is increased when mixed with hydrophilic materials [20] and in acidic environment due to the increased solubility of CHX in low pH [163]. A 24-hour burst of CHX release from dental materials followed by a rapid decrease has also been noted [21], as with most of the antibacterial substances [173].

Table 1: Summary of *in vitro* studies with CHX added into adhesives.

Study	Type of test	Type of adhesive	Application of adhesive	CHX Type	CHX wt%	Teeth / sticks per group	Storage time & medium	Adhesive interface & geometry
Yiu et al. 2012 [159]	μTBS (1 mm / min)	2-step experimental total-etch 50% ethanol / 50% monomers	15´´ 2 layers 10´´ air 20´´ LP	CHX diacetate	2%	5 / 40	24 h / 12 months - Artificial saliva	0.81 mm^2 sticks
Nishitani et al. 2013 [157]	μTBS (1 mm / min)	1-step self-etch AQ Bond Plus, AQP Sun Medical, Shiga, Japan + experimental	20 ´´ 1 layer 10´´ air 20´´ LP	CHX diacetate	0.5% 1% 2% 5%	3 / 10	24 h	0.7 mm^2 sticks
Zhou et al. 2009 [160]	μTBS (1 mm / min)	2-step self-etch Primer of Clearfil SE, Kuraray, Tokyo, Japan	(no data available)	CHX diglugonate	0.05% 0.1% 0.5% 1%	4 halves / 32-40	24 h / 12 months - 0.9% NaCl+ sodium azide	0.6 mm^2 hourglass
Zhou et al. 2010 [162]	μTBS (1 mm / min)	2-step self-etch Primer of Clearfil SE, Kuraray	According to manufacturer	CHX diglugonate	0.05% 0.1% 0.5% 1%	8 halves	1 week – distilled ater 37°C	0.6 mm^2 hourglass
Pomacóndor-Hernández et al. 2013 [170]	μTBS (0.5 mm / min)	1-step self-etch Scotchbond™ SE (replacement liquid A	(no data available)	(no data available)	2%	2-3 halves / 21-26	24 h / 3 months / 6 months - Water	0.8 mm^2 sticks

André et al. 2015 [22]	µTBS (1 mm / min)	1-step self-etch Peak® universal bond (0.2% CHX), Ultradent, Cologne	According to manufacturer	CHX diacetate	0.2%	≈3 / 10	24 h / 1 week / 12 months – Artificial saliva	1 mm^2 Sticks
de Munck et al. 2009 [36]	µTBS (1 mm / min) Zymography	Optibond™ FL, Kerr Dental, CA, USA 1-step self-etch Clearfil SE, Kuraray + experimental	According to manufacturer	CHX diacetate	0.05%	< 1 half / 2-4	1 week / 3 months / 6 months / 12 months - Water	1 mm^2 sticks
Stanislawczuk et al. 2014 a [144]	µTBS (0.5 mm / min)	1-step self-etch Ambar, FGM, Joinville, Brasil 1-step self-etch XP Bond Plus, Dentsply De Trey, Konstanz	According to manufacturer	CHX diacetate	0.01% 0.05% 0.1% 0.2%	2,5 / 55-63	24 h / 12 months - Water	0.8 mm^2 sticks
Stanislawczuk et al. 2014 b [158]	UTS / water sorption / solubility / CHX release						24 h / 1 month - Water	
Da Silva et al. 2015 [168]	µTBS (0.5 mm / min) Degree of conversion / water sorption / solubility	Experimental total-etch adhesives	2 layers 5´´ air 20´´ LP	CHX diacetate	2%	2 / 36-40	24 h / 6 months / 12 months - Water	1 mm^2 sticks
Cadenaro et al. 2009 [167]	Degree of conversion / E-modulus	Experimental resin blends with increasing hydrophilicity corresponding to 1-, 2, and 3-step adhesives		(no data)	1% 5%			

LP: light polymerization

2.4.3 Inhibition of bacterial action

Despite the fact that restorative materials have evolved regarding their basic mechanical, physical and bonding properties, an innovation would be the exhibition of "therapeutic effect". Modern restorative materials should be able to interact with dental tissues and the oral environment in a favorable manner – the so-called bioactive materials – and among all possible interactions, their antibacterial action is highlighted as one of the most important theurapeutic effects. Inhibition of bacterial action via incorporation of antibacterial substances in many dental materials, such as composite resins, resin cements, glass-ionomer cements, provisionary cements and adhesives is extensively discussed [5,69,94,5,174]. However, a review of the Cochrane Collaboration concludes that there is not enough clinical data to assess the ability of antibacterial restorative materials to prevent dental caries [94]. Despite the fact that adhesives possess an antibacterial effect themselvesdue to their low pH, this is limited to 24 – 48 hours [175] and their acidity is neutralized by their contact with the tooth tissues [5]. Therefore, materials with a longer-lasting anti-bacterial effect were developed, mainly by incorporation of antibacterial substances in their composition, which can be i) releasing, soluble antibacterial agents, ii) non-releasing co-polymerized antibacterial agents and iii) inorganic fillers [69,5]. The main advantage of soluble antibacterial agents, such as CHX, is that they can easily be released from the restorations to the oral environment. Immobilization of polymerizable antimicrobials such as quartenary ammonium salts (MDPB being the most popular) is a different approach, offering long-lasting activity. Antibacterials which are added into the materials in forms of fillers are silver and zinc oxide [173,176]. The antibacterial substances which are released from the materials used as carriers may challenge their kinetics of release or affect the physical properties of their carriers. Inevitably, their antibacterial activity decreases over time [69]. Issues concerning the physical properties of the carrier-materials and their release potential also arise for the non-releasing antibacterials that are in-situ polymerized. Moreover immobile antibacterials can only kill bacteria which come in contact with the adhesive [5]. Regarding antibacterial fillers, and especially silver, polymerization may be negatively influenced by them, let alone their poor color appearance [177].

Resin cements containing 3-4% CHX exhibited CHX release for 5 weeks and antibacterial action for 2 weeks, while no CHX release was detected when its concentration in the cement was 2% or lower [103]. No inhibition zone against bacteria was produced by CHX-containing resin after 2-week storage [20], showing that CHX concentration was not enough. When CHX was incorporated in ion-exchanging materials, such as glass-ionomer cements, its antibacterial activity against *S. mutans* increased to 90 days [164,154,178] and their inhibition zones were not dependent upon CHX content [174]. *In vivo*, CHX-containing glass-ionomer cements decreased the microbial count in dentine under the restoration after 7 days [179] and after 3 months [180], but a CHX-containing glass-ionomer cement pit-and-fissure sealant did not increase caries reduction in 12 months [181]. There was no published clinical study investigating the antibacterial effect of CHX in adhesives.

2.5 Caries models

Experimental tests to simulate dental caries have greatly varied since *Magitot, 1878* [182], *Miller, 1905* [183] and *Pickerill, 1919* [184] reported their first attempts to generate cares *in vitro*. Many methods have since then appeared in the literature [69], but are all derivations of two basic systems: bacterial cultures with nutrient systems, known as bacterial models or omission of bacteria [14,185,176] and chemical systems, known as chemical or static models [186,187], which use either acidic gels or buffered solutions [186].

The ultimate goal of the caries models is to produce a caries-like lesion corresponding to the three zones of an early enamel lesion; from inner to outer being the translucent zone, the dark zone and the body of lesion [188]. It is demonstrated that dentine caries produced by caries models resembles natural dentine caries both histologically and microradiographically, since both include subsurface mineral loss, producing different zones [189]. Others state that artificial dentine lesions represent usually only a single demineralisation phase and this contrasts with the natural way of caries production which consists of multiple de- and remineralisation cycles [186]. When it is attempted to mimic lesions observed *in vivo* with caries models, several factors such as substrate type, lesion type and depth, severity of cariogenic challenge and type of microflora may influence the

reactivity of hard tissue, and hence development of the lesion [186]. Fundamental requirements for an effective caries model are: pH control, pH cycling reproducing de- and remineralisation phases, simulation of intra-oral sugar effects for the demineralisation phase, adjustment of saliva effect and of sugar clearance for the remineralisation phase and choice of nutrition medium being exactly adjusted to the bacteria under investigation [190].

Clinical studies are regarded as the ultimate way to gather scientific evidence on the clinical effectiveness of a restorative treatment [191]. However, by producing caries-like lesions *in vitro*, factors implicated in caries aetiology can be separately investigated, a large number of samples can be studied and ethical issues connected to animal trials are set aside [187]. Compared to clinical studies, caries model tests are often less expensive and results are produced much quicker. Since variations in dietary patterns in real-life are moderate [192], caries models which standardize the sugar intake, are able to provide results that correspond well with the majority of dietary patterns. The purpose of a caries model is to separate a complex system into more simple parts, so as to study defined aspects of caries under controlled experimental conditions. Most of the studies performed with antibacterial agents in adhesives involved evaluation of their effect in with the agar diffusion methods, assessment of bacterial growth through colonies counting, minimum inhibitory concentration or bactericidal, biofilm accumulation or bacterial adherence tests [69]. All the fore-mentioned methods provide however an indirect assessment of the antibacterial potential in the oral cavity. Since experimental adhesives cannot be tested *in situ* and since ethical issues arise with animal studies, let alone their differences to human teeth, bacterial microcosm models may offer simulated oral cavity conditions [69]. The first study designed to test anti-caries agents in a caries model, was conducted by *Pigman and Newbrun, 1962* [193]. Models can be of great value in predicting behaviours, but by definition a model will always differ from the natural situation; the question is to what extent. For that reason, the extent of resemblance of artificial caries-like lesions against natural ones is an issue under discussion.

In general an early caries lesion is observed clinically as a white opaque spot, being softer than the surrounding sound enamel. Lesions produced by caries model have the macroscopical appearance of initial caries and encircle the restorations [194]. *In vivo* initial

enamel lesions do not have a surface layer but develop this mineral-rich layer later on [195]. Absence of the dark zone in experimental caries is also discussed [194]. It is also not clear if demineralisation is combined with erosion in caries model systems, even in bacterial models, since both tooth defects are related to hypomineralized tissues but the etiology of mineral loss differs. In cases of erosion, enamel is demineralised by direct contact with acids and is primarily a surface phenomenon, while caries is formed by the action of acids produced by bacteria, and begins as a subsurface lesion that eventually leads to a pit in the tooth surface [196]. However, chemical caries models present more parameters which may influence the similarity of these lesions to natural caries, like viscosity of the fluids, [194] and are not able to simulate factors such as biofilm concentration, saliva flow and collagen degradation [186]. Plaque thickness built around restorations at bacterial models, using carbohydrate broth inoculated with *S. mutans*, results to different potential of penetration through marginal gaps and thus different caries induction at cavity walls [197]. Bacterial caries models result in caries-like dentine lesion with similar surface hardness, lesion depth, and calcium and phosphate ion concentration as natural lesions, thereby providing a more realistic simulation of oral conditions than chemical caries models or even *in situ* experiments, the latter of whichcan only have a limited duration of time [186]. Bacterial caries model are also able to create an infected outer layer and an affected inner layer like in natural dentine caries [186]. Metabolic and pH behavior of plaque produced by bacterial caries models is typical of natural plaque [198] and *de Campos et al. 2015* [199] suggested an optimal period of 8 days for producing non-cavitated caries-like lesions.

2.6 Bond strength tests

Bond strength testing is accomplished by manufacturing adhesive interfaces, in most cases tooth – material specimens, which is loaded to failure with either shear or tensile load or in push-out mode. By definition, the ideal bond strength test should be easy, in order to have low technique-sensitivity and be reproducible and fast, in order to permit loading of multiple specimens [191]. According to the extent of the adhesive area, bond strength testing methods can be divided into “macro” and “micro” tests, referring to bonded area below 2 mm^2 [200]. After *Okumo et al. 1970* [201] introduced the idea of μ-tensile bond

strength (μTBS) and *Sano et al. 1994* [202] tested it in human and bovine dentine, this method has been widely used and accepted as an "ideal technique for evaluating the long-term durability of resin-hard-tissue bond" [203]. μTBS is calculated as the tensile load at failure divided by the adhesive are of the bonded interface. Although a consensus or standardized approach does not exist in dentistry, bond strength testing remains useful and necessary for the control of new products and study of experimental variables [200]. Among its benefits is that it allows for measurement of high bond strengths without cohesive failure in dentine, it subjects specimens to uniform loading, and permits multiple measurement to be performed from a single tooth.

In contrast to clinical studies, laboratory testing such as a bond strength test, allows for quick gathering of data with relative ease and minimum cost, simultaneously testing more groups with one study set-up, measuring one parameter while keeping the other variables constant, incorporating aging methods in the study and directly comparing experimental or new materials with that of the current "gold standard" [191]. The final objective however of an *in vitro* test, such as bond strength testing, should obviously be the prediction of the eventual clinical outcome according to the gathered laboratory data. It is noted that *in vitro* immediate μTBS are higher than *in vivo* bond strengths recreated in the same study [153]. That resemblance was not however observed in μTBS values after *in vitro* (5000 thermocycles) and *in vivo* aging (6 months in oral function) [153]. It is also shown that there is an association between clinical outcomes and laboratory results of bond strength studies [204].

3. Aim of the study – Null hypotheses (H_0)

The aim of the study was to test CHX adhesives for their ability to inhibit formation of secondary caries-like lesions around Class V composite restorations when loaded biologically with *S. mutans* in an artificial caries model and for their potential to bond to dentine despite CHX addition, to maintain bond strength after 6- and 12-month storage and to withstand adhesive bond deterioration after biological loading. The following questions were placed:

1. Is experimental or industrial addition of CHX in the adhesives able to inhibit secondary caries formation around Class V composite restorations?
2. Is it possible for the CHX adhesives to have bond strength values at baseline, after 6- and 12-month storage same to the control group?
3. Can CHX adhesives maintain their bond strength after 6- and 12-month storage compared to baseline?
4. Is there a difference between artificial saliva and distilled water used as storage media in 6- and 12-month storage?
5. Can CHX adhesives withstand bond strength reduction after biological loading with *S. mutans*?

Null hypotheses (H_0):

1. Experimental or industrial addition of CHX in the adhesives is not able to inhibit secondary caries formation around Class V composite restorations.
2. It is not possible for CHX adhesives to have bond strength values at baseline, after 6- and 12-month storage same to the control group.
3. CHX adhesives cannot maintain their bond strength after 6- and 12-months storage compared to baseline.
4. There is no difference between artificial saliva and distilled water as storage media in 6- and 12-month storage.
5. CHX adhesives cannot withstand bond strength reduction after biological loading with *S. mutans*.

4. Materials & Methods

4.1 Teeth collection & storage

Upon approval of the Ethical Committee of the Medical Faculty of Justus Liebig University Giessen (AZ 143/09), n=224 healthy, intact upper and lower human 3rd molars were collected, immediately after extraction, cleaned with a scaler (H5 Hygienist/U15 Towner Scaler, Hu-Friedy, Frankfurt) under water irrigation and stored in 0.5% Chloramin-T solution (Chloramin T trihydrate, Carl Roth, Karlsruhe) in 5-7ºC for up to 30 days. In case longer storage was needed, teeth were refrigerated (-15ºC) in distilled water until further use. After visual examination under 3X magnifying dental loupes, teeth which were identified with signs of caries, visible fractures or damage during extraction were excluded from the study.A single operator performed all experimental steps. After collection, teeth were divided into five experimental groups according to the adhesive protocol used (Figure 1, page 28).

4.2 Adhesive systems

Five different adhesive bonding protocols were used, including a 3-step bonding system, two experimental CHX adhesives and a 2-step etch-and-rinse CHX adhesive (Table 2, page 26). The experimental adhesives were built on the basis of the 3-step bonding system, according previous literature [160,162]. Application procedure is demonstrated in Table 3, page 27. During adhesive application brush tip was allowed to soak in the solutions and was then rubbed against dentine for 10 seconds. For μ-tensile test specimens, application time for all liquids was doubled in order to cover the larger area of the exposed dentine surface. Primer was air-dried in order to allow for sufficient solvent evaporation and air-thinning was performed for the bonding agent, until no visible liquid movement. No rinsing was performed after application of CHX as dentine surface pre-treatment. Polymerization was performed with a LED polymerization unit for 20 seconds according to manufacturer's instructions (1200 mW/cm^2 light intensity, Elipar™, 3M Healthcare, Seefeld).

Table 2: Information of materials used according to manufacturer's data.

Product – Manufacturer	Type	Composition (% by wt.)	LOT
Adper Scotchbond™ Multipurpose Adhesive System, 3M Healthcare	3-step etch-and-rinse bonding system	**Scotchbond™ Etchant**: 55-65% Water, 30-40% Phosphoric Acid, 5-10% Synthetic Amorphous Silica **Primer:** 40-50% Water, 35-45% 2-HEMA, 10-20% Copolymer of acrylic and itaconic acids **Bonding:** 60-70% BISGMA, 30-40% 2-HEMA, <0.5% Triphenylantimony	516827 N510460 N515442
Peak® Universal Bond with 0.2% Chlorhexidine, Ultradent, Cologne	2-step etch-and-rinse bonding system	**Ultra-etch:** <45% Phosphoric Acid **Adhesive:** <20% Ethyl Alcohol, ≤16% 2-HEMA, ≤6% Methacrylic Acid, <0.3% Chlorhexidine di(acetate), 7.5% Fillers	B8ZG1
Gluco-CHeX 2%, Cerkamed, Stalowa Wola, Poland	Chlorhexidine Digluconate	2% Chlorhexidine gluconate	1806131
Filtek™ Z250, 3M Healthcare	Composite Resin	75-85% Silane Treated Ceramic, 1-10% BISEMA6, 1-10% UDMA, 1-10% BISGMA, <5% TEGDMA, <5% Aluminum Oxide, <0.5% Benzotriazol, <0.2% EDMAB	N512895 N561790 N608865 N635023

Table 3: Application directions of the adhesives used.

Bonding system used	Group	Preparation of CHX adhesives	Application Steps
Adper Scotchbond™ Multipurpose Adhesive System, 3M Healthcare	CTRL	-	1, 2, 3, 4, 5, 6, 7
	DENT	-	1, 2, 9, 3, 4, 5, 6, 7
	PRIM	Mix 0.5 µL of 2% CHX digluconate and 9.5 µL Scotchbond™ Primer = 5% v/v CHX PRIMER	1, 2, 3 (CHX PRIMER), 4, 5, 6, 7
	BOND	Mix 0.5 µL of 2% CHX digluconate and 9.5 µL Scotchbond™ Bonding = 5% v/v CHX BOND	1, 2, 3, 4, 5 (CHX BOND), 6, 7
Peak® Universal Bond with 0.2% Chlorhexidine, Ultradent	PEAK	CHX industrially admixed	1, 2, 8, 4, 7

[1] Etch enamel (30 s) and dentine (15 s) with phosphoric acid, [2] Rinse for 30 s and dry, [3] Apply primer with an applicator brush to enamel and dentine for 10 s, [4] Air-dry gently for 5 s from 10 cm distance, [5] Apply Bonding with an applicator brush to enamel and dentine for 10 s, [6] Air-thinning, [7] Light-cure for 20 s, [8] Apply adhesive with applicator sponge and scrub for 10 s, [9] Apply 2% CHX on dentine with an applicator sponge for 10 s and air-dry.

For the experimental adhesives 2% chlorhexidine digluconate (Gluco-Hex 2% Solution, Cerkamed) was admixed into the primer or bonding agent (Table 3). Final solutions contained 0.1% CHX. Adhesive and CHX were thoroughly mixed with a 2-mm sized brush applicator for 20 seconds and the mixture was allowed to set for 10 seconds. Fresh quantity of CHX adhesive was prepared for each tooth. Following groups were formed: 1) control group (CTRL), 2) 2% CHX dentine pre-treatment (DENT), 3) 0.1% CHX in primer (PRIM), 4) 0.1% CHX in bonding agent (BOND), 5) Peak® Universal Bond with 0.2% CHX (PEAK).

4.3 Study design

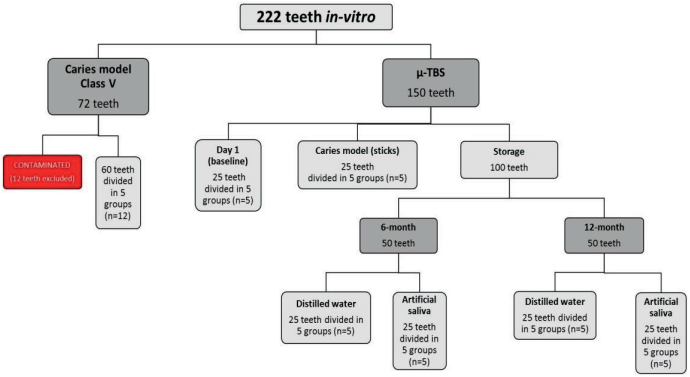

Figure 1: Schematic demonstration of the study design and steps of the two tests performed i) caries model with Class V restorations and ii) μTBS test at baseline, after biological loading and after 6- and 12- month storage (n=number of teeth).

Table 4: Number of specimens in each group. Teeth are regarded as specimens for the Class V caries model, while sticks are the specimens for μTBS test.

Group	**Caries Model (number of teeth)**	**μ-tensile bond strength test (number of sticks)** **n=5 teeth for each group*					
	Caries Model Class V	Day 1 (baseline)	Caries Model Sticks	6-month water	6-month saliva	12-month water	12-month saliva
CTRL	12	99	102	115	93	96	84
DENT	12	105	109	96	106	92	92
PRIM	12	108	99	111	106	88	106
BOND	12	106	107	98	99	85	115
PEAK	12	103	105	90	102	81	81
TOTAL	60	521	522	510	506	442	478

4.4 Specimens

4.4.1 Fabrication of specimens for caries model (Class V caries model)

Standardized buccal Class V cavities (4-5 mm in width mesio-distally, 2-3 mm height, 2 mm depth) (Figure 2, page 31) with margins located 50% in enamel and 50% in dentine or cementum were prepared with a cylindrical round-end diamond bur (Revelation Diamond #881-014C, SS-White Burs, Pennsylvania, USA) and a high-speed handpiece (Alegra TE-95, W&H Dentalwerk, Bürmoos, Austria) under water irrigation. Consequently a 2-mm depth-indicator bur (Diamond Bur FG 2mm, Meisinger, Neuss) was used with a contra-angle 1:5 handpiece (Synea WK-99 LT, W&H Dentalwerk) in order to standardize cavity depth. No bevels were made and axial were parallel and sharp, with no undercuts.Cavity dimensions were checked with a periodontal probe (Qulix Periodontal Probe, Hu-Friedy) and inner walls were smoothed with a cylindrical round-end diamond bur (Piranha Diamond #881-010F, SS-White Burs). Bur was replaced after 5 preparations or in case of signs of bur damage. In case of pulp exposure, tooth was discarded and replaced by another in its group.

Cavities were restored according to the adhesive protocols (Table 3, page 27) and finally with composite resin (Filtek™ Z250, 3M Healthcare), placed in a two diagonal layers and polymerized for 40 sec each with a LED polymerization unit (Elipar™, 3M Healthcare). Excess material at restoration margins was removed with a scaler (H5 Hygienist/U15 Towner Scaler, Hu-Friedy) and restorations were polished with Al_2O_3-coated polishing discs in successive roughness (Sof-Lex™ Discs and Sof-Lex™ Wheels, 3M Healthcare), in order to eliminate composite overhangs and obtain an absolutely composite-free margin.

4.4.2 Storage and thermocycling

Restored teeth were stored in distilled water in 37ºC (Incubator IP20 Function Line, Heraeus, Hanau) for 2 weeks in order to stabilize water sorption from the composite. Specimens were then subjected to 10000 thermocycles (±5°C and ±55°C with 15'' dwell time and 15'' transfer time) (Thermocycler, Thermo Fisher Scientific™, Waltham, Massachusetts, USA) (Figure 3, page 31).

4.4.3 Impressions before caries model

After thermocycling the first set of impressions with vinylpolysiloxane impression material was taken. Tray adhesive (VPS Tray Adhesive, 3M Healthcare) was applied on plastic trays (Miratray®-Mini, Hager Werken, Duisburg) and impressions were taken with the single-step double-mix technique with putty (Panasil® Putty, Kettenbach, Eschenburg) and light-body impression material (Panasil® Initial Contact Light, Kettenbach) according to manufacturer's instructions. Impressions were then casted with a polyurethane precision model die-material (AlphaDie MF Ivory, Schütz Dental, Rosbach) under pressure (2 bars) in a pressure pot (Polyclav®, Dentaraum, Ispringen) following manufacturer's instructions. Replicas were removed after 1 hour and were cleaned using a thin scalpel (Surgical Disposable Scalpel, B Braun, Melsungen) for removal of excess die material and a toothbrush for polyvinylsiloxane remnants. The same procedure was repeated for the second set of impressions after caries model, resulting in two sets of polyurethane replicas which proceeded to scanning electron microscopy, with this non-destructive method which maintained the original specimens (Figure 3, page 31).

4.4.4 Preparation before insertion into caries model

Apical root thirds were removed with a slow-speed diamond saw (Isomet 1000 Precision Saw, Buehler, Uzwil, Switzerland) with a diamond disc (Isomet Diamond Wafering Blades 15LC Diamond [127 x 0.4 mm], Buehler) at 975rpm, in order to expose the pulp complex to the disinfecting agent. Remaining pulp tissue was removed with a scaler (H5 Hygienist/U15 Towner Scaler, Hu-Friedy). Teeth were then mounted on chewing simulator metal plates (custom-made plates, Festo Systemtechnik, Denkendorf) with wax (Supradent-Wachs, Chemisches Dental-LaborOppermann-Scwedler, Pluradent, Offenbach am Main) and were immersed into 70% ethanol solution (Pharmacy of the University Clinic) for 2 hours [205]. Every 30 minutes the solution was carefully stirred in order to shake up bubbles and achieve full disinfection. Teeth were then transferred into the sterilized reaction chamber and were positioned at its Teflon base (Bretthauer, Dillenburg) by means of sterilized tweezers (PluLine St Nr. 43083, Pluradent) under a Clean Bench (Clean bench, Thermo Fisher Scientific™) in order to avoid contamination. Teeth chamber was

previously filled with sterilized distilled water, in order to avoid specimen damage in case of an accidental drop (Figure 2).

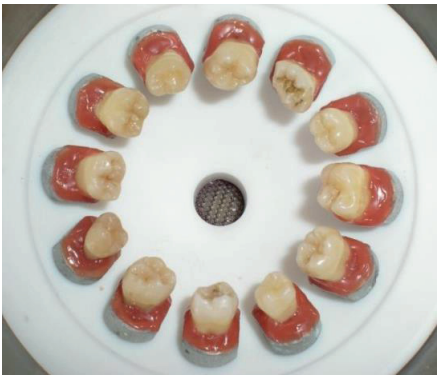

Figure 2: Placement of 12 specimens on chewing simulator plates in the reaction chamber of the caries model.

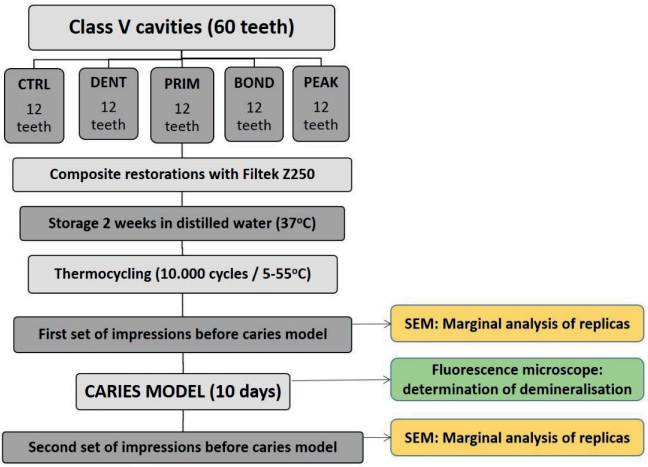

Figure 3: Schematic demonstration of experimental steps for caries model with class V restorations.

4.4.5 Calibration for μTBS

Calibration was performed with five human third molars, restored with Optibond™FL, Kerr Dental (LOT: Prime 5682054, Adhesive 5662314) and Filtek™ Z250, 3M Healthcare (LOT: N561790), according to manufacturers' instructions. This adhesive is regarded as a gold-standard regarding its bonding performance [191]. Based on the methodology described in *Tilch, 2015* [206], specimen preparation, sectioning in sticks (Isomet 5000 Linear Precision Saw, Buehler) and bond strength testing parameters were modified until a standard deviation of 30-50% among sticks of the same tooth was reached. Those standardized parameters were applied at the μTBS main study.

4.4.6 Fabrication of specimens for μTBS

For the preparation of μTBS specimens, roots from 150 third molars were removed and mid-coronal dentine was exposed in a microtome (Isomet 1000 Precision Saw, Buehler) (Figure 4). Teeth were mounted on microtome table with screws and the section was made in the middle of the crown using the microtome blade (Isomet Diamond Wafering Blades 15LC Diamond [127 x 0.4 mm], Buehler) at speed 975 rpm and with a weight of 75 gr.

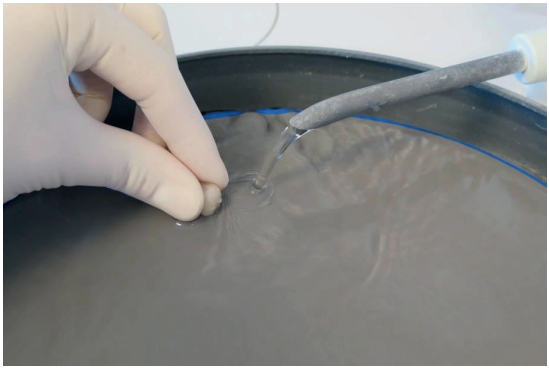

Figure 4: Exposed mid-coronal dentine was polished and a standardized smear layer was formed before the application of the adhesives.

Exposed dentine was polished in a grinding machine (Beta Grinder-Polisher, Buehler) (Figure 4, page 32) with silicon carbide sandpaper in roughness P 600 – Grit 360, followed by decreasing roughness P 1200 – Grit 600 (Silicon Carbide Grinding Paper Grit 360 and Grit 600, Buehler Met II, Buehler), under water irrigation. Dentine surfaces were dried and checked under light for enamel remnants. Subsequently, dentine was further polished with P 1200 – Grit 600 sandpaper for 60 sec manually forming "8-routes" in order to remove debris and to create a standardized and even smear layer zone.Teeth were randomly divided in 5 groups and subsequent application of the adhesives followed (Table 3, page 27). First layer of composite resin (Filtek™ Z250, 3M Healthcare) was applied and was homogenously thinned to approximately 0.5 mm (Comporoller 5300, Kerr Dental). Consecutive composite layers of 1 mm thickness were placed, building up composite height up to approximately 6 mm. Each layer was separately polymerized for 40 seconds with a LED polymerization unit with 1200 mW/cm^2 light intensity (Elipar™, 3M Healthcare). Specimens were stored in distilled water in 37^{o}C (Incubator IP20 Function Line, Heraeus) for 24 hours, in order to balance water intake of the composite [207].

Teeth were then mounted on a microtome table with wax (Supradent-Wachs, Chemisches Dental-Labor Oppermann-Scwedler, Pluradent) with the composite build-up facing downwards. Specimens' total height was measured with a periodontal probe (Qulix Periodontal Probe, Hu-Friedy), in order to adjust the cutting depth and produce sticks of adequate length. Diamond blade (127 mm X 0.4 mm) (Isomet Diamond Wafering Blades 15LC Diamond, Buehler) of precision microtome (Isomet 5000 Linear Precision Saw, Buehler) was cleaned and sharpened prior to sectioning.

Specimens were sectioned vertically buccolingually, were then rotated 90^o and sectioned againmesiodistally (Figure 5, page 34). Both sections were made with direction from the apex to the crown. First cut was made 1.5 – 2 mm from the edge and external slices were discarded, since composite was bonded to enamel. Approximately 18-25 sticks were fabricated from each tooth. Microtome settings were: Rotating speed: 3450 rpm / Cutting speed: 2.5 mm/min / Section length: 14 mm / Specimen size: 0.716 mm / Sections: 10 / Slice size: 0.381 / Cutting depth: 6-7 mm (measured for each specimen). Sticks were removed from the underlying composite using a thin scalpel (Surgical Disposable Scalpel,

B Braun), were measured using a digital caliper (ABSOLUTE 500-196-20 digital caliper, stainless steel, +/-0.001'' accuracy, 0.0005'' resolution, Mitutoyo Germany, Neuss) and were then either immediately loaded or stored in sealed tubes until bond strength testing, according to the group they belonged. Sticks dimensions were 0.68 mm X 0.68 mm, resulting in rectangle-shaped bonded area of 0.46 mm^2 (±0.04 mm^2). Inclusion criteria were: i) a macroscopically intact bonding area between tooth and composite, ii) adequate amount of tooth or composite to enable fixation on the microtome table, iii) void-free composite build-up at the adhesive area. Sticks were excluded from the study when the following drop-out criteria were met: i) inadequate length of composite and of dentine (> 3 mm), ii) voids at the adhesive zone or in the composite, iii) signs of dentine caries or any other flaw macroscopically visible, iv) incorrect dimensions of the adhesive area, v) non-rectangle adhesive surface. Bond failure during sectioning was evaluated as pre-test failure and was regarded as "zero" value (Figure 5).

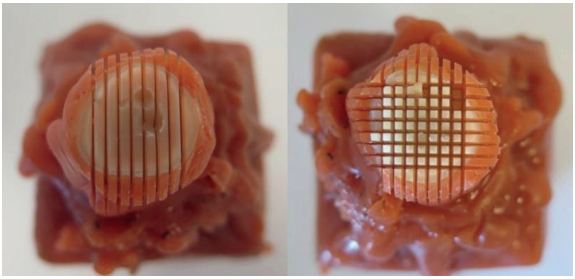

Figure 5: Production of sticks by sectioning of tooth in two perpendicular directions. Composite-dentine sticks which broke during sectioning were regarded as pre-test failures (right). Composite-enamel sticks were excluded.

4.4.7 Preparation for 6- and 12-month storage for µTBS

Sticks planned for long-term storage were stored in sealed tubes at 37ºC (Incubator IP 20 Function Line, Heraeus) for 6 or 12 months, in distilled water or artificial saliva, according to the storage protocol. Artificial saliva was produced as described below at preparation for caries model, page 41. Storage media were not renewed during storage time. Despite the fact that all containers were tightly closed, 12-month storage containers were refilled with storage medium at 6 months, due to evaporation because of the high temperature in the incubator. No refill was needed for the 6-month groups.

4.4.8 Preparation for caries model before µTBS (µTBS caries model)

Sticks were not removed from sectioned teeth which were planned for biological loading in the caries model before µTBS, and thus sectioned teeth were mounted as a whole on chewing simulator plates(custom-made plates, Festo Systemtechnik) with wax (Supradent-Wachs, Chemisches Dental-Labor Oppermann-Scwedler, Pluradent) (Figure 5, page 34). Following to that, they proceeded to disinfection for 60 min (Braunol 7,5 gr Povidon-Jod, B Braun). Teeth were then transferred into the sterilized reaction chamber of the caries model under a Clean Bench (Clean bench, Thermo Fisher Scientific™) in order to avoid contamination (Figure 6).

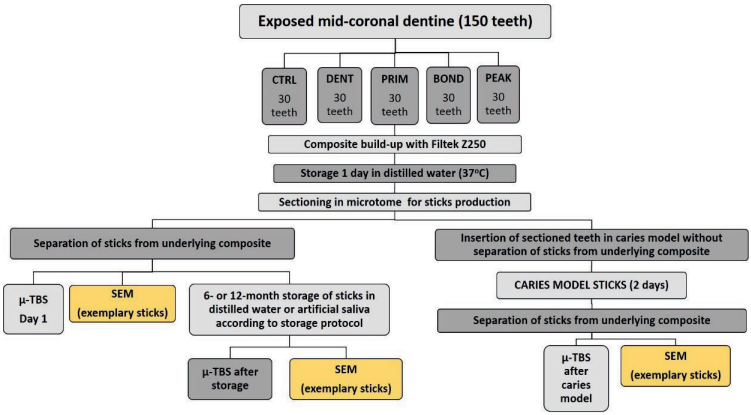

Figure 6: Schematic demonstration of experimental steps for µTBS.

4.5 Caries model

4.5.1 Description of caries model

A fully automated caries model was used in the present study, which was developed by *Ritzmann, 2008* [190] and further updated at the Department of Paediatric Dentistry, Justus-Liebig University of Giessen [185] (Figure 7, page 38). The model consists of five containers i. Reaction chamber, ii. Waste container, iii. Reservoir-container, iv. Artificial saliva container, v. Nutrition medium container connected with tubes (Platinum-treated silicone pump tubes, autoclavable, wall thickness 1.6 mm, inside 4.8 mm, outside 8.0mm, Carl Roth) with the mediation of three drip-systems (Glass dripping system manufactured by the Chemical Institute, University Erlangen-Nürnberg); between the nutrition medium container and the reservoir container, between the reservoir container and reaction chamber and between the artificial saliva container and the reaction and four pumps (Cyclo II Pumps, Carl Roth) which enable fluid movement through the tubes (Figure 8, page37). Tubes are equipped with male/female plug-in metal connectors (metallic tube extensions conical for Luer- Lock (LLW) female P337.1 (21023012) and (LLM) male P341.1 (31027022), Carl Roth) to allow for easy mantling/dismantling. By installation of drip-systems reverse flow of the solutions is avoided and bacterial colonization of the containers is prevented. All connected parts were assembled before each experimental cycle and could be easily disassembled in order to be separately cleaned and sterilized or checked for damage. All parts of the caries model were placed in an incubator (IPS Memmert, Memmert, Schwabach) at 37°C, except from the container for artificial saliva and for nutrition medium, which were placed outside the incubator for practical reasons. A personal computer (operating system Windows XP) was connected with the caries model and collectedall the information of the experimental circles, saved the data and operated the pumps automatically according to the 24-hour biological protocol (Table 7, page 51) by means of a software (LeC Operating Software for Relay Module 8X-serial, Conrad Electronics SE, Hirschau). The software operated according to the desired duration of the experiment - 2 days for the biological loading of the sticks before μTBS and 10 days for the biological loading of Class V composite restorations. Operation of caries model was controlled thrice per day (connections, pH and temperature control, level of media in containers to avoid flooding or insufficiency) and waste container was changed every two

days. During the 10-day caries model, change of artificial saliva container after 5 days was required for the quantity of media to suffice. In order to double the number of specimens which were tested simultaneously, two independent caries models could be connected with the personal computer and run at the same time.

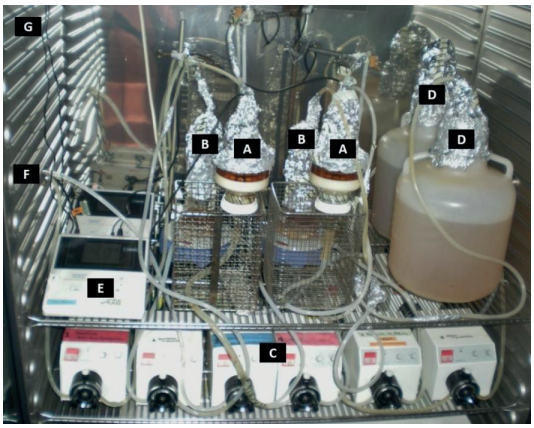

Figure 7: Caries model set up with two independent caries models. A: reaction chamber, B: reservoir-container with *S. mutans*, C: pumps, D: waste container, E: pH and temperature measuring device, F: input lines for artificial saliva and nutrition medium, which are located outside the incubator, G: connection of the caries model with the personal computer.

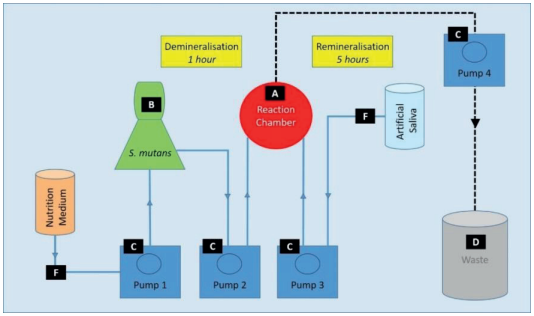

Figure 8: Schematic drawing of caries model set up in association to figure 9. A: reaction chamber, B: reservoir-container with *S. mutans*, C: pumps, D: waste container, F: input lines for artificial saliva and nutrition medium.

4.5.2 Containers

i. Reaction Chamber

The reaction chamber (300-4100 Reusable Filter Holder with Receiver, Thermo Fisher Scientific™ Nalgene ™ Labware, Rochester, NY, USA) (Figure 9, page 40) is a sterilized, plastic container, where teeth are placed and provided with media for the growth of the bacterial biofilm and the development of caries-like lesions and with artificial saliva for the remineralisation phases. Inside the chamber, a Teflon base (Bretthauer GmbH) is placed (Figure 9, page 40), where fixed specimens on the chewing simulator metal plates (custom-made plates, Festo Systemtechnik) are positioned. The screwable cap of the reaction chamber (Carl Roth) contains two air-filters (Pressure Compensation Filter PTFE 0.20 μm, Duran® Group, Mainz), two input lines (Carl Roth); one for the insertion of the bacterial solution for the demineralisation phase and one for the artificial saliva for the remineralisation phase (Figure 9, page 40). The pH electrode (Blueline N1048 1M-DIN-ID with temperature sensor Serial No: A133114003, SI Analystics, Mainz) is placed in the reaction chamber through a Telfon holder (Bretthauer GmbH) at the centre of the cap, is connected with the measuring device inside the incubator (pH Measuring Instrument Lab870, SI Analystics) and consequently to the personal computer. This way pH and temperature are monitored continuously throughout the experiment (MultiLab® pilot v4.7.2, WTW, Weilheim). At the bottom of the reaction chamber one output line (Carl Roth) for the waste, is also installed. The reaction chamber is mounted on a metallic basket in order to be stabilised, to avoid tilting and allow for easy handling during sterilisation (Figure 7, page 37).

ii. Waste Container

The polypropylene waste container (Nalgene™ 10 l Container with vented closure, Thermo Fisher Scientific™) is connected with the bottom of reaction chamber with a tube (Carl Roth) and collects all the waste produced during the experiment (Figure 7, page 37). This container is capped with a properly vented closure with an air-filter (Duran® Group) and is sterilized, in order to avoid a reverse contaminationthrough the connecting tubes. Even if not full, every two days the waste container was changed to avoid an external contamination.

iii. Reservoir-Container

In order to maintain a constant amount of bacteria throughout the experiment a sterilized reservoir-container (500 ml Erlenmeyer wide-neck flask, diameter 50 mm, Duran® Group) is used (Figure7). On the top of the Erlenmeyer flask a sponge serves as a cap, through which bacteria can be injected without removing it. At the beginning of every experimental circle, the bacterial solution is formed in the reservoir-container by insertion of the nutrition medium into the reservoir-container through a fixed tube (Carl Roth) on the sponge-cap of the Erlenmeyer flask (Duran® Group), following by injection of bacteria through the sponge. At the bottom of the Erlenmeyer flask (Duran® Group) an output line for the bacterial solution is installed, which after 6 hours of bacterial prolliferation can be pumped through a tube (Carl Roth) into the reaction chamber. A magnetic bar (Rotilabo® Economy 25 mm magnetic bars, Carl Roth) is placed inside the Erlenmeyer flask (Duran® Group), and the reservoir-container is positioned on amagnetic stirrer (IKA® - Werke, Staufen) to achieve constant stirring of the bacterial solution, at the lowest speed. Four fine-pore stones (Klarwasser Bio Filter RöhrchenfürAquarien, Dennerle, Vinningen) are also placed inside the reservoir-container in order to support mechanical settlement of bacteria and thus avoid significant reduction of their concentration (Figure10, page 40).

iv. and v. Artificial Saliva and Nutrition Medium Bottles

Both media are maintained in 20 l glass bottles (Duran® Group) to make sterilization of large amounts of solutions possible. Glass bottles are placed in suitable metallic baskets (Systec, Linden) to allow for easy handling during transportation and sterilization. The 20 l containers are placed outside the caries model incubator in order to provide simple handling during the assembling and disassembling of the caries model (Figure 10, page 40).Through connecting tubes (Carl Roth) installed at the screwable cap (Systec) of the bottles, artificial saliva is directly inserted into the reaction chamber for the remineralisation phase, while nutrition medium is first inserted into the reservoir-container, in order to promote bacterial growth, and then the bacterial solution is inserted into the reaction chamber for the demineralisation phase (Figure 7, 8, page 37). The cap is also equipped with an air-filter (Duran® Group) in order to facilitate pressure compensation during sterilization and with a metallic pipeline (Systec) for the placement of the temperature sensor during sterilization.

4.5.3 Pumps

In order to achieve movement of the solutions though the tubes, four pumps (Cyclo II Pumps, Carl Roth) are installed (Figure7, 8, page 37).

Figure 9: The screwable cap (left) and the Teflon base (right) of the reaction chamber. Chewing simulator plates with waxed specimens are placed in the 12 holes of the Teflon base. The central opening holds the pH-electrode.

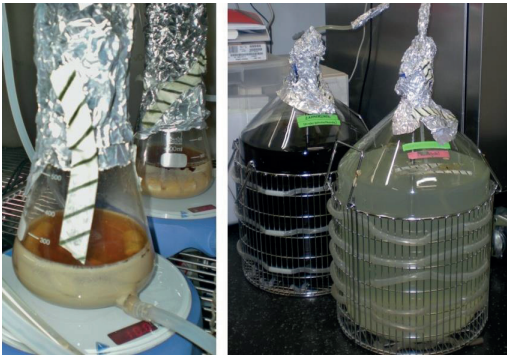

Figure 10: Containers of the caries model. Reservoir-containers with *S. mutans*, placed on magnetic stirrers during a double caries model (left). Note the biofilm accumulation at the inner walls of the flask after a few days of operation and the white fine-pore stones, which are visible through the bacterial solution. Artificial saliva (clear) and nutrition medium (dark) 20 l glass bottles are placed outside the incubator due to limited space (right).

The following connections are made (Figure 8, page 37): i) from the artificial saliva to the reaction chamber (Pump 1), ii) from the nutrition medium to the bacterial reservoir-container (Pump 2), iii) from the reservoir-container to the reaction chamber (Pump 3), iv) from the reaction chamber to the waste container (Pump 4).

4.5.4 Artificial Saliva

A mineral solution with pH=7 and phosphate buffer (2.2 mmol / l KH_2PO_4, 4.59 mmol / l K_2HPO_4) was used as artificial saliva in the caries model, in order to simulate the remineralisation phase in the oral cavity. 20 l artificial saliva was produced according to [208](BBL ™ Trypticase TM Peptone,Becton, Dickinson and Company, Sparks, MD, USA / di-kaliumhydrogenphosphate, Calciumchloride dihydrate, Kaliumdihydrogenphosphate, Natriumhydrogenphosphate, Magnesium chloride hexahydrate, Kaliumchloride, Carl Roth) using 20 l distilled water (Milli-Q® water purification system, LS Orbital Sanitary Process Equipment, Schwechat, Austria), while exact quantities of powders were determined with a calibrated laboratory balance (Kern PBS-PBJ, Balingen). Artificial saliva was produced twice for every caries model cycle, due to the multiple rinsings with artificial saliva according to the biological protocol (Table 7, page 51). Its preparation took place in 20 l glass bottles (Duran® Group) which were sterilized with their cap partially closed in order to allow steam movement during the sterilization cycle. To avoid contamination of the sterilized half-closed container during removal from the autoclave, cap was protected with aluminium foil (Aluminium folieRotilabo 30 μm, Carl Roth). Immediately after removal, cap was tightly screwed and the glass bottle was stored in cold-storage room (4^{o}C) of the Medical Microbiology of the University Clinic of Giessen and Marburg. Sterilization settings were chosen so as to prevent qualitative degradation of the solution. Change of artificial saliva glass bottle during the fifth day of the caries model cycle was performed by dismantling the metal connector of the empty glass bottle, flame sterilizing and attaching it at the new artificial saliva bottle over a flame (BIC® Lighter, Society BIC.ClinchyCedex, France). In order to maintain sterility of the connectors, when needed, sterilized plastic caps (Plastic stops Combi - Stopper, B Braun) were used as further protection after dismantling and before mounting of the connectors.

4.5.5 Nutrition medium

The bacterial solution used for the demineralisation phase consists of bacteria and nutrition mediumfor bacterial growth. The nutrition medium was produced according to the manufacturer's instructions, by diluting 28.4 gr of Schaedler Broth powder (BBL™ Schaedler Broth, Becton, Dickinson and Company, LOT: 3240102 and LOT: 4022232) into 1 l distilled water (Milli-Q® water purification system, LS Orbital Sanitary Process Equipment), until completely dissolved and was composed of pancreatic digest of casein, peptic digest of animal tissue, papaic digest of soybean meal, dextrose, yeast extract, sodium chloride, dipotasium phosphate, hemin, L-cystine and TRIS (hydroxymethyl) aminomethane. The preparation of the nutrition medium took also place in 20 l glass bottles (Duran® Group); their handling, sterilization and storage being same as with artificial saliva glass bottles (page 41). Nutrition medium was not renewed during the caries model cycle.

4.5.6 Bacteria

Many types of microorganisms inhabit the oral cavity and those implicated in the carious process form complicate biofilms. However, in order to design a simple yet effective *in vitro* caries model, *S. mutans* (DSMNr: 20523, Leibniz Intitute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig) was used as a monobacterial culture. Freeze-dried bacteria, stored in portioned glycerin cultures in -80ºC, were used and cultivated for insertion in the caries model (Figure 11, page 44), as exhibited in detail in Table 5. Two overnight cultures were cultivated, as back-up in case of contamination or insufficient bacterial growth (Figure 12, page 44). After determination of the optical density(Spectrophotometer Bio UV / Visible geneszs 10S, Thermo Fisher Scientific™), the bacterial solution with OD_{600nm} closest to ~1 proceeded to purity controland was injected in the caries model using sterilised single-use syringes (Syringes Omnifix-F 1m mL, B Braun / Needles Sterican 21G, B Braun). All routine procedures took place under a Clean Bench (Clean bench, Thermo Fisher Scientific™) and instruments were flame sterilized (Safety Burner Fireboy Eco, Integra Biosciences Deutschland, Biebertal). Dilutions were performed with the help of mechanical pipettors (Sartorius mLine® Biohit Pipettes / 20-

200 µl and 100-1000 µl, Sigma-Aldrich, Darmstadt) and an automated stirrer (Mixer Vortex Genie-2, Sigma-Aldrich) in order to obtain homogenous solutions.

Table 5: Cultivation of bacteria for insertion into the caries model.

Step	Process	Materials	LOT
Bacterial culture (Figure 11, page 44)	Unfreezing of *S. mutans,* inoculation of blood agar base with 1 inoculation loop *S. mutans* and incubation at 37°C, under anaerobic conditions for 48 hours.	-Columbia Agar Plates with sheep blood plus, OXOID, Wesel	167234
		-Freeze-dried *S. mutans* (DSMZ 20523)	
Overnight culture (Figure 12, page 44)	Dilution of 1 inoculation loop *S. mutans* in 20 ml Schaedler-Broth and incubation at 37°C, under unaerobic conditions for 12 hours.	-BBL™ Schaedler Broth, Becton, Dickinson and Company	3240102 and 4022232
Dilution	Control of bacterial growth via opacity of the solutions, further 1:10 dilution with Schaedler-Broth and incubation at 37°C, under unaerobic conditions for 8 hours		
Control of bacterial proliferation	Measurement of optical density (OD_{600nm}) at 600 nm using a negative control (20 ml Schaedler Broth) (Table 8, page 62).	-Spectrophotometer Bio UV/Visible Genesys 10S, Thermo Fisher Scientific™	
Injection in caries model	Injection of 1 ml bacterial solution into the reservoir-container in order toproliferate for 8 hours before the caries model start.	-Needles Sterican 21G, B Braun -Syringes Omnifix-F 1m mL, B Braun	
Purity control (Figure 13, page44)	Dilution of 100 µl bacterial solution up to 10^{-6} with 1X PBS and aerobculture in BHI-plates for 48 hours.	-Phosphate-buffered saline (PBS) -Bacto Brain Heart Influsion/BHI Plates, Becton, Dickinson and Company	4191852

Figure 11:*S. mutans* (DSM Nr: 20523) culture after 48 hours incubation. 1 inoculation loop is taken in order to dilute the bacteria for the overnight culture.

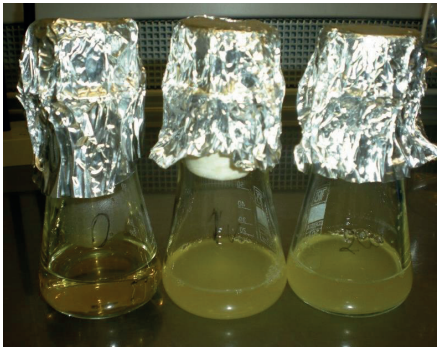

Figure 12: Control of bacterial growth via opacity after overnight culture. 0 (left): negative control, 1 (middle): Nº 1 bacterial solution, 2 (right): Nº 2 bacterial solution.

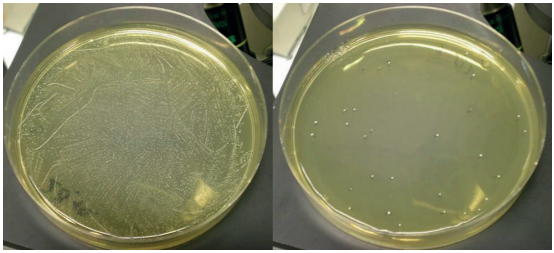

Figure 13: Purity control before each caries model cycle. It is impossible to count the colonies at 10^{-1} plate (left), while 28 *S. mutans* colonies are visible at plate with bacterial solution diluted up to 10^{-5}.

4.5.7 Microbial count and purity control

At the beginning of each caries model cycle and after the end of the experiment, microbial colony count and purity control of the bacterial solutions were performedby diluting the solutions up to 10^{-6} with 1X PBS, pH=7.4 (Natrium chloride, Kalium chloride, KH_2PO_4 anhydrous, Na_2HPO_4 x 2 H_2O). For the purity control at the beginning, the bacterial solution which was injected in the caries model was used, while purity control at the end of the caries model was performed with the bacterial solution drained from the reaction chamber, after the last demineralisation phase. After 48 hours, individual, visible colonies were counted usually on the 10^{-5} or 10^{-6} plates, as the colonies were not dense and thus easier to distinguish *S. mutans* or any other external species. Colour, odour and morphology of cultures made identification of *S. mutans* possible through optical observation. Purity control at the end of the experiment confirmed the presence/absence of external contamination. *B. cereus* was usually detected at the purity controls at the end of the caries model (Figure 14), but since it is not acid-producing [124], it has no influence on the experiment.

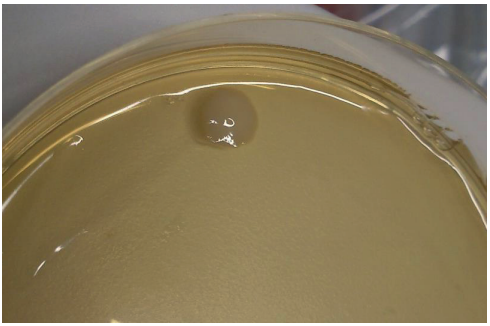

Figure 14: *B.cereus* colony usually detected at the purity control at the end of a caries model cycle.

4.5.8 pH-measurement

pH and temperature in the reaction chamber were constantly monitored during each caries model cycle by means of a pH-electrode with temperature sensor (Blueline N1048 1M-DIN-ID with temperature sensor Serial No: A133114003, SI Analystics) which was connected to the measuring device (pH Measuring Instrument Lab870, SI Analystics) and from there to the personal computer. pH electrode was calibrated before each experiment with standard buffer solutions according to DIN 19 266 (Buffer Solution pH=4.01 ± 0.01 in Glass Ampoules, LOT: 120116A, and Buffer Solution pH=6.87 ± 0.01 in Glass Ampoules, LOT: 111616A, Schott, Mainz) which corresponded to the expected pH values. Calibration was performed according to manufacturer's instructions under a Clean Bench (Clean bench, Thermo Fisher Scientific™). Immediately afterwards, pH electrode was disinfected with 70% ethanol solution (Pharmacy of the University Clinic) for 3 minutes, cleaned with sterile distilled water and was inserted in the reaction chamber through an opening at its cap designed to hold the pH electrode (Teflon holder, Bretthauer). Graphic presentation of pH and temperature variations was performed by software MultiLab® pilot v4.7.2 (WTW), and were a visual aid in controlling the progress of the experiment, monitoring malfunctions or leakage and evaluating the results afterwards (Figure 15).

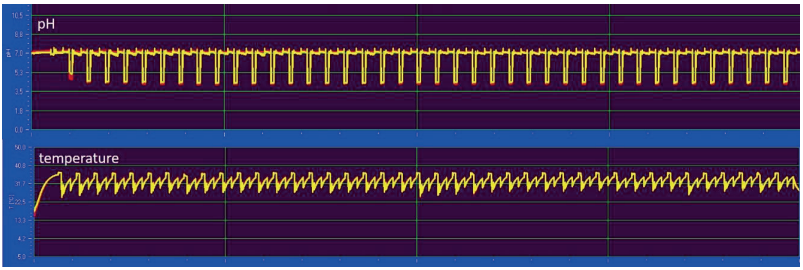

Figure 15: Continuous control of pH (up) and temperature in °C (down) inside the reaction chamber throughout biological loading in two independent caries models (caries model N° 1: yellow, caries model N° 2: red). Downward peaks correspond to pH decrease during demineralisation. Caries model runs undisturbed when pH curves are symmetrical and even.

4.5.9 Preparation of caries model

Preparation before every caries model test included i) sterilization of the model (Autoclave Systec VX-75, Systec / Milli-Q® water purification system, LS Orbital Sanitary Process Equipment) (Table 6) and assembling, ii) preparation and sterilisation of the solutions (artificial saliva and nutrition medium for bacteria) (see pages 41-42) iii) cultivation of bacteria (Table 5, page 43), iv) purity control of bacterial solution (see page 45), v) calibration of pH-electrode (see page 46).

Table 6: Sterilization programms

Type of container-medium	Process	Sterilization Temperature (ºC)	Sterilization Time (min)
Waste container (10l), reservoir-container, reaction chamber, tubes	Sterilization of the containers or tubes in the beginning (dry)	121	20
Waste container (10 l), reservoir- container, reaction chamber, tubes	Sterilization of the waste within the containers or tubes at the end (wet)	121	15
Artificial saliva and nutrition medium glass bottles (20 l)	Sterilization of media before each experimental cycle	121	15

All parts of the caries model were separately packed and prepared for dry sterilization. Plug connections were covered with aluminum foil (Aluminium folieRotilabo 30µm, Carl Roth) and then tubes and caps of containers and glass bottles were double-packed with aluminum foil. Wet sterilization was performed at the end of each caries model cycle and for during the caries model for the waste container. Caries model was disassembled; tubes and containers with were sterilized with their infectious content and proceeded to further cleaning and preparation for the next caries model cycle.

4.5.10 Biological protocol

Specimens – either being composite restorations or sticks - were loaded in the caries model according to a biological protocol (Table 7, page 51), which determined the alternation of demineralisation / remineralisation phases and the duration of specimens' incubation with *S. mutans*. The biological protocol was adjusted before the start of the caries model cycle, according to the desirable experimental conditions and was controlled by the operating program of the caries model (Conrad Electronics SE) [190]. The biological loading protocol consisted of alternating demineralisation and remineralisation phases, induced by bacterial solution (pH=4.2 - 4.3) and artificial saliva (pH=7) consecutively. Teeth placed into the reaction chamber (Figure 2, page 31) were incubated in the demineralising (bacterial solution) and remineralising media (artificial saliva), which were provided by bath, instead of using a drip technique, in order to obtain a more realistic simulation of the oral environment (Figure 16, page 49).

Eight hours before the beginning of each caries model series *S. Mutans* (DSM Nr.: 20523) was injected in the reservoir-container using sterilised single-use syringes and allowed to proliferate in approx. 250 ml nutrition medium, before the first demineralisation phase. This offered the bacteria the extra time to adapt and reproduce. First demineralisation begins with drainage of approximately 250 ml bacterial solution from the reservoir-container into the reaction chamber via pump 2 and lasts for 1 hour. At the same time a new amount of nutrition medium is pumped into the reservoir-container via pump 1 and further bacterial proliferation continues parallel. After 6 hours of proliferation, bacterial solution can be drained again for the next demineralisation phase. Upon completion of the demineralisation phase, bacterial solution is drained into the waste container via pump 4. Acidic remnants from the bacterial solution cannot be immediately neutralized, due to low phosphate concentration in the artificial saliva, and thus a neutral pH value cannot be reached. Therefore rinsing the reaction chamber twice with approximately 250 ml mineral solution for 2 minutes and for 28 minutes is mandatory, by pumping artificial saliva via pump 3. The remineralisation phase starts with the first rinsing and lasts for 5 hours, including both rinsing phases. Aftereach rinsing with artificial saliva and upon completion of the remineralisation phase the bacterial solution is drained into the waste container via

pump 4. Drainage of the bacterial solution of the last demineralisation phase took place in a separate glass bottle (500 ml Erlenmeyer wide-neck flask, diameter 50 mm, Duran® Group) which was attached through a three-way valve (Metal valve Luer Lock female LLW to male LLM, side female LLW, Carl Roth) with the tube connecting the reaction chamber with the waste container. Bacterial solution was collected and proceeded to purity control at the end of the caries model cycle (see page 45). Demineralisation (1 hour) and remineralisation phases (5 hours) interchanged and were repeated 4 times within 24 hours, resulting in 4 hours / day incubation of specimens with *S. mutans* (Table 7, page 51).

Biological loading in Class V caries model and µTBS caries model operated under the same biological protocol. However, composite restorations were loaded for 10 days, resulting in 40 demineralisation / remineralisation phases or 40 hours incubation with *S. mutans*, and loading of sticks was limited to 2 days, with 8 demineralisation / remineralisation phases (Figure 16) or totally 8 hours of incubation with *S. mutans* due to the fragility of the sectioned sticks. When pH of the first demineralisation did not reach the critical value range (for enamel 5.0 – 5.5) an additional demineralisation phase took place at the end of the caries model cycle in order to reach the total amount of demineralisation phases planned, namely 40 for Class V caries model and 8 for µTBS caries model.

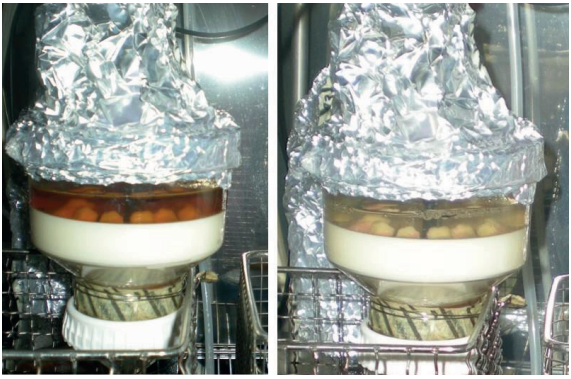

Figure 16: Reaction chamber during demineralisation (left) and remineralisation phase (right) filled with the appropriate medium.

Five caries model series were planned for the Class V caries model and four for the μTBS caries model. A maximum of 12 teeth could be simultaneously inserted into the caries model and specimens were in every case randomized. Since bond strength testing should follow immediately after the end of the μTBS caries model cycle, up to 5 teeth with sectioned sticks were simultaneously tested. Regarding Class V caries model, two independent caries models were able to run parallel. Therefore two caries model sets were assembled in the incubator and a total of 24 specimens were loaded at once. Although each caries model was autonomous, their operation was coordinated, to allow for easier control of the alteration between the phases.

4.5.11 Follow-up processing after caries model

Upon completion of the caries model cycle specimens were removed from the reaction chamber with tweezers (PluLine St Nr. 43083, Pluradent) and Class V caries model specimens (Figure 17) were disinfected with 70% ethanol solution (Pharmacy of the University Clinic). Sectioning along the vertical tooth axis and through the composite restoration followed (Isomet 1000 Precision Saw, Buehler) resulting in two tooth halves, which were stored in distilled water. μTBS caries model sticks (Figure 18, page 51) were disinfected with Braunol (Braunol 7.5 gr Povidon-Jod, B Braun) for 30 minutes. They were then rinsed with distilled water and were removed from the chewing simulator plates (Festo Systemtechnik). Disinfected sticks were then separated from the underlying composite using a scalpel and were stored in distilled water until bond strength testing, in order to avoid dessication.

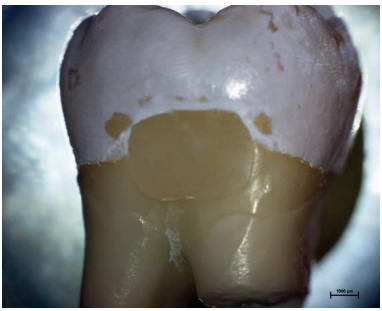

Figure 17: Class V caries model specimen after the 10-day biological protocol. Demineralisation in enamel and substance loss in dentine are macroscopically visible around the restoration.

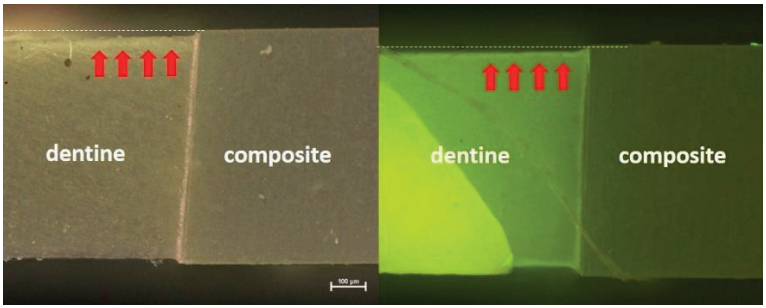

Figure 18: µTBS caries model sticks after the 2-days biological protocol under microscope due to illustration reason. Demineralisation of dentin is visible due to its opacity (left: light microscope 15X magnification) or due to its fluorescence (right: fluorescence microscopy 15 X magnification). Substance loss due to demineralisation is the distance between the dotted white line and dentin surface in both images.

Table 7: Biological protocol for the caries model in 24 hours.

Demineralisation 1 hour	1	Demineralisation 1 hour	3
Rinsing with mineral solution 2 minutes		Rinsing with mineral solution 2 minutes	
Rinsing with mineral solution 28 minutes		Rinsing with mineral solution 28 minutes	
Remineralisation 4.5 hours		Remineralisation 4.5 hours	
Demineralisation 1 hour	2	Demineralisation 1hour	4
Rinsing with mineral solution 2 minutes		Rinsing with mineral solution 2 minutes	
Rinsing with mineral solution 28 minutes		Rinsing with mineral solution 28 minutes	
Remineralisation 4.5 hours		Remineralisation 4.5 hours	

4.6μ-Tensile Bond Strength (μTBS)

Bond strength of sticks was evaluated in μ-tensile at day 1 (baseline), after 6- and 12-month storage in distilled water and artificial saliva and after biological loading in caries model.

4.6.1 μTBS at baseline

μTBS was performed at Bond Strength Testing Machine Syndicad TC-550, Munich with its accompanying operating software (TC-550 Zug-/Druck-Messsoftware V3_1, Munich). Self-calibration and distance specification between table plates which would hold the sticks, took place automatically each time the device was turned on. Test parameters for loading in μ-tensile mode were adjusted at the testing machine: Units: Newton (Strength), Specimen: rectangle (a. 0.610 / b. 0.610), Force Direction: Tensile Force, Max: 40 N, Speed: 1 mm / min. After 24 hours storage in distilled water (baseline), sticks were allowed to dry on blotting paper to remove excess moisture and were then placed one by one on the metallic plates. One edge of the specimen (e.g. dentine) was placed at one plate and the other edge of the specimen (e.g. composite) was placed at the opposing plate, keeping the adhesive interface between the plates. Both ends of each stick were fixed at both sides with flowable composite resin (Dyract Flow, Dentsply De Trey) which was allowed to flow up to 0.5 mm distance from the adhesive interface (Figure 19, page 53) and was then polymerized for 40 seconds (Bluephase G2 Curing Unit, IvoclarVivadent, Schaan, Lichtenstein / light intensity: 1200 mW/cm^2). The adhesive interface had no contact with the plates and distance between the plates was set at 1 mm. In order to ensure horizontal placement and even tensile force distribution, avoiding simultaneous shear strain, metallic plates were of equal thickness. Sticks were loaded at a speed of 1 mm / min until fracture, maximum force was recorded by the software in Newton (N) (Figure 20, page 53) and inserted by the operator in an Excel worksheet (Excel for Windows), where conversion in Mega Pascal (MPa) took place. Type of bond failure (adhesive, cohesive in composite, cohesive in dentine, mixed in dentine and in adhesive area, mixed in composite and in adhesive area, mixed in dentine, composite and adhesive area)was assessed under light and 4X magnification (Magnifier Glass Lamp 1.75/4X, Model No: 8093. Bulb: 12W, MBFZ toolcraft, Spalt) by a single examiner. Selected specimens were investigated under fluorescence microscope (AZ 100 Macroscope, Nikon, Tokyo, Japan) (Figure 21, page 54).

Fractured sticks were stored in sealed tubes (SafeSeal Reagiergefäß 0.5 ml, Sarsted) in distilled water. Upon completion of loading the sticks of a single tooth, mean bond strength value was calculated for each tooth, using Excel for Windows and standard deviation was checked to be within the acceptable limits of 30-50% of the mean value [200]. In case this condition was not met, tooth was excluded from the study and replaced with another in the group.

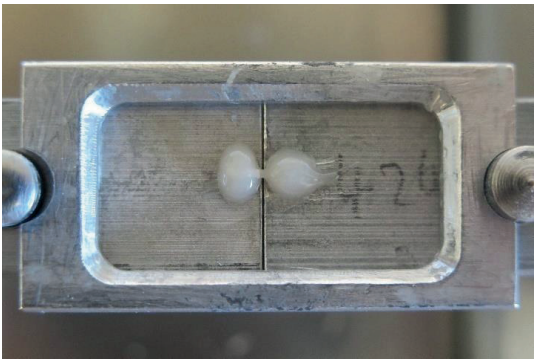

Figure 19: Placement of the stick on the metal plates of the bond strength testing machine and fixation with flowable composite resin.

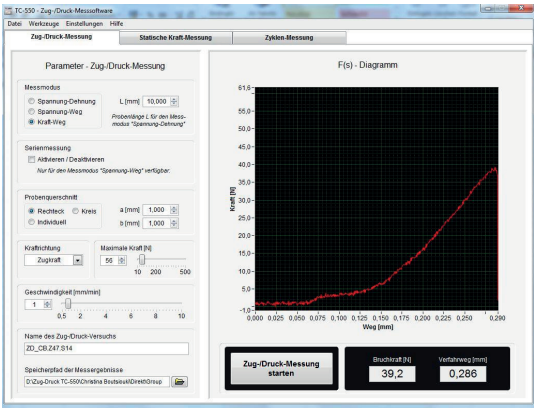

Figure 20: Screenshot of µTBS software TC-550 in operation. Parameters can be adjusted at the left side, while the red curve shows the tensile loading of the stick until bond failure. Maximum force applied is displayed in N.

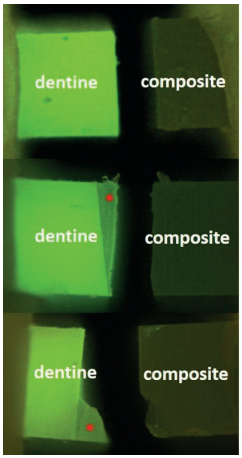

Figure 21: Failure modes under fluorescence microscope in 15X magnification for illustration reasons. Adhesive failure (up), cohesive failure in composite (middle) and mixed failure in composite and in adhesive interface. Composite remnants on the adhesive interface are marked with red asterisk.

4.6.2 µTBS after 6- and 12-month storage

Stored sticks proceeded to µTBS after the designated 6- or 12-month storage duration.They were rinsed with distilled water and were blot-dried before being fixed on the bond strength testing device and loaded until fracture, as described above. Sticks which broke during storage time were regarded as pre-test failures and evaluated as “zero”. Sticks which broke during handling were regarded as pre-test failures and were excluded from the evaluation.

4.6.3 µTBS after caries model

After 2 days biological loading with *S. mutans* and repeated demineralisation and remineralisation phases teeth proceeded to µTBS within the next 24 hours.Sticks were blot-dried, fixed on the bond strength testing device and loaded until bond failure as described above. No stick broke during biological loading in caries model. Sticks which broke during separation from the underlying composite or during handling were regarded as drop-outs and were excluded from the evaluation.

4.7 Microscopic evaluation

Microscopic evaluation after Class V caries model and after biological loading and storage of sticks was performed by means of fluorescence microscopy and scanning electron microscopy, by a single examiner (Figure 3, page 31).

4.7.1 Fluorescence Microscopy

The two tooth halves which were produced after sectioning (see page 50) proceeded to microscopic evaluation. Overview images of restoration halves with adjacent enamel or dentine (n=120) were first taken under light microscope (AZ 100 Macroscope, Nikon) (Figure 22, page 56), and then under fluorescence microscope (AZ 100 Macroscope, Nikon) using a FITC filter (excitation filter 450 – 490 nm, blocking filter 515 – 565 nm) (Figure 22, page 56). Overview images were standardised with the following parameters: objective 1X, zoom 1X and exposure 100-400 ms, therefore resulting in 6x magnification (calculated by multiplying objective 1x * zoom 1x * ocular 10x * tube factor 0.6x). In order to evaluate enamel – composite and dentine – composite margins, images at 72X magnification (objective 3x * zoom 4x * ocular 10x * tube factor 0.6*) were further taken under fluorescence microscope and measurements followed using NIS-Elements AR 4.00.07 (64 bit) for Windows XP, with pixel size 0.9 μm. For every specimen two enamel – composite images and two dentine – composite images were taken, resulting in 240 images. Since depth of fluorescence corresponds to the depth of demineralisation, and demineralised tissue fluoresces stronger than healthy tissue, demineralisation depth, tooth substance loss due to demineralisation and total demineralisation were calculated at restoration margins and at 300 μm and 500 μm away from the margins. Since composite volume is not affected by biological loading [70], in order to measure tooth substance loss, a reference horizontal line was drawn which corresponded to the initial height of enamel or dentine, with regards to the composite restoration top surface. Total demineralisation was calculated as the sum of demineralisation and substance loss due to demineralisation (Figure 23, page 56). Marginal gap width and marginal gap depth at enamel and at dentine were also measured. Marginal gap width was measured as the distance between composite and enamel or dentine at the tooth surface, while marginal gap depth was calculated as the distance between the deepest point of the marginal gap and the meeting point of the line

starting from the deepest point and the line connecting the composite and tooth tissue edges (or marginal gap width line) (Figure 23).

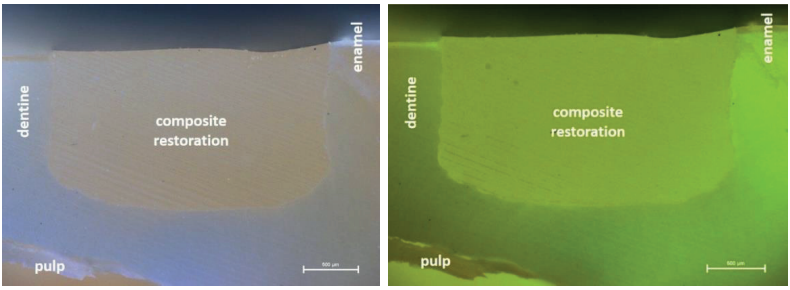

Figure 22: Overview image of the restoration after caries model under light microscope (left) and fluorescence microscope (right) (AZ 100 Macroscope, Nikon, 6X magnification).

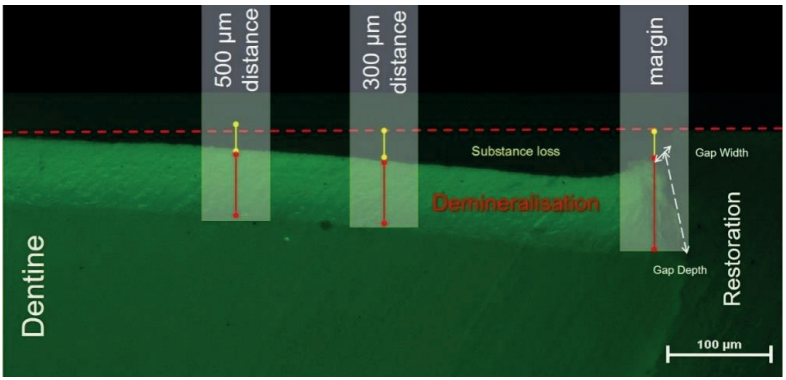

Figure 23: Evaluation of fluorescence microscope capture at restoration margins. The following parameters are determined: substance loss due to demineralisation (gelb), demineralisation depth (red), marginal gap width (white), marginal gap depth (white-stripped).

4.7.2 Scanning Electron Microscopy (SEM)

Class V restorations

Marginal gap analysis was performed at enamel and dentine margins of all Class V composite restorations in order to compare marginal deterioration and gap formation before and after biological loading in the caries model (Figure 3, page 31). For this reason 120 polyurethane replicas were fabricated by first (N=60 before caries model) and second (N=60 after caries model) set of impressions (see page 30). First set of replicas (before caries model) was demonstrated with letter A and second set of replicas (after caries model) was demonstrated with number B, following specimen numbering. Each replica was fixed on aluminium stub (Nr. G301, Plano GmbH, Wetzlar) with a carbon conductive cement (Leit-C nachGöcke, Plano GmbH, Wetzlar) and was allowed to dry overnight. Specimens were gold-spattered under argon gas vacuum (Sputter Coater, Polaron, SC502, Fisons Instruments, Ipswich, UK) with pressure 10 Pafor approximately 75 seconds each. In case gold-spattering was incomplete, the process was repeated for the same replica. Gold-spattered replicaswere then inserted into Scanning Electron Microscope (SEM) (SEM Amray Model 1610 Turbo, Amray, Bedford, MA, USA).

Overlapping and continuous images at enamel and dentine margins were taken at 200X magnification (acceleration voltage 10 kV) using Software Digital Image Scanning System 5 (DISS 5, point electronic, Halle (Saale)) and were then processed and saved using Digital Image Processing System 2.9 (DIPS 2.9, point electronic). Approximately 7-10 SEM images were taken at each side (enamel or dentine margins), and were stitched pairwise in order to illustrate the total length of restoration margin. Digital stitching was performed by means of Fiji is just ImageJ Software (Freeware, https://fiji.sc) (Figure 24).

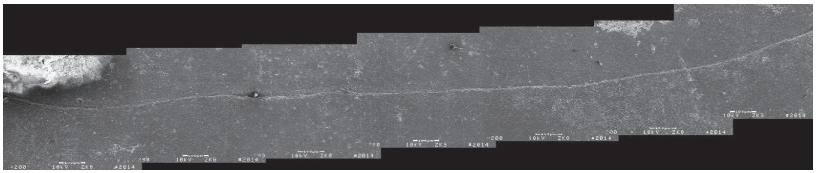

Figure 24: Stitched SEM images (200X magnification) demonstrating the dentin / composite margin before biological loading.

Stitched images of enamel and dentine restoration margins proceeded to quantitative marginal gap analysis, according to the criteria described by *Schmidt, 2013* [209]. By means of computer software KHKs_jQuantiGap (Prof. Dr. med. dent. Karl-Heinz Kunzelmann, Ludwig-Maximilians-University Munich; http://www.dent.med.uni-muenchen.de/~kkunzelm/htdocs/6_software-imagej-quantitative_margin_analysis.html) margins were evaluated as follows: perfect margin, overhang, underfilled, gap, fracture, not evaluable (Figure 25). As a result, quality of enamel/ composite and dentine/ composite margins was assessed as a percentage of perfect or imperfect margin length / total length.

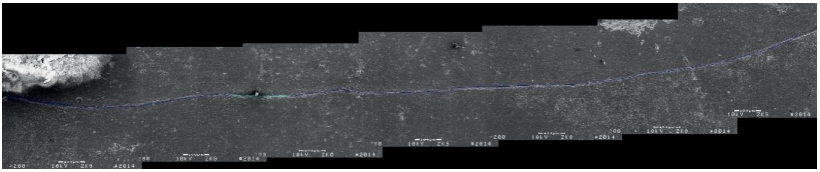

Figure 25: Color-coded evaluation of dentin / composite margin of figure 24. Dark blue corresponds to perfect margin, while turquoise shows marginal gap.

µTBS sticks

Exemplary intact, not µTBS loaded sticks, proceeded to qualitative SEM evaluation at baseline, after 6- and 12-month storage and after caries model in order to study the adhesive interface. 9 - 10 sticks were chosen from each group (Figure 6, page 35). The surface to be evaluated was manually polished on sandpaper with decreasing roughness (360 – 600 – 1200 – 2000 Grit Silicon Carbide Grinding Paper Grit 360, Buehler Met II, Buehler) under water irrigation, in order to attain a standardised surface. The opposite surface (other than the composite-dentine interface under investigation) was marked with a waterproof marker to help positioning of the stick. Half of the sticks, namely 4 – 5 sticks of each group were planned for qualitative evaluation of the adhesive area zones (Figure 26, page 60) and the other half proceeded to removal of their inorganic content, in order to assess characteristics of the hybrid layer such as thickness of the hybrid zone, or composite tag formation and maintenance throughout storage (Figure 27, page 60). Sticks were immersed in 4% NaOCl solution (diluted from 12% NaOCl, Carl Roth) for 20 minutes, rinsed with distilled water

and placed in 20% HCl (diluted from 37% HCl, Sigma-Aldrich) for 30 minutes and rinsed again with distilled water. This way partial removal of the inorganic content of dentine was achieved. Sticks which were planned for evaluation of the hybrid zone and their composite tags, proceeded to further handling with 37% HCl for 6 hours, until dentine was completed dissolved. All sticks were then dehydrated, by immersion in ascending ethanol-series (60 – 70 – 80 – 90 % for 20 minutes each, 100% for 1 hour). Finally, sticks were inserted in 1,1,1,3,3,3-Hexamethyldisilazane (Merk Schuchardt, Hohenbrunn) for 10 minutes, in order to obtain a preferably complete desiccation and were allowed to dry further overnight [210]. Same procedure for dehydration was followed for all sticks, with or without removal of the inorganic content, since specimens had to be anhydrous and electrically conductive, in order to be observed in SEM. Fixing and gold-spattering of all sticks followed, as described above for the polyurethane replicas (see page 57). Images for all sticks were taken field by field in various magnifications from 150 X - 2000 X.

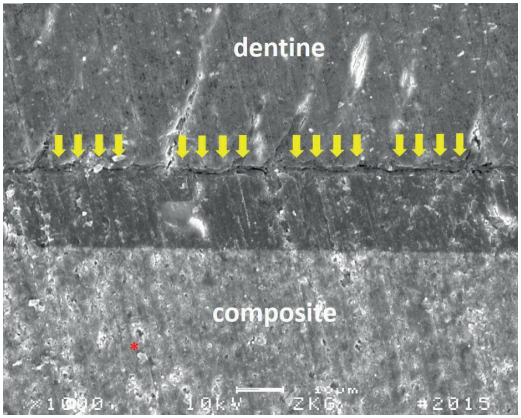

Figure 26: Qualitative evaluation of the adhesive area (yellow arrows) with SEM at 1000X magnification. Red mark showing composite resin fillers.

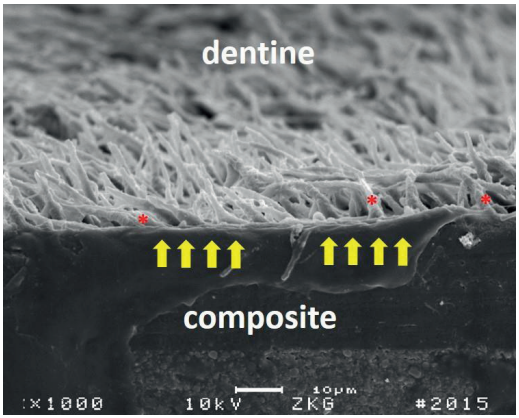

Figure 27: Qualitative evaluation of hybrid zone (yellow arrows) and composite tags (red marks) with SEM at 1000X magnification, after removal of the inorganic content. Picture corresponds to the same specimen as figure 26.

4.8. Statistical analysis

Description of the results was performed with mean value, standard deviation, min. value, max. value and median. Statistical analysis was performed with Statistical Package for Social Sciences – SPSS, version 15.0 (SPSS, Chicago, IL, USA) for Windows. Level of significance was set at $p<0.05$.

4.8.1 Caries model

Fluorescence microscope evaluation

Differences within the same group between the 5 randomized caries model series, were checked with Mann-Whitney *U* test. Normal distribution of the obtained data was checked with Kolomogorov-Smirnov test. Comparisons between enamel and dentine, margins and 500 μm away from the margins, or between 300 μm and 500 μm away from the margins, were performed with Student's T-Test. Analysis of variance was performed with One-way ANOVA and *post-hoc* analysis Fisher's Least Significant Difference (LSD) test was conducted to explore the presence of significant differences between specific comparisons for each variable.

SEM evaluation

Normal distribution was checked with Kolomogorov-Smirnov test. Comparisons between values before and after caries model were performed pairwise for every adhesive and every variable with the non-parametric Friedman Test. Differences between the tested adhesives were checked with One-way ANOVA followed by *post-hoc* analysis with Fisher's Least Significant Difference (LSD).

4.8.2 μTBS

Normal distribution of the obtained μTBS values was checked with Kolomogorov-Smirnov test. Analysis of variance was performed with One-way ANOVA and in cases of significant results, post-hoc test for multiple comparisons followed with Fisher's least significant difference (LSD). Significant differences between failure modes were investigated with Mann-Whitney *U* test.

5. Results

Table 8: Information on caries model series. Nr 1 – 5: 10-day biological loading of Class V restorations. Nr 6 – 9: 2-day biological loading before μTBS

Nr	OD 600nm	Concentration CFU/ml of *S. mutans* injected in the beginning / end of caries model	DEM – REM (hours/ pH)	Comments
1	0.958	800.000 *S.mutans* 11.000.000 *S.mutans*	40 / 4.2 - 218 / 7	Pump 2 failed during the experiment and specimens remained in artificial saliva for ~ 10 hours. External species detected during purity control at the end: *Pseud. Aeruginosa* (due to pH-electrode change), *B. Cereus.* Normal odor during operation.
2	1.006	200.000 *S.mutans* 3.000.000 *S. Mutans* 1.000.000 *B. cereus*	40 / 4.2 - 200 / 7.2	Caries models Nr. 2 and Nr. 3 operated simultaneously. External species detected during purity control at the end: *B. Cereus.*
3	1.006	200.000 *S.mutans* 5.000.000 *S. Mutans* 1.000.000 *B. cereus*	40 / 4.3 - 200 / 7	Caries models Nr. 2 and Nr. 3 operated simultaneously. External species detected during purity control at the end: *B. Cereus.*
4	0.882	1.440.000 *S.mutans* 30.000.000 *S. Mutans* 10.000.000 *B. cereus*	40 / 4.2 - 200 / 7.1	Caries models Nr. 4 and Nr. 5 operated simultaneously. External species detected during purity control at the end: *P. Aeruginosa* (on plates 10^{-1} – 10^{-5}), *B. Cereus*.
5	0.882	1.440.000 *S.mutans* 10.000.000 *S. Mutans* No *B. cereus*	40 / 4.2 - 200 / 7	Caries models Nr. 4 and Nr. 5 operated simultaneously. External species detected during purity control at the end: *P. Aeruginosa* (on plates 10^{-1} – 10^{-5})
6	1.025	480.000 *S.mutans* 131.000*S. Mutans* 10.000 Listeria	8 / 4.3 - 16 / 7.1	External species detected during purity control at the end: *L. monocytogenes*and micrococci at plate 10^{-6}. *Caries model cycle was excluded.*
7	1.039	1.440.000 *S.mutans* 50.000.000 *S. Mutans* 20.000.000 *B. cereus*	8 / 4.7 - 16 / 7	Analysis with MALDI-TOF to confirm presence of *S. mutans* due to size variations on the plates. External species detected during purity control at the end: *B. Cereus.*
8	0.977	360.000 *S.mutans* 4.000.000*S. Mutans* 6.000.000 *B. cereus*	8 / 4.5 - 16 / 7.1	Caries model Nr. 8 and Nr. 9 operated simultaneously. External species detected during purity control at the end: *B. Cereus.*
9	0.977	360.000 *S.mutans* 50.000.000*S. Mutans* 80.000.000*B. cereus*	8 / 4.4 - 16 / 7	Caries model Nr. 8 and Nr. 9 operated simultaneously. External species detected during purity control at the end: *B. Cereus.*

5.1 Caries model

Table 8 in page 62 summarizes the concentration of *S. mutans* colonies which were injected in the caries model before each caries model series and the concentration of *S. mutans* and of *B. Cereus* which were measured on BHI-plates (Becton, Dickinson and Company) after the end of the 10-day biological loading. The plating was performed in terms of purity control. In case contamination with external bacterial species was detected, caries model series was excluded from the study, if the external bacteria were acid-producing or had an influence on *S. mutans*. *P. Aeruginosa* (Pseudomonas Aeruginosa) was detected on BHI-plates (Becton, Dickinson and Company) during purity control at the end of caries model nr. 1 (possibly due to the need for pH-electrode change during the experiment), nr. 4 and 5 (possibly due to handling during assembling, since both caries models were assembled and operated simultaneously) (Table 8). Caries model nr. 6 which was contaminated with *L. monocytogenes* (Listeria monocytogenes) was excluded from the study (Table 8). Mean pH was 4.2 during demineralisation and 7 during remineralisation for Class V caries model, while 4.5 during demineralisation and 7 during remineralisation for μTBS caries models. Artificial plaque was detected around all restoration margins. No premature restoration loss was recorded during operation of caries model, due to failed retention.

5.1.1 Fluorescence microscope results

Descriptive statistic (mean value and standard deviation, SD) of demineralisation, substance loss due to demineralisation and total demineralisation as the sum of the two aforementioned variables, at restorations margins, at 300 μm and at 500 μm away from the margins, in enamel and in dentine, as well as marginal gap depth and width in both tooth tissues are presented in Tables 9, 10, 11, pages 65, 66, 67. Rounding of descriptive statistic data was performed to zero decimal places, since measurements were performed at pixel size of 0.9 μm. Data for all variables tested were normally distributed ($p>0.05$, Kolomogorov-Smirnov). Despite specimens were randomized, no significant difference was reported for none of the variables between the 5 Class V caries model series, which were compared in pairs with Mann-Whitney ($p>0.05$), although *S. mutans* counts differed (Table 8, page 62).

Between enamel and dentine

As expected, significant differences were noted in demineralisation, substance loss and total demineralisation, marginal gap depth and marginal gap width between enamel and dentine for all tested adhesives, at restoration margins, at 300 μm and at 500 μm away from the margins ($p<0.05$, T-Test), except from total demineralisation for PEAK at 500 μm away from the margins, which exhibited small statistical significance ($p=0.054$, T-Test). All other comparisons showed significantly higher values in dentine ($p<0.05$, T-Test).

Between restoration margins and 500 μm away from the margins

As expected, demineralisation, substance loss and total demineralisation at restoration margins was significantly higher for all tested adhesives in comparison to corresponding variables at 500 μm away from the margins ($p<0.05$, T-Test), except from total demineralisation for CTRL in enamel ($p=0.144$, T-Test) and total demineralisation for BOND in dentine ($p=0.21$, T-Test).

Between 300 μm and 500 μm away from the margins

No significant difference was noted for demineralisation, substance loss and total demineralisation between 300 μm and 500 μm away from the restoration margins for all tested adhesives ($p>0.05$, T-Test), exhibiting that despite distance away from the margins was randomly chosen, it had no significant impact on the results.

Between the adhesives

No significant differences between the adhesives were noted for total demineralisation values at 300 μm and at 500 μm away away from enamel or dentine margins ($p>0.05$, ANOVA) (Tables 9, 10, pages 65, 66). Results at enamel and dentine margins are separately discussed below.

- Enamel margins

Significant differences were exhibited between the tested adhesives for the variables total demineralisation (TOTAL) at enamel margins (p=0.03, ANOVA), for demineralisation and substance loss at dentine margins (p=0.003, ANOVA), and for marginal gap depth in enamel (p=0.029, ANOVA). Further analysis with*post hoc* test LSD showed that PRIM (p=0.007, mod. LSD), BOND (p=0.012, mod. LSD) and PEAK (p=0.008, mod. LSD) exhibited total demineralisation values in enamel margins, which were significantly higher than the CTRL. DENT showed however no statistical difference with any of the groups (p>0.05, mod. LSD) (Table 9 and Appendix I, page 129).

Table 9: Results of enamel demineralisation (µm, [SD]) after biological loading of Class V restorations for 10 days in the caries model.

Enamel			
Demineralisation µm, [SD]	DEM	SUB	TOTAL
	At restoration margins		
CTRL	55 [21]	6 [8]	61 [19] $^{A, B, C}$
DENT	66 [18]	7 [15]	73 [23]
PRIM	69 [16]	13 [17]	81 [16] ^{A}
BOND	74 [12]	6 [12]	80 [14] ^{B}
PEAK	68 [15]	15 [20]	82 [19] ^{C}
	300 µm away from the margins		
CTRL	48 [16]	7 [13]	55 [12]
DENT	36 [17]	24 [34]	60 [28]
PRIM	39 [23]	20 [21]	58 [14]
BOND	51 [15]	18 [14]	69 [10]
PEAK	41 [25]	14 [17]	54 [21]
	500 µm away from the margins		
CTRL	50 [15]	3 [11]	53[13]
DENT	38 [18]	15 [27]	54 [25]
PRIM	40 [16]	22 [28]	62 [19]
BOND	47 [19]	18 [17]	65 [23]
PEAK	37 [23]	13 [22]	50 [32]

Demineralisation (DEM), substance loss due to demineralisation (SUB) and total demineralisation (TOTAL) in enamel after biological loading in the caries model for 10 days. TOTAL = DEM + SUB. Adhesives exhibiting statistically significant differences are marked with the same capital letters.

- Dentine margins

No significant differences were noted for total demineralisation (TOTAL) in dentine. However adhesives showed significantly worse demineralisation (DEM) indentine margins in comparison to CTRL; DENT (p=0.001, mod. LSD), PRIM (p=0.07, mod. LSD), BOND (p=0.000, mod. LSD) and PEAK (p=0.006, mod. LSD). On the contrary substance loss due to demineralisation (SUB) at dentine margins was significantly higher for CTRL in comparison to BOND (p=0.004, mod. LSD) and PEAK (p=0.023, mod. LSD) (Table 10 and Appendix II, page 129).

Table 10: Results of dentinedemineralisation (µm, [SD]) after biological loading of Class V restorations for 10 days in the caries model.

Dentine			
Demineralisation µm, [SD]	DEM	SUB	TOTAL
	At restoration margins		
CTRL	34 [25] A,B, C, D	105 [38] E, F, G	144 [21]
DENT	74 [19] A	76 [40] G	150 [37]
PRIM	65 [29] B	93 [34] K	158 [28]
BOND	77 [18] C	58 [34] E, K	135 [31]
PEAK	68 [39] D	67 [47] F	134 [39]
	300 µm away from the margins		
CTRL	48 [21] G, H, J	65 [26]	113 [33]
DENT	75 [21] G	46 [31]	122 [38]
PRIM	69 [16] H	62 [19]	131 [22]
BOND	61 [20]	50 [24]	111 [33]
PEAK	70[30] J	56 [38]	126 [32]
	500 µm away from the margins		
CTRL	52 [16]	52 [24]	104 [21]
DENT	71 [35]	42 [36]	114 [63]
PRIM	66 [10]	53 [27]	119 [25]
BOND	64 [14]	51 [14]	116 [24]
PEAK	65 [31]	35 [34]	101 [50]

Demineralisation (DEM), substance loss due to demineralisation (SUB) and total demineralisation (TOTAL) in dentine after biological loading in the caries model for 10 days. TOTAL = DEM + SUB. Adhesives exhibiting statistically significant differences are marked with the same capital letters.

- Marginal gap width & depth

Concerning marginal gap depth in enamel, CTRL showed significantly higher values in comparison to every CHX adhesive; specifically with DENT (p=0.009, mod. LSD), PRIM (p=0.007, mod. LSD), BOND (p=0.007, mod. LSD). No difference was exhibited for marginal gap width in enamel or in dentine and for marginal gap depth in dentine (p>0.05, ANOVA) (Table 11).

Table 11: Results of marginal gap formation (µm, [SD]) in enamel and in dentine after biological loading of Class V restorations for 10 days in the caries model.

Marginal gaps				
	Enamel		Dentine	
Marginal gap in µm, [SD]	Marginal gap depth	Marginal gap width	Marginal gap depth	Marginal gap width
CTRL	35 [66] $^{A, B, C}$	8 [14]	59 [37]	26 [18]
DENT	0 [0] ^{A}	0 [0]	55 [42]	30 [17]
PRIM	0 [0] ^{B}	0 [0]	68 [25]	37 [16]
BOND	0[0] ^{C}	0[0]	72 [58]	26 [17]
PEAK	5 [17]	10 [31]	69 [37]	30 [16]

Adhesives exhibiting statistically significant differences are marked with the same capital letters.

5.1.2 Marginal analysis with SEM

Descriptive statistic (mean value % and standard deviation, [SD]) regarding marginal quality before and after biological loading in caries model, in enamel and in dentine, are presented in Tables 12, 13, page 69). Data for all variables tested were normally distributed ($p>0.05$, Kolomogorov-Smirnov).

Before and after caries model

Significant differences were exhibited in enamel for the variable "perfect" before and after caries model for all tested adhesives ($p<0.05$, Friedman), as the percentage of perfect margins decreased after biological loading (Table 12, page 69). On the contrary, no such difference was demonstrated for dentine, except for adhesives BOND ($p=0.021$, Friedman) and PEAK ($p=0.001$, Friedman) (Table 13, page 69). At the same time, percentages for the variable "gap" increased significantly after biological loading ($p<0.05$, Friedman). Marginal gaps in dentine were significantly increased after caries model for PRIM ($p=0.021$, Friedman) and PEAK ($p=0.001$, Friedman) (Table 13, page 69).

Between the adhesives

Significant differences between the adhesives were only exhibited for the variable "gap". No difference was noted between the tested adhesives, regarding the percentage of perfect margins in enamel or in dentine, before or after caries model ($p>0.05$, ANOVA) (Tables 12, 13, page 69). DENT exhibited significantly lower enamel gap values compared to all other groups ($p=0.001$, ANOVA). Regarding dentine margins, DENT showed significantly lower gap percentage compared to the CTRL ($p=0.025$, mod. LSD).

Table 12: Marginal analysis before and after caries model in enamel for variables perfect margin, overhand and gap, demonstrated in % percentage mode.

Mean value % of total margin length [SD]	Before caries model			After caries model		
	Perfect	Over hang	Gap	Perfect	Over hang	Gap
CTRL	83 [14]^{A}	1 [0]	14 [4]^{A}	8 [2]$^{A, a}$	0 [0]	98 [6]$^{A, a}$
DENT	95 [7]^{A}	1 [0]	2 [0]^{A}	78 [4]$^{A, a, b, c, d}$	4 [0]	52 [5]$^{A, a, b, c, d}$
PRIM	81 [20]^{A}	1 [0]	17 [2]^{A}	6 [2]$^{A, b}$	4 [0]	93 [14]$^{A, b}$
BOND	87 [19]^{A}	0 [0]	12 [1]^{A}	2 [2]$^{A, c}$	0 [0]	100 [0]$^{A, c}$
PEAK	78 [27]^{A}	4 [1]	16 [2]^{A}	19 [12]$^{A, d}$	18 [14]	81 [18]$^{A, d}$

Variables underfilled, fracture and not evaluable are not included since they range ≈0% for all tested adhesives. Adhesives exhibiting statistically significant differences before and after caries model (horizontal) are marked with the same capital letters, while significant differences between the adhesives (vertically) are marked with same lowercase letters.

Table 13: Marginal analysis before and after caries model in dentine for variables perfect margin, overhand and gap, demonstrated in % percentage mode.

Mean value % of total margin length [SD]	Before caries model			After caries model		
	Perfect	Over hang	Gap	Perfect	Over hang	Gap
CTRL	49 [2]^{a}	19[6]	46 [2]^{a}	33 [6]	0 [0]	63 [2]
DENT	69 [2] ^{a}	5 [1]	25 [2]^{a}	45 [2]	7 [1]	44 [2]
PRIM	60 [3]	8 [1]	31 [2]^{A}	29 [2]	2 [0]	67[19]^{A}
BOND	68 [9]^{A}	1 [0]	30 [2]	44 [19]^{A}	5 [0]	50 [19]
PEAK	76 [17]^{A}	6 [1]	36 [14]^{A}	27 [21]^{A}	9 [1]	63 [22]^{A}

Variables underfilled, fracture and not evaluable are not included since they range ≈0% for all tested adhesives. Adhesives exhibiting statistically significant differences before and after caries model (horizontal) are marked with the same capital letters, while significant differences between the adhesives (vertically) are marked with same lowercase letters.

5.2 Calibrationfor µTBS

Comparison of µTBS data from operator of the present study (mean value 54.6 MPa ± 14.7) was performed against an already calibrated operator (mean value 65.1 MPa ± 25.2). Data were normally distributed (Kolomogorov-Smirnov, $p>0.05$). Comparison exhibited statistically higher bond strength values compared to the already calibrated operator ($p<0.001$, ANOVA).

5.3 µTBS

Mean values and standard deviations of the descriptive statistic for µTBS of the adhesives at day 1 (baseline), after µTBS caries model and after long-term storage in different storage media, is reported in Tables 14, 15, 16, pages 71, 72, 73. Since data were normally distributed ($p>0.05$, Kolomogorov-Smirnov), significant differences were calculated with ANOVA, mod. LSD between the adhesives and within each adhesive regarding µTBS values for day 1, after µTBS caries model, and after long-term storage.

5.3.1 Comparison between the adhesives

Day 1

CTRL exhibited significantly higher µTBS in comparison to PRIM ($p=0.000$, mod. LSD), BOND ($p=0.002$, mod. LSD) and PEAK ($p=0.000$, mod. LSD), while DENT exhibited better performance compared to PRIM ($p=0.006$, mod. LSD) and PEAK ($p=0.004$, mod. LSD) (Table 14, page 71 – upper case). Moreover, DENT showed significantly less adhesive fractures compared to both aforementioned groups; with PRIM ($p=0.022$, Mann-Whitney) and PEAK ($p=0.002$, Mann-Whitney). No difference in fractures was evident between CTRL and DENT, or between PRIM, BOND and PEAK ($p>0.05$, ANOVA) (Figure 28, page 74).

Caries Model

CTRL and BOND showed the lowest values after biological loading in caries model (Table 14). CTRL demonstrated significantly lower μTBS compared to DENT (p=0.000, mod. LSD), PRIM (p=0.008, mod. LSD) and PEAK (p=0.000, mod. LSD). Same behavior was noted by BOND in comparison to DENT (p=0.000, mod. LSD), PRIM (p=0.003, mod. LSD) and PEAK (p=0.001, mod. LSD) (Table 14 – upper case). DENT (p=0.029, Mann-Whitney), PRIM (p=0.002, Mann-Whitney) and BOND (p=0.001, Mann-Whitney) exhibited less adhesive and more cohesive fractures than CTRL, while PEAK showed significantly more adhesive fractures than PRIM (p=0.04, Mann-Whitney) and BOND (p=0.023, Mann-Whitney) (Figure 29, page 74).

Table 14: Comparison of μTBS values between day 1 (baseline) and after biological loading in caries model.

Groups	Day 1		Caries Model	
	Nr. of sticks	MPa, [SD]	Nr. of sticks	MPa, [SD]
CTRL	99	58,82 [19,55] $^{A, B, C, a}$	102	30,44 [16,92] $^{F, G, H, a}$
DENT	105	54,00 [18,22] $^{D, E, a}$	109	45,55 [17,7] $^{F, I, a}$
PRIM	108	45,70 [16,05] $^{A, D, a}$	99	40,13 [13,41] $^{G, J, a}$
BOND	106	49,60 [18,59] $^{B, a}$	107	32,37 [13,08] $^{I, J, K, a}$
PEAK	103	45,22 [15,41] $^{C, E, a}$	105	40,83 [14,85] $^{H, K, a}$

Statistically significant differences between the groups (vertically) are marked with same upper case letters, while differences before and after biological loading (horizontally) are marked with same lower case letters.

Water storage

Within the adhesives, μTBS decreased significantly between baseline and 6 months or baseline and 12 months ($p<0.01$, ANOVA). No significant interaction between CHX adhesives was noted after 6-month storage in water ($p>0.05$, ANOVA), except from PRIM and BOND ($p=0.002$, mod. LSD). PEAK exhibited significantly less adhesive failures than CTRL ($p=0.001$, mod. LSD), PRIM ($p=0.001$, mod. LSD) and BOND ($p=0.02$, mod. LSD). Concerning 12-month storage, only μTBS of DENT was significantly higher to CTRL, PRIM, BOND and PEAK ($p=0.000$, mod. LSD) (Table 15 – upper case). Adhesive fractures of DENT were also significantly lower in comparison to all aforementioned groups ($p=0.000$, Mann-Whitney) (Figure 30, page 75).

Table 15: Comparison of μTBS values between day 1 (baseline) and after 6- and 12-month storage in distilled water.

Groups	Day 1		6 – Month Storage Water		12 – Month Storage Water	
	Nr. of sticks	MPa, [SD]	Nr. of sticks	MPa, [SD]	Nr. of sticks	MPa, [SD]
CTRL	99	58,82 [19,95] A, B, C, a	115	33,84 [18] a	96	23,59 [14,79] K, a
DENT	105	54,00 [18,22] D, E, a	96	37,94 [13,07] F, a	92	39,14 [16,29] K, L, M, N, a
PRIM	108	45,70 [16,05] A, D, a	111	33,26 [13,97] a	88	26,94 [11,57] L, a
BOND	106	49,60 [18,59] B, a	98	29,84 [13,55] F, a	85	23,68 [8,84] M, a
PEAK	103	45,22 [15,41] C, E, a	90	34,30 [14,14] a	81	22,02[11,49] N, a

Statistically significant differences between the groups (vertically) are marked with same upper case letters, while differences after 6- or 12-month storage within each group (horizontally) are marked with same lower case letters.

Saliva storage

Within the adhesives, μTBS decreased significantly between baseline and 6 months or 12 months ($p<0.05$, ANOVA), except for PRIM which showed no significant reduction after 12 months compared to baseline ($p>0.05$, ANOVA). DENT exhibited significantly higher μTBS compared to PRIM ($p=0.013$. mod LSD) and BOND ($p=0.000$, mod. LSD) after 6-month storage in saliva. PEAK also demonstrated higher bond values against PRIM ($p=0.037$, mod. LSD) or BOND ($p=0.001$, mod. LSD), although its difference with PRIM was at the borderline (PEAK=34.3 MPa and PRIM=33.26 MPa) (Table 16). No significant interactions where noted between bond failure mode at 6-months ($p>0.05$, Mann-Whitney). After 12 months, more significant interactions presented, as CTRL had significantly lower μTBS in comparison to DENT ($p=0.002$, mod. LSD), PRIM and BOND ($p=0.000$, mod. LSD), as well as significantly more adhesive failures ($p<0.001$, Mann-Whitney).As CTRL and PEAK showed the same lowest μTBS values ($p=1.000$, ANOVA), PEAK exhibited significantly lower values to DENT, PRIM BOND as well (Table 16 – upper case).

Table 16: Comparison of μTBS values between day 1 (baseline) and after 6- and 12-month storage in artificial saliva.

Groups	Day 1		6 – Month Storage Saliva		12 – Month Storage Saliva	
	Nr. of sticks	MPa, [SD]	Nr. of sticks	MPa, [SD]	Nr. of sticks	MPa, [SD]
CTRL	99	58,82 [19,95] A, B, C, a	93	36,35 [13,67] a	84	32,12 [16,84] K, L, M, a
DENT	105	54,00 [18.22] D, E, a	106	38,11 [13,96] G, H, a	92	41,39 [14,63] K, N, a
PRIM	108	45,70 [16,05] A, D, a	106	31,62 [14,68] G, I, a	106	43,29 [14,43] L, O
BOND	106	49,60 [18,59] B, a	99	29,45 [14,08] H, J, a	115	43,26 [18,83] M, P, a
PEAK	103	45,22 [15,41] C, E, a	102	37,52 [16,41] I, J, a	81	33,89 [17,01] N, O, P, a

Statistically significant differences between the groups (vertically) are marked with same upper case letters, while differences after 6- or 12-month storage within each group (horizontally) are marked with same lower case letters.

Fracture modes

Mode of bond failures is demonstrated in Figures 28 - 31.The failure pattern is described in % percentage terms. Some pre-test failures appeared after 12-month storage. After biological loading, DENT (p=0.029, Mann-Whitney), PRIM (p=0.002, Mann-Whitney) and BOND (p=0.001, Mann-Whitney) exhibited significantly less adhesive fractures than CTRL.PEAK exhibited significantly less adhesive failures than CTRL (p=0.001, mod. LSD) after 6 months in water. Adhesive fractures of DENT after 12-month storage, were significantly lower in comparison to all other groups in water (p=0.000, Mann-Whitney) or in saliva (p<0.001, Mann-Whitney).

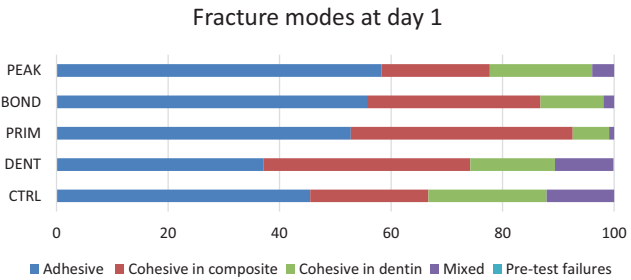

Figure 28: Mode of bond failure at day 1 (baseline). Colors correspond to different types of fractures, which are presented in percentage %.

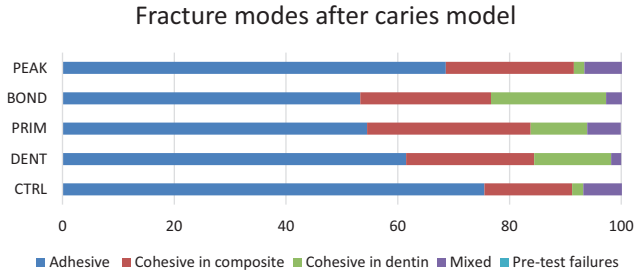

Figure 29: Mode of bond failure after caries model. Colors correspond to different types of fractures, which are presented in percentage %. Adhesive failures have increased compared to baseline.

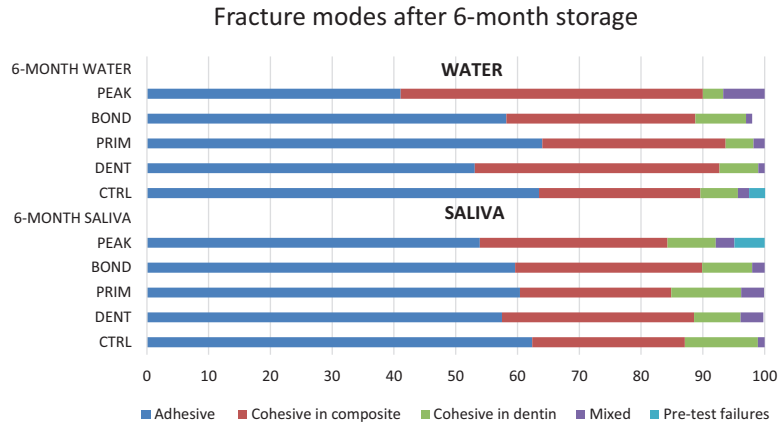

Figure 30: Mode of bond failure after 6-month storage in distilled water and artificial saliva. Colors correspond to different types of fractures, which are presented in percentage %. Few pre-test failures are observed in CTRL in water and in PEAK in saliva.

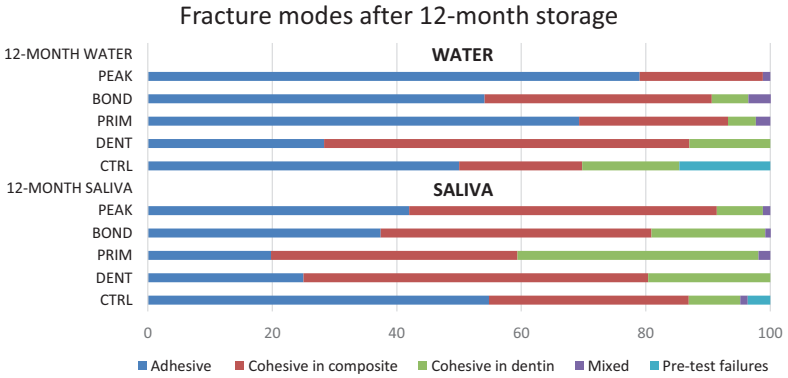

Figure 31: Mode of bond failure after 12-month storage in distilled water and artificial saliva. Colors correspond to different types of fractures, which are presented in percentage %. Pre-test failures were only observed in CTRL, in both storage conditions.

5.3.2 Comparison within the adhesives

CTRL

CTRL exhibited, as expected, significantly higher μTBS values at baseline, compared to biological loading, after 6- and 12-month storage in water or saliva (p=0.000, mod. LSD). Comparison between 6- and 12- months in saliva showed no statistical difference (p=1.000, mod. LSD).

DENT

DENT exhibited significantly higher μTBS values at baseline, compared to biological loading, after 6- and 12-month storage in water or saliva (p=0.000, mod. LSD), exactly as the CTRL group (CTRL). Comparison between 6- and 12- months in water or saliva showed no statistical difference (p=1.000, mod. LSD).

PRIM

PRIM showed significantly lower μTBS values after μTBS caries model (p=0.038, mod. LSD). Significant μTBS reduction in comparison to baseline values was also noted after 6-month storage in water (p=0.028, mod. LSD) or saliva (p=0.000, mod. LSD) and after 12-month storage in water (p=0.000, mod. LSD). No difference was reported for 12-month saliva storage (p=1.000, mod. LSD). Storage medium had no effect on μTBS values after 6-months (p=1.000, ANOVA), but 12-month storage in saliva demonstrated significantly higher μTBS values compared to water (p=0.000, mod. LSD). Values were significantly higher at 6-month compared to 12-month storage in water (p=0.028, mod. LSD) and in saliva (p=0.000, mod. LSD).

BOND

BOND exhibited significantly lower μTBS values after μTBS caries model (p=0.000, mod. LSD) and after 6-month storage in water or saliva, and after 12-month water storage (p=0.000, mod. LSD), like PRIM. 12-month storage in saliva also exhibited statistically lower values compared to baseline (p=0.032, mod. LSD). μTBS values were significantly higher at 6-month compared to 12-month storage in water (p=0.000, mod. LSD). No such difference was shown for the same comparison in saliva (p=1.000, mod. LSD).

PEAK

PEAK exhibited significantly lower μTBS values after μTBS caries model (p=0.000, mod. LSD) and after 6-month storage in water or saliva, and after 12-month water storage (p=0.000, mod. LSD), like the previous CHX adhesives. 12-month storage in saliva also showed lower μTBS values (p=0.000, mod. LSD), like BOND. Same with BOND were the significant differences exhibited between 6- and 12-month storage in water (p=0.000, mod. LSD) and saliva (p=1.000, mod. LSD).

5.3.3 Comparison between storage media

No significant difference was noted between storage media at 6-months (p=1.000, mod. LSD) for any of the adhesives under investigation. At 12-month storage, no difference was reported for DENT (p=1.000, mod. LSD), while PRIM, BOND and PEAK exhibited statistically lower μTBS values in water compared to saliva (p=0.000, mod. LSD). On the other hand, CTRL exhibited significantly lower values in water storage, but level of significance was lower (p=0.011, mod. LSD) (Table 17).

Table 17: Comparison of μTBS values (MPa, [SD]) between storage media after 6- and 12-month storage in distilled water or artificial saliva.

Groups	6 – Month Storage Water	6 – Month Storage Saliva	12 – Month StorageWater	12 – Month Storage Saliva
CTRL	33,84 [18]	36,35 [13,67]	23,59 [14,79] ^{A}	32,12 [16,84] ^{A}
DENT	37,94 [13,07]	38,11 [13,96]	39,14 [16,29]	41,39 [14,63]
PRIM	33,26 [13,97]	31,62 [14,68]	26,94 [11,57] ^{B}	43,29 [14,43] ^{B}
BOND	29,84 [13,55]	29,45 [14,08]	23,68 [8,84] ^{C}	43,26 [18,83] ^{C}
PEAK	34,30 [14,14]	37,52 [16,41]	22,02[11,49] ^{D}	33,89 [17,01] ^{D}

Statistically significant differences between storage media for each storage duration (horizontally) are marked with same upper case letters.

5.3.3 Qualitative SEM evaluation

Qualitative SEM evaluation was performed in exemplary samples at 1000X magnification. Presence of resin tags, width of hybrid layer and quality of the adhesive interface was compared within each group between different loading and storage conditions. CTRL exhibited evident degradation of its adhesive zone, expecially after 6-month storage in water and 12-month storage in saliva (Figure 32, page 79). This corresponded well with the μTBS values shown in Table 15, page 72 and Tabe 16, page 73, showing a significant decrease of μTBS overtime in both storage media ($p<0.05$, mod. LSD). Adhesive interface quality was preserved in DENT up to 6 months, but resin tags collapsed after 12-month storage in both storage media (Figure 33, page 80). However, a significant decrease in μTBS values was monitored both in 6- and in 12-month storage ($p<0.05$, mod. LSD) (Table 15, page 72 and Table 16, page 73) but adhesive fractures decreased in 12 months (Figure 31, page 78). Similarly, PRIM presented a well-preserved adhesive interface up to 6 months, which was degraded especially after 12-month storage in water (Figure 34, page 81). However, a significant decrease in μTBS values was monitored in 6-month storage ($p<0.05$, mod. LSD) (Table 15, page 72 and Table 16, page 73) but not for 12-month storage in saliva ($p>0.05$ ANOVA) (Table 16, page 73), which corresponds well with the qualitative SEM evaluation. Although μTBS showed significant decrease overtime after storage for BOND and PEAK ($p<0.05$, mod. LSD) (Table 15, page 72 and Table 16, page 73), resin tags in BOND were well preserved after 12-month in water (Figure 35, page 82) and PEAK showed no alteration regarding its adhesive interface in SEM pictures after 6-month storage in both media. Resin tags in PEAK disappeared completely after 12 months (Figure 36, page 83).

CONTROL GROUP (CTRL)

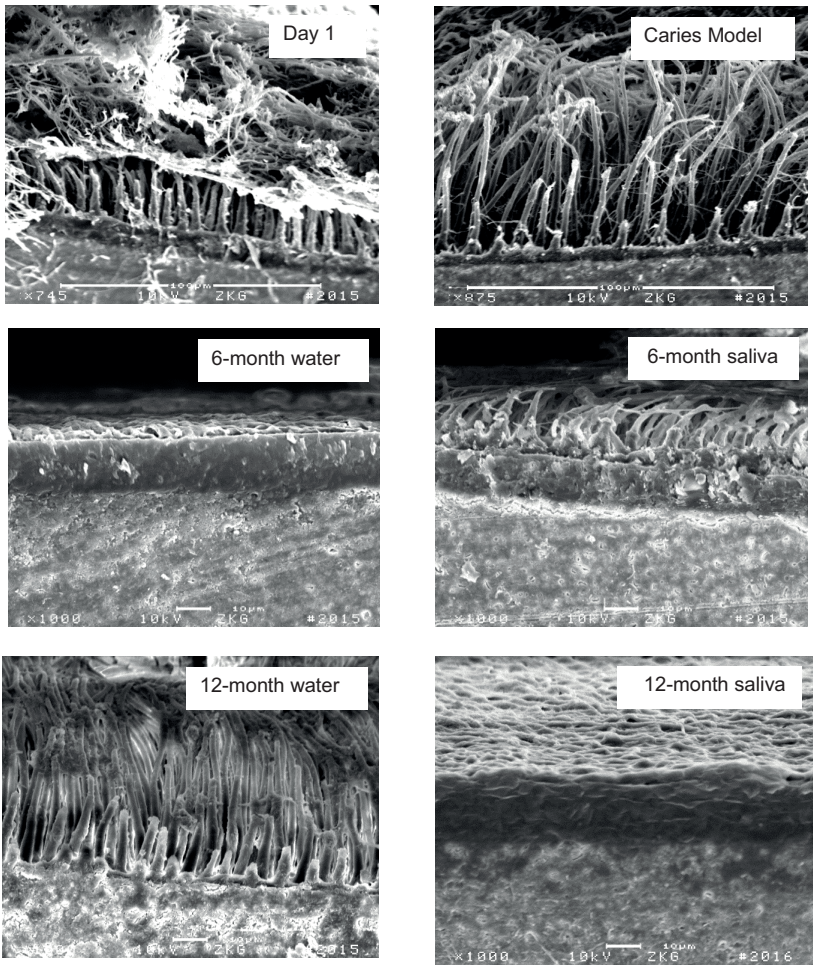

Figure 32: SEM pictures 1000X showing adhesive interfaces in CTRL group for Day 1 (baseline), Caries Model, 6-month water storage, 6-month artificial saliva storage, 12-month water storage, 12-month artificial saliva storage. Degradation of resin tags is evident after storage in both media.

2% CHX AS DENTINE PRE-TREATMENT(DENT)

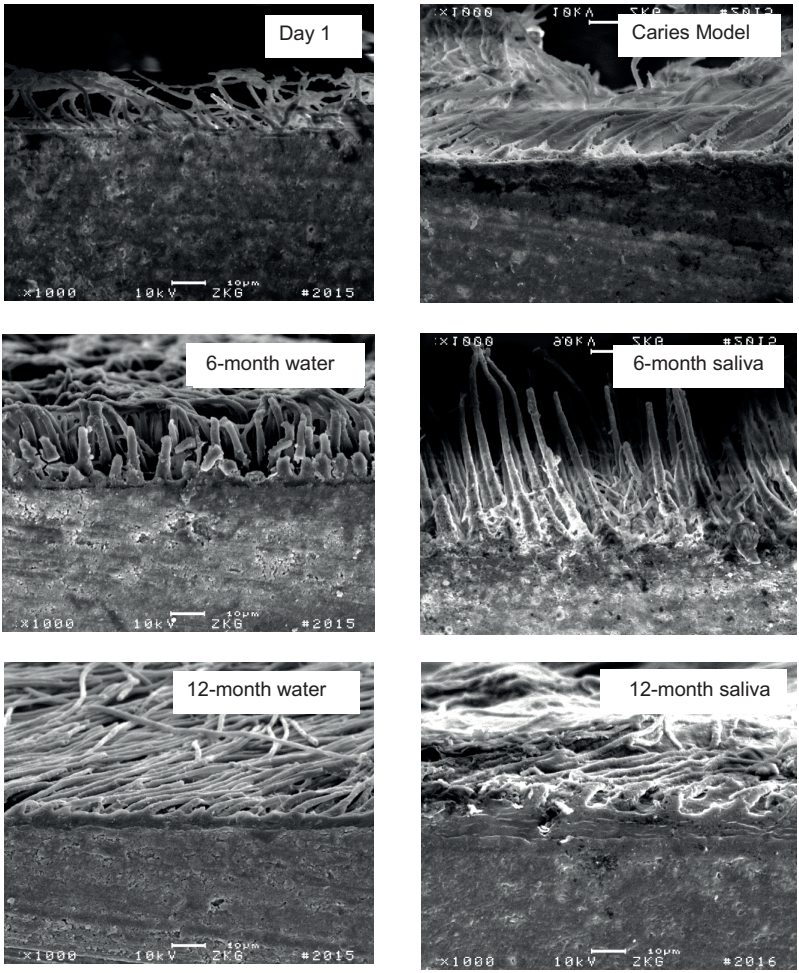

Figure 33: SEM pictures 1000X showing adhesive interfaces in DENT group for Day 1 (baseline), Caries Model, 6-month water storage, 6-month artificial saliva storage, 12-month water storage, 12-month artificial saliva storage. Resin tags were preserved up to 6-month storage, but they collapsed after 12 months storage in distilled water or artificial saliva.

0.1% CHX IN PRIMER (PRIM)

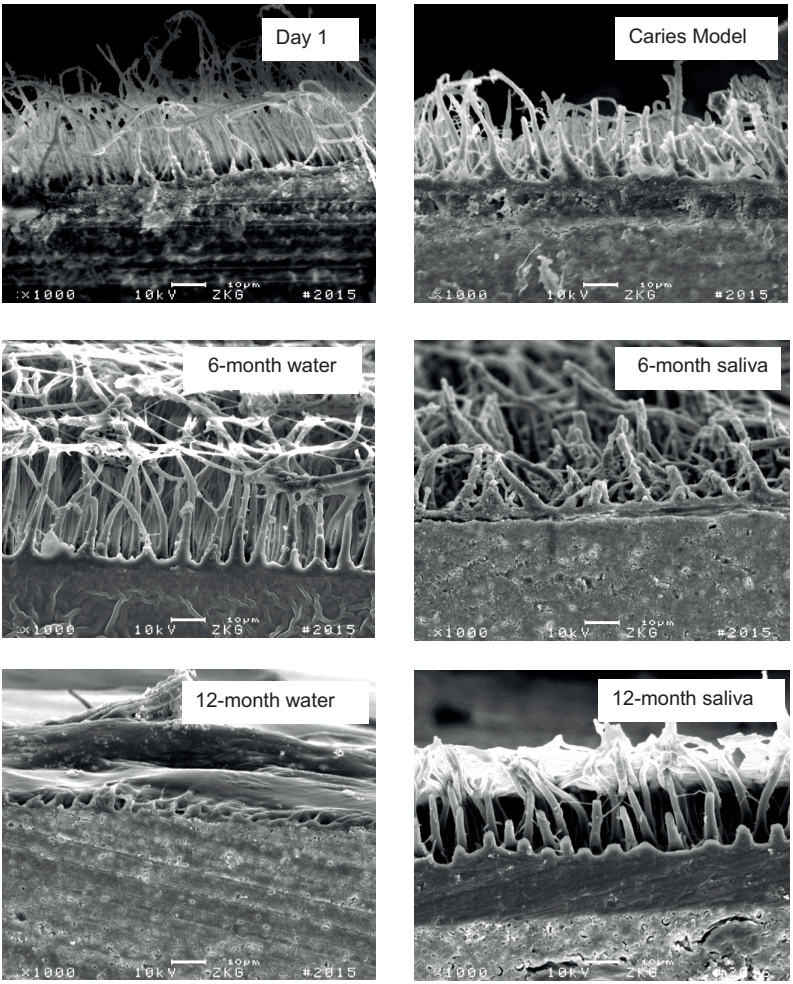

Fig. 34: SEM pictures 1000X showing adhesive interfaces in PRIM group for Day 1 (baseline), Caries Model, 6-month water storage, 6-month artificial saliva storage, 12-month water storage, 12-month artificial saliva storage. Resin tags are well preserved after 6 months and only after 12-month storage in artificial saliva. 12-month storage in water destroyed the tags.

0.1% CHX IN BONDING RESIN (BOND)

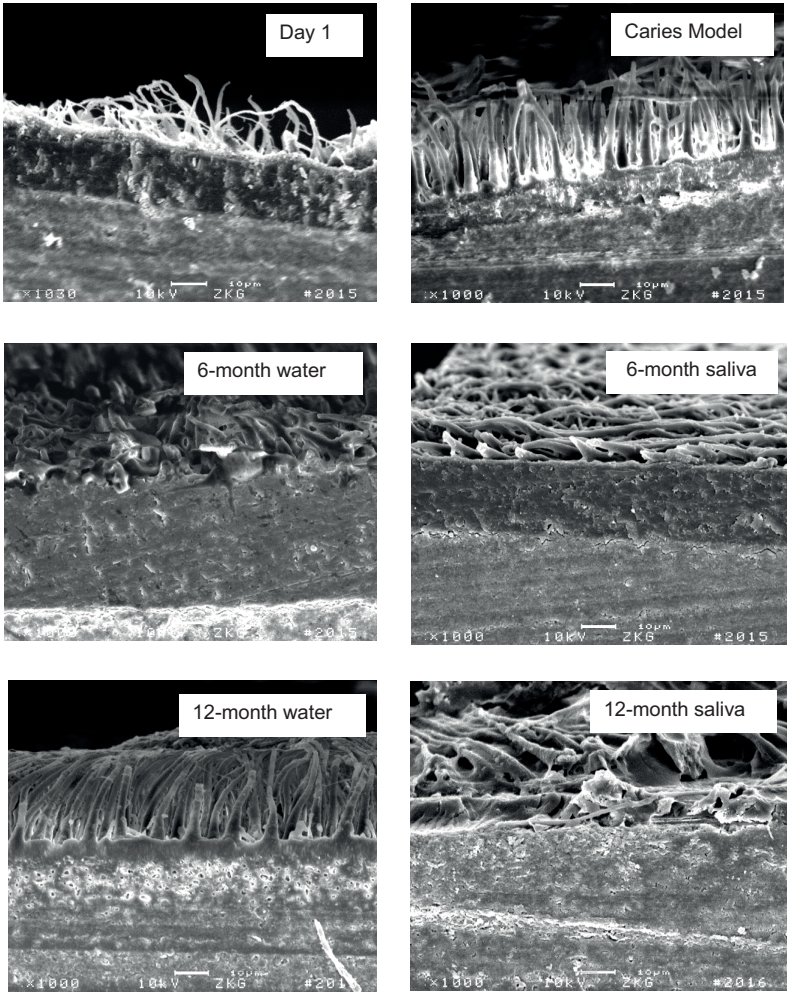

Figure 35: SEM pictures 1000X showing adhesive interfaces in BOND group for Day 1(baseline), Caries Model, 6-month water storage, 6-month artificial saliva storage, 12-month water storage, 12-month artificial saliva storage. Resin tags collapsed after 6- or 12-month storage in saliva, but were well preserved after 12 months in distilled water.

PEAK BONDING AGENT WITH 0.2% CHX (PEAK)

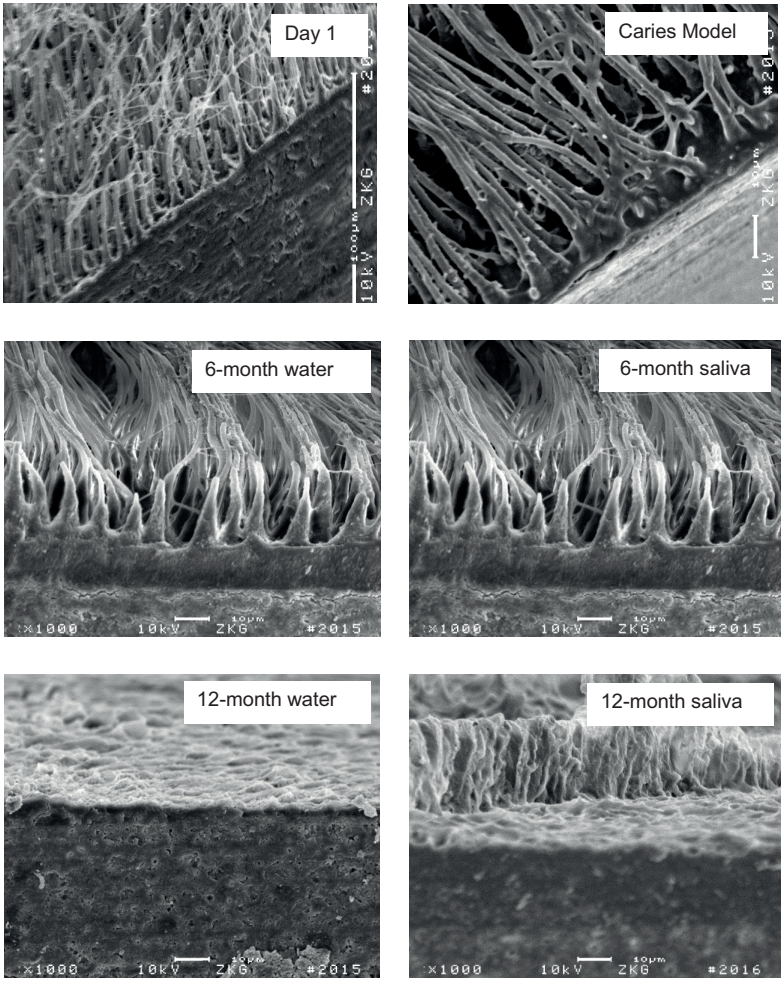

Figure 36: SEM pictures 1000X showing adhesive interfaces in PEAK group for Day 1 (baseline), Caries Model, 6-month water storage, 6-month artificial saliva storage, 12-month water storage, 12-month artificial saliva storage. Resin tags were preserved up to 6 month storage but disappeared in 12-month storage samples.

6. Discussion

6.1 Discussion of material and methods

6.1.1 Addition of CHX in adhesives

Incorporation of CHX in dental materials has been investigated since 1983 [154], as a way of avoiding the addition of one more step of separate CHX application on dentine, during the adhesive procedure. Since addition of CHX in restorative composites is related to serious side-effects in their physicomechanical properties [154,165,163,20], it was decided to alternatively test CHX addition in adhesive systems. CHX diglugonate was admixed to the primer and bonding agent of a commercially available adhesive according to *Zhou et al. 2009* [160,162]. By loading the adhesives with CHX, these could act as CHX carriers deeper into the adhesive zone, hypothetically offering a double benefit: i) a potentially slower CHX release due to its increased depth and deeper localization, and thus longer duration of antimicrobial action and ii) closer proximity to the source of endogenous proteases (MMPs and CCs), therefore increasing its anti-collagenolytic action. Moreover, the adhesive interface is the weakest link of the composite restorations and thus more vulnerable to bacterial attack. Therefore, loading the adhesives with antimicrobials instead of the restorative materials could be advantageous in terms of localized action. On the contrary, the ability of an antibacterial adhesive to inhibit progression of secondary caries is directly proportional to the contact area between biofilm and the adhesive [211], and adhesives, unlike composite resins have a limited exposed area at the tooth-restoration interface.

6.1.2 How much CHX is too much

Since literature presents controversial results regarding degree of conversion [157,167], elasticity [167], water sorption [158,168] and bond strength [170,162,159,168,144] when CHX is admixed into adhesives in concentrations up to 5%, a safe concentration of 0.1% CHX was chosen as one of the lowest concentration evaluated in the literature (Table 1, page 17-18). In that direction, alteration of the physicomechanical properties of the experimental adhesives PRIM and BOND would be minimal or even avoided. On the other hand, it is unclear whether this very low – but safe – CHX concentration would be able to

induce an antibacterial and anti-proteolytic effect. According to *Gendron et al. 1999* [110], a much lower CHX concentration of 0.0001% is needed to suppress MMP-2 activity, 0.002% for MMP-9 and 0.02% for MMP-8, the latter not being covered by the experimental adhesives of the study. These values however correspond to the appropriate CHX concentration at the site of action and not to the CHX concentration initially delivered on dentine or admixed with the adhesives. Moreover, since CHX concentration changes overtime due to kinetics, it is questionable how long could CHX be delivered, even in those minimum amounts, regardless of its initial concentration. On the contrary, after application of CHX on dentine or after its release from the adhesives, it can be bound to dentine [105] due to its excellent substantivity, which is not affected by its concentration either being 0.2% or ten times higher (2%) [115]. Since CHX is classified as a soluble agent [69,5], potential of release of admixed CHX from the adhesives raises no arguments.In order to obtain an antibacterial effect, CHX levels should however be higher than those discussed for its anti-proteolytic activity. Even then, CHX release could be monitored for up to 5 weeks [103] and decrease in bacteria counts up to 3 months [180], both observation times being within the timeframe of the present experiment for CHX antibacterial and antiproteolytic action. However, since the chemical integrity of the adhesive is a critical factor in the adhesive procedure, it seems logical not to overload adhesives with CHX in order to achieve higher release. CHX industrially added (PEAK) reached a higher final concentration of 0.2% according to the manufacturer compared to the experimental CHX adhesives, PRIM and BOND (Tables 2, page 26 and Table 3, page 27). In order to comply with literature, which shows that 2% CHX as dentine pre-treatment suppresses collagenolytic activities in dentine [77,15,80,16,110,117,84,109,17,118–125,63,64,126, 127,120,128], same concentration was used in the present study.

6.1.3 Caries model set-up

A mono-bacterial, automated caries model (Figure7, 8, page 37) was used in the present study, according to the biological protocol (Table 7, page 37) established by earlier studies [185,209]. Simulation of demineralisation was achieved by *S. mutans*, which is regarded as the main pathogen that causes dental caries [88]. Therefore it is widely used in artificial mouth set-ups [212,213,185,209,176]. Among its positive characteristics regarding *in vitro*

studies, is that as facultative anaerobic bacteria, they are able to grow in both aerobic and anaerobic conditions, which allows for caries models to function without the need of air exchange, thus eliminating communication of the caries model with the external environment and possible external contaminations. *S. mutans* are also easily recognizable in cultures from their arrangement “like a row of pearls” [190], allowing for an ease way to perform purity control before and after each caries model cycle. *B. Cereus* (Bacillus Cereus) which was visually detected on BHI-plates (Becton, Dickinson and Company) during purity control at the end of the most caries model series (Figure 14), is resistant to disinfection with alcohol [214,215] and to acidic pH [124] but there is no evidence that it can influence tooth demineralisation. Therefore caries model series where *B. cereus* was detected, were not repeated.Although bacterial counts differed between caries model series (Table 8, page 62), no significant difference was noted between demineralisation values of randomized specimens from different caries model series ($p>0.05$, Mann-Whitney), thus exhibiting that the biological protocol was repeatable. Simulation of secondary caries-like lesions, as close as possible to the clinical situation, was achieved through constant alteration between de- and remineralisation phases [216], which caused interchangeable pH values imitating Stephan’s curve [217]. Remineralisation was induced by artificial saliva which neutralized bacterial acids. Prerequisites for its action were adequate concentration of calcium and phosphate ions and sufficient duration of time [216], both of which were met according to the biological protocol (Table 7, page 51) and the composition of the artificial saliva.It is important to acknowledge that *in vitro* studies have limitations because they cannot simulate all the complexity of an *in vivo* environment, such as tooth brushing, dietary alterations and different sugar intake among individuals, bacterial concentration and salivary flow. However literature supports that bacterial counts are not related with caries activity or caries risk [91] and that variations in dietary patterns in real-life are moderate [192]. The computer-controlled caries model set-up used in the present study gathered a lot of favorable characteristics which bring the simulation of secondary caries production as close to the intraoral conditions as possible; among them interchangeable de- and remineralisation phases, continuous culture of *S. mutans*, possibility of recharging containers under aseptic conditions, controlled flow of artificial saliva and nutrition medium, availability of two reaction chambers operating independently, continuous pH

monitoring, intraoral sugar clearance before each remineralisation phase, exposure of all specimens to the same pH and temperature, plaque accumulation and biofilm formation. Moreover, attention was given to the following details. Bacteria were allowed to proliferate in the reservoir container (Figure 10, page 40) for 6 hours before inserting them into the reaction chamber. This time interval allows for the production of the appropriate amount of bacteria, which would result in sufficient acid production for the induction of demineralisation. Since bacterial concentration was not high enough from the first demineralisation phase at the beginning of a caries model, this first demineralisation, was excluded and repeated at the end of the caries model cycle, in order to end up with totally 40 (for the Class V caries model) or 8 (for the μTBS caries model) sufficient demineralisations. In order for a demineralisation to be considered sufficient, pH of the bacterial solution should be 4.2 – 4.3. This enables sufficient demineralisation of both enamel and dentine. Each demineralisation lasted for 1 hour and every day specimens were incubated with *S. mutans* for 4 hours. This induces secondary caries-like lesions comparable to clinical situations, while smooth surface caries, like Class V secondary caries, are also induced at even shorter demineralisation periods [190]. A problem with *in vitro* caries models is a rather destructive demineralisation of the whole tooth surface as well as erosion of more soluble dental biomaterials such as cements working according to acid / base reactions [218]. Therefore, a potassium buffer (potassium dihydrogen phosphate buffer and dipotassium hydrogen phosphate buffer) was used in the caries model setup as described already previously [176]. Finally the effect of intraoral sugar clearance was simulated by rinsing the reaction chamber three times with artificial saliva after each demineralisation and before the following remineralisation phase begins. This action removed nutrition medium and bacterial remnants from the reaction chamber, which would otherwise impede pH rise.

6.1.4 Specimen preparation

Regarding Class V cavities, they were chosen because they are simple to prepare and restore, no special skills are needed and specimen standardisation is obtained easily. In contrast to MOD-cavities preparation depth is easier to standardise [219], while compared to MOD or Class I cavities, removal of excess material at occlusal restoration margins is

not controllable due to the complex occlusal morphology. Finally, the microscopic evaluation of a three-surface restoration (versus a single-surface restoration) is would create additional difficulties. Class V cavities were prepared only on buccal surface. Preparation of cavities on lingual surfaces would immediately double the number of specimens, reduce the experimental costs and shorten the duration of the experiment, but it was not preferred, due to the fact that bacterial concentration could be different on buccal and lingual sides, leading to inhomogeneous biological loading and to false results. Class V restorations are preferred when assessing the effectiveness of adhesives since they do not provide any mechanical retention, they challenge the materials by their high C-factor (5 bonded surfaces / 1 free surface) [220], their margins are located in enamel as well as in dentine, preparation is minimal and restoration technique is easy therefore reducing operator variability. Clinically, secondary caries occurs more often in the cervical areas of restorations [10] and biofilm tends to form and mature at the cervical area of the tooth [92]. From that point, Class V lesions are a common clinical finding and such restorations are frequently placed.

Although the study on which the development of the caries model was based [190], suggested placing composite restorations without prior etching, so as to provoke a larger marginal gap for more bacteria to gather and proliferate, in the present study, adhesives were used according to the directions of the manufacturer. This offers a realistic simulation of the clinical procedure and avoids extreme biological challenge. The experimental adhesives, which were built on the base of the control adhesive (CTRL), were also used according to the same instructions. Composite was placed in two diagonal layers (incremental technique), which were separately polymerized for 40 sec each, so as to control the polymerization side-effects, being polymerization shrinkage stress and cusp deflection, and thus improve marginal adaptation [220]. In that way, marginal gap formation was limited to that during controlled aging via thermocycling. No evidence of the number of cycles likely to be experienced *in vivo* was found, but an estimate of approximately 10000 cycles per year is suggested [221]. Class V restorations were high-gloss finished to prevent bacteria from adhering on the restoration and therefore altering the bacterial concentration during the experiment. During polishing and finishing material excess was carefully removed from restoration margins, in order not to block marginal gap formation and gathering of bacteria.

Regarding μTBS, similar studies have been performed with either sticks or hourglass-shaped beams. Sticks are most commonly encountered (Table 1, page 17-18), and thus preferred in order to generate comparable data. Regarding the extent of the adhesive area, it is shown that the smaller the interface, the lesser the risk for cohesive failures [200]. The adhesive area in the present study was smaller compared to similar studies (Table 1, page 17-18). A sufficient number of sticks was included in each group in order to provide accurate results. Compared to other CHX adhesive studies (Table 1, page 17-18), number of specimes per group was notably higher.

6.1.5 Impressions before and after caries model

Impressions of Class V restorations taken before and after caries model, provided an effective and accurate replicating technique [222] in order to ensure dimensional stability and allow for multiple evaluations or long term storage. In cases where teeth are inserted in SEM, dehydration *in vacuo* may exhibit false positive results in space measuring between restoration and dentine [98] thus margin dimensions may alter in absence of moisture [223]. The materials used for the impressions (polyvinylosiloxanePanasil® Putty and Panasil® Initial Contact Light, Kettenbach) and replica fabrication (polyurethane AlphaDie MF Ivory, Schütz Dental) were used in previously established caries model studies [185,148,209,176]. The casting was performed under pressure in a pressure pot to avoid bubble formation.

6.1.6 μTBS test

μTBS was chosen as an easy and well-documented bond strength technique. In contrast to macro tensile bond strength test, micro bond strength values tend to be 2X – 4X higher, because the defect concentration in the small adhesive interface is lower [224,191]. Similarly, smaller specimens are more durable than larger ones, due to lower possibility of presenting a critical-sized defect, aligned in a crack opening orientation relative to the applied load [200]. Adhesive interface of the present study was 0.46 mm^2, while published studies in the same field ranged from 0.6 – 1 mm^2. Smaller sticks are generally harder to manufacture due to many drop-outs but bond strength results correspond to the true strength of the adhesive, since cohesive failures are rare [200]. Loading speed was set to 1 mm / min which is also widely used in literature. Another alternative also widely used

would be 0.5 mm / min. Handling of sticks during sectioning and before insertion in the bond strength machine may alter bond strength values. Attention was given so as to prevent sticks from dehydrating, which would make them more brittle and would decrease their bond strength values. During μTBS loading, sticks were covered from all sides with flowable composite, which was allowed to flow as close to the adhesive interface as possible, without covering it and were in that way tightly fixed on the metallic plates, in order to avoid vibrations during tensile loading. Another factor related to specimen fixation on the plates of the testing maching is degree of polymerization of the fixation composite. Insufficient polymerization may lead to incorrect fixation of the stick allowing for minor movement, while excessive polymerization may lead to increased hardness of the composite and thus inability to absorb vibrations during loading. Polymerization time was for this reason standardized at 40 seconds. Disinfection of the sticks before insertion into μTBS caries model was performed with Braunol instead of 70% ethanol solution which was used for Class V caries model, in order to avoid harming the adhesive area or dessicating dentine. Regarding mode of failure, categorization is related to the level of magnification, as a failure that is labeled as adhesive under low magnification, can be listed as cohesive or mixed when evaluated under an optical microscope, where small composite or dentine remnants on the adhesive interface would be visible. While this may result in less adhesive failures, there is no standardization of the level of magnification for μTBS studies. In the present study, the level of magnification was in accordance with relevant literature. Moreover due to the large number of specimes (2979 sticks) it was not possible to evaluate failure mode under an optical microscope.

6.1.7 Aging methods

In order to mimic the aging process in the intraoral environment, Class V restorations were thermocycled prior to insertion in caries model. Thermomechanical cycling challenges restorative materials due to volumetric changes related to thermal expansion during temperature increase and contraction during its decrease, which happens at different rates due to different coefficients of thermal expansion [225]. It has been demonstrated that when thermocycling is used in combination with long-term water storage, it may be useful in forecasting the decline in strength of resin-dentinebonds created *in vivo* [226]. In the

present study, thermocycling was conducted before Class V caries model and water storage served as an aging method for μTBS sticks.

Thermocycling was used in order to provoke a marginal opening around Class V restorations which would allow for sufficient bacterial concentration and accelerate caries formation around marginsduring the 10-day cariogenic challenge. However, a direct shift from cold (5ºC) to hot (55ºC) israrely encountered in the oral cavity. Thermal changes of dietary origin are often smoother, and maximum tooth surface temperature is approximately 47ºC (extremes being 0ºC – 67ºC) [227]. The thermal shock that materials underwent before insertion in the caries model aimed in marginal opening which would provide nestling for the bacteria. In other words, if restorations were inserted in the caries model with intact margins, no proper *S. mutans* concentration would be reached and no proper demineralisation would be attained. This is also the case in clinical situations, as described by *Kidd et al. 1992*, marginal gaps are the prerequisite for the development of secondary caries [9]. The number of thermal cycles (10 000) was in agreement with previous studies performed with the same caries model [185,209,190,176], although marginal changes can already be seen after 2000 cycles [228].

The most used aging protocol in CHX studies is storage in artificial saliva or water [109]. CHX adhesives were mostly stored in water (Table 1, page 17-18). It has been demonstrated that use of water instead of Ca- and Zn-containing artificial saliva as an aging medium may underestimate the hydrolytic activity of MMPs [82]. In the present study, storage in water did not exhibit greater bond strength values either at 6- or at 12-months, but on the other hand, no Zn was contained in the artificial saliva used for storage [185,209]. It should however be noted, that sticks – and not teeth – are stored for *in vitro* purposes, imposing a great challenge on dentine – composite bond, which is directly exposed in storage solution. Therefore, no direct correlation of *in vitro* storage time and duration of clinical performance of the tested adhesives can be made. When conducting a long-term storage study, following parameters which could contribute to heterogenic results, should be kept in mind: type different water solutions used (distilled or deionized water), type of specimens stored (sticks or teeth samples), temperature, water pH and number of times the solution was renewed or refilled [109]. Finally, in order to prevent

bacterial growth during storage, antibacterial solutions such as sodium azide, chlorhamine or antibiotics are suggested [2], but since a potential interference with resin – dentine bond degradation is demonstrated [109], no such solution was used in the present study, so as to examine the sole effect of CHX.

6.2 Discussion of the results

It is hypothesized that dental adhesives with CHX could i) minimize secondary caries progression due to the antibacterial action of CHX and ii) inhibit adhesive bond degradation over time due to its anti-proteolytic effect. For the first hypothesis, it is mandatory that CHX will be released from the adhesive possibly through the adhesive interface and act extrinsically around the restoration margins. On the other hand, the second hypothesis requires that CHX will remain within the hybrid layer, exhibiting its protective activity against collagenolytic enzymes intrinsically. Since the amount of added CHX is in every case limited in terms of protection of the physichomechanical properties of the adhesives, it is logical that these two scenarios are antagonistic. The possible interference of external CHX addition with the adhesives' components and the mechanism of CHX release – either towards dentine or towards the restoration – are critical factors which determine the behavior ofthe CHX adhesives studied.

6.2.1 Caries model

Despite the fact that CHX reduces the number of *S. mutans* when applied as dentine pre-treatment [102], CHX adhesives were partially able to protect restoration margins from demineralisation in the caries model (Null hypothesis 1).

- **Enamel margins**

Specifically around enamel margins of Class V composite restorations, CHX addition in the adhesives (in primer; PRIM, in bonding; BOND, or industrially added in a 2-step adhesive; PEAK), resulted in significantly higher total demineralisation values, compared to the control group without CHX ($p<0.05$, mod. LSD) (Table 9, page 65), showing that not only did it not manage to protect the margins, but it made the situation worse. This could be explained in two ways, which represent two totally different directions; either due to the inability of CHX adhesives to bond efficiently to enamel due to their altered chemistry,

thus initially leaving a greater marginal gap for further biological degradation by *S. mutans* or due to the hermetic closure between enamel and composite [4], which does not allow CHX to be released outside from the restoration [204]. Marginal analysis with SEM shows a definite deterioration of enamel margins after caries model for PRIM, BOND and PEAK, as there is a significant difference between the percentage of perfect margins before and after caries model, and the percentage of gaps before and after caries model ($p<0.05$, Friedman) (Table 12, page 69). This strengthens the first scenario, supporting the inefficiency of CHX adhesives to bond to enamel. Moreover, negative results for PEAK, can be explained by the fact that as demonstrated in a recent study, this adhesive could only inhibit anaerobic bacteria and not facultative anaerobic bacteria, such as *S. mutans* [22]. Another possible explanation would be the short duration of CHX release, as a study about CHX-containing copolymers shows [21]. This bonding insufficiency is however not noted for dentine pre-treatment with CHX (DENT), as it exhibited significantly higher percentage of perfect enamel margins after caries model (78%), compared to all the other groups (2% - 9%) ($p=0.001$, ANOVA) along with the lowest percentage of gaps (52%) compared to the other adhesives (81% - 100%) ($p=0.001$, ANOVA). These results indicate that 2% CHX as dentine pre-treatment offers an advantage in protection of enamel margins against secondary caries. Release of CHX through the margins where its protective effect was exhibited, may be due to marginal gap induction during water storage and thermocycling, and due to the fact that enamel margins were not bevelled. Although Table 11, page 67 shows that not only 2% CHX as dentine pre-treatment (DENT), but also CHXfrom the primer (PRIM), or from the bonding agent (BOND) managed to significantly decrease marginal gap depth in enamel ($p<0.01$, mod. LSD) due to demineralisation, the measurements refer to a single point of the restoration margins, where the tooth was sectioned in two halves. Therefore, since SEM marginal analysis (Tables 12, 13, page 69) involved the total length of restoration margins, can be regarded as more accurate. The fact that DENT, PRIM and BOND significantly decreased marginal gap depth and not marginal gap width (Table 11, page 67), shows that CHX was possibly able to inhibit bacterial activity when in close proximity with bacteria or when its concentration was higher, e.g. when bacteria managed to reach at the adhesive area where it was applied.

Despite the fact that none of the CHX adhesives inhibited secondary caries formation in enamel, 2% CHX dentine pre-treatment managed tolimit marginal gap formation in enamelcompared to the other adhesive protocols in the study.

- **Dentine margins**

Regarding dentine margins, CHX addition in adhesives did not affect total demineralisation compared to the control group (CTRL) ($p>0.05$, ANOVA) (Table 10, page 66), thus showing no secondary caries inhibition in dentine. The control group (CTRL) (49% before vs 33% after), dentine pre-treatment with CHX (DENT) (69% before vs 45% after) and addition of CHX in the primer (PRIM) (60% before vs 29% after) maintained their percentage of perfect dentine margins after biological loading ($p>0.05$, Friedmann), however PRIM showed significantly more gaps (31% before vs 67% after) after caries model($p=0.021$, Friedman), which is controversial, but can be partially explained from its high standard deviations (Table 13, page 69). Therefore, since CHX adhesives showed similar behaviour to the control group (CTRL), it can be concluded that they do not exhibit favourable behaviour regarding caries inhibition around dentine margins, either due to the inability of CHX to be released outside of the restoration, or due to its low concentration and therefore short-term releasealready during storage of specimens and thermocycling, procedures which lasted for 3 weeks. Industrial addition of CHX (PEAK) was also not able to protect dentine margins from deterioration and gap formation after caries model, since both the percentage of perfect margins (76% before vs 27% after) and gaps (36% before vs 63% after) showed significant differences before and after caries model ($p=0.001$, Friedman) (Table 13, page 69) and a possible explanation is already discussed for enamel margins.

Neither experimental, nor industrial addition of CHX in the adhesives, or dentine pre-treatment with 2% CHX could provide protection against secondary caries or marginal deterionration in dentine.

6.2.2 μTBS

After 1 day

Experimental (PRIM, BOND) or industrial addition of CHX in adhesives (PEAK) decreased immediate bond strength to dentine, compared to the control group (CTRL) ($p<0.01$, mod. LSD) (Null hypothesis 2). This is in contrast with previously published studies, which showed that immediate bond strength was not affected by admixing CHX in adhesives [159,162,168,158,144], or even by replacing an adhesive component (liquid A) by 2% CHX [170]. However the aforementioned studies have tested small samples (36 – 43 sticks / experimental group) (Table 1, page 17-18) corresponding roughly to 1/3 of the sample size in the present study, thus questioning the accuracy and reproducibility of the results. According to a critical review of micro bond strength tests [200] *in vitro* bond strength studies require ‘‘a sufficient number of specimens for its testing condition for statistical analysisbecause of the probabilistic strength distribution’’. Small numbers of samples may cause heterogeneity of the bond strength results, due to consequently higher standard deviations [109]. Moreover, these studies have tested CHX addition in self-etch adhesives [162,144] and when total-etch adhesives were used [146,159], these were 2-step adhesives, meaning primer and bonding agent were delivered from the same bottle. Since the present study separately evaluated CHX addition in the primer or the bonding step of 3-step total-etch adhesive, comparison with the existing literature would be ineffectual. A single published study which included an CHX 3-step total-etch adhesive also demonstrated that CHX addition did not affect immediate μTBS to dentine [36], but had even smaller sample size than the studies mentioned before (2 – 4 sticks / group) (Table 1, page 17-18), and cannot be compared with the results of present study, which tested 99 – 108 sticks / group regarding immediate μTBS (Table 4, page 28). Differences were also found in the adhesive interface dimensions and at the speed of tensile loading (Table 1, page 17-18), both of which are related to the bond strength testing methodology, further justifying the disagreement.

There was no difference in bond strength values between experimental (PRIM or BOND) and industrial CHX addition (PEAK) ($p>0.05$, ANOVA).There is no published data in the literature concerning this comparison, but it is demonstrated that after PEAK application, as

well as with other 2-step total-etch adhesives of the same study, MMP activation was not prevented, despite the addition of 0.2% CHX in its composition [83]. Pre-treatment of dentine with 2% CHX did not alter the ability of the total-etch adhesive to bond to dentine ($p>0.05$, ANOVA), exhibiting that it is possible to obtain similar bond strength values with the control group (CTRL) (Null hypothesis 2). The finding correlates well with the existing literature, which shows no harmful effect on immediate bond strength after 2% CHX dentine pre-treatment [109,7,131,137,138,121,129,122,146,229]. Hybrid layer and resin tag formation in BOND appeared degraded, as evaluated qualitatively in exemplary SEM pictures (Figure 35, page 82). In agreement with literature [125], dentine treated with 2% CHX before the application of either a total-etch adhesive had clear presence of a hybrid layer and resin tags remained unaffected. No clear difference in failure pattern was observed between the control and the CHX adhesives, and this is also in agreement with literature findings [36].

According to the results of the present study, it is preferred in terms of immediate bond strength protection, to add an extra step in the adhesive procedure by separately pre-treating dentine with 2% CHX, than admixing CHX into adhesives.

Effect of biological loading

Despite the fact that the oral cavity is exposed daily to cariogenic challenge, the effect of bacteria on bond strength is rarely evaluated. It was investigated whether *S. mutans* would negatively affect dentine bond stability and whether CHX adhesives could prevent it. The decreased bond strength after biological loadingin all groups under investigation ($p<0.05$, ANOVA) (Table 14, page page 71) (Null hypothesis 5) correlated well with the literature, which has shown that cariogenic bacteria can degrade dental resin composites and adhesives [70] and negatively affect μTBS [230]. However qualitative evaluation of the hybrid layer at exemplary specimens of each CHX group, did not show any alteration in the appearance of the hybrid layer or presence of resin tags (Figure 32 – 36, pages 79-83). On the contrary *Borges et al. 2014* [231], demonstrated opposite results, which showed no reduction of adhesive bond strengths after a 4 hour / day cariogenic challenge. However the latter study did not took into account the alternating between demineralisation and

remineralisation phases, and although duration of demineralisation was the same with the present study, the cariogenic challenge did not simulate oral cavity conditions.

Bond strength of CHX adhesives after cariogenic challenge varies. DENT, PRIM and PEAK exhibited significantly better performance compared to the control group (CTRL) ($p<0.01$, mod LSD) (Table 14, page 71) (Null hypothesis 5), pointing out the protective effect of CHX against bacterial degradation of the adhesive bond. Up to now, there is no other published data concerning biological loading of CHX adhesives.

CHX adhesives did not protect their bond to dentine. However, when 2% CHX is used as dentine pre-treatment, added in primer or industrially added in adhesive, loss of adhesion was less extended.

Effect of 6- and 12-month storage

Literature shows that long-term storage in water or other aging media may affect the durability of the dentine bonds. The loss of stability of the adhesive interfaces overtime was related to the loss of stability of the polymer components, due to water penetration through nanoleakage channels, resulting in lower bond strengths and interfacial failure [73,66]. Adding to that, MMPs and CCs are activated by water and contribute to further enzymatic degradation of the adhesive area [8,109,30,232,44]. μTBS of all 6- and 12-months groups after aging provided evidence of a detrimental effect imposed by water or saliva on dentine bonds, except from PRIM after 12-months storage in saliva ($p>0.05$, ANOVA) (Table 16, page 73) (Null hypothesis 3). CHX adhesives are faced with the limitation of uncontrolled release lasting for a short period of time [21]. The positive behavior of CHX added in the primer can be attributed to the fact that the primer may have acted as a carrier of CHX inside the complex network of the hybrid layer and since it was not in situ polymerized [49], CHX could be released. Addition of CHX in the primer of a 3-step total-etch adhesive and storage in water for 12 months [36], demonstrated same outcome with the present study, however concentration of CHX was different. There is no exact literature to support the findings of the present study, however addition of 2% CHX in a 2-step total-etch adhesive showed reduction of bond degradation after 12 months in artificial saliva [159]. The difference to the present study is that CHX was added in a primer – bonding agent mixture and in 20-times higher concentration (2% vs 0.1%) [159], therefore no direct

comparison can be made between the studies. Experimental total-etch adhesives with addition of 2% CHX have also exhibited positive results after 12-month storage [168], but published research on the topic is generally scarce. It is however confirmed that bond strength results of CHX adhesives depend on the type of adhesive system used, and it is suggested that CHX should be used in combination with total-etch adhesive [139], as in the present study.

Dentine pre-treatment with 2% CHX does not produce better bonding values after 6 or 12 months and this is confirmed by literature for a time interval from 6 months up to 2 years [123,133,136,140,147,122]. Other studies which may exhibit favorable bond strength results of CHX as dentine pre-treatment in the beginning indicate that the effect is lost after long-term storage [109,149], despite CHX's substantivity. These results are in agreement with the present study, since 2% CHX as dentine pre-treatment (DENT) showed significantly worse μTBS values after storage, compared to baseline ($p<0.05$, Mann-Whitney). This can be explained by the fact that CHX eventually leaches out of the hybrid layer due to its electrostatic nature of binding with water acting as the desorption medium [113] and even when CHX is tracked inside the hybrid layer after 8 weeks [16,115] or 5 years [142] the concentration may still be very low to exhibit a therapeutic result. If CHX was released by the adhesives during the storage time in the storage media solution, no effect in dentine should be expected. Last but not least, even if CHX deactivates MMPs and CCs initially, re-activation of the enzymes by water or acidic metabolic products by microorganisms in the storage media, and therefore further deterioration of the bond strength, is also possible in the long run and could explain the absence of significant differences after storage.

Clinical studies agree with the findings of the present study, regarding the absence of long-term effect of CHX application on dentine, since enzymatic degradation by MMPs and CCs is not the only reason for bond failure and cannot be counteracted in total by CHX. Presence of CHX does not eliminate the negative impact of water sorption, ultimately leading to bond degradation. Even if collagen matrix can be preserved by MMP- and CC-inhibitors, loss of integrity through the degradation of the adhesive component itself will remain [7]. Data from clinical studies with CHX exhibit no benefit from use of CHX as

dentine pre-treatment in 120 non- restorations for up to 18 months [151] and in 70 restorations for up to 3 years [150]. On the other hand, clinical application of CHX is limited in Class I restorations with enamel margins [63,64], which provide an excellent marginal seal. The action of CHX against enzymatic degradation may be more evident at dentine – composite interface, where bonding is challenging. Even when Class V restorations with margins in enamel as well as in dentine are monitored [150,152], those were non-carious lesions [151,150]. Despite the fact that there is an association between clinical outcomes and laboratory results of bond strength studies [204], this is just one factor that influences the effectiveness of dentine adhesion. It should be noted that *in vitro* storage time does not correspond to the duration of clinical monitoring *in vivo*, since exposure of the resin-dentine sticks during laboratory storage is more challenging, therefore the 6- or 12-month results of the present study. Moreover, the duration of the clinical study may not be long enough for the adhesive bond of the control group (CTRL) to deteriorate in such an extent that a significant difference with the CHX pre-treatment would be exhibited.

Industrial addition of 0.2% CHX in the adhesive (PEAK) did not offer any benefit after 6- or 12-month storage, and achieved significantly lower values after 12-months compared to CHX application as dentine pre-treatment (DENT) ($p<0.05$, mod. LSD) (Table 16, page 73).The outcomes of the present study for 6-month storage correlate well with existing literature for the same adhesive [23]. There is no published data regarding its 12-month behavior. Generally, 2-step etch-and-rinse adhesives, such as PEAK, have performed less favorably in clinical circumstances than the conventional 3-step approach [191]. *In vitro* studies have corroborated this performance, relating their poorer performance to their higher hydrophilicity and reduced hybridization potential within the hybrid layer [191].

Bonding efficiency did not alter at 6-month storage in water or artificial saliva for CHX adhesives compared to the control group (CTRL), possibly due to the fact that the time interval was relatively small to induce significant changes. Bond strength values at 12 months storage differed significantly among the tested CHX adhesives ($p<0.05$, ANOVA). 2% CHX dentine pre-treatment (DENT) showed better inhibition of bond degradation at 12 months storage in water, compared to every other adhesive ($p<0.05$, ANOVA). After 12

months in artificial saliva, 2% CHX dentine pre-treatment (DENT), CHX in primer (PRIM) or in bonding agent (BOND) ($p<0.01$, mod. LSD) showed better μTBS values compared to the control group (CTRL) (Table 16, page 73). This showed that when CHX was added with one of the three aforementioned ways, it managed to suppress collagenolytic effects in dentine compared to the control group (CTRL) (comparison between the adhesives), but not in such an extent so that it could significantly protect bond strength throughout the long-term storage (comparison within the adhesive).

Fracture analysis revealed cohesive and adhesive fractures of the CHX adhesives with a tendency to fail more adhesively over time (Figure 28 – 31, page 74-75). This is in agreement with literature [123,137,36]. On the contrary another study showed that storage in artificial saliva decreased the number of adhesives failures over time [77], although not significantly [138]. Despite the fact that number of pre-test failures increases with time in long-term storage studies [142], this was mainly observed at the control group (CTRL) in the present study. CHX adhesives had very few or no pre-test failures after long-term storage (Figure 30, 31, page 75).

Addition of CHX in the primer managed to inhibit bond strength degradation after 12 months storage in saliva, compared to baseline. Since the shelf-life and stability of this experimental formulation was not evaluated in the present study, further research should be planned in that direction.

Effect of storage medium

The majority of storage studies – also those with CHX adhesives – use either water or artificial saliva as storage medium. In the present study both storage solutions were used at 6- and 12-month storage, in order to perform a comparison under the same circumastances. No other published study exists on CHX adhesives and their long-term storage in both media. Results of the present study showed that the effect of storage solution was not significant during 6-month storage for every adhesive tested ($p=1.000$, mod. LSD). However, differences between 12-month water and 12-month saliva bond strength data were demonstrated for PRIM, BOND and PEAK. Those performed significantly worse when stored in water ($p<0.001$, mod. LSD) (Null hypothesis 4). Control group (CTRL) followed the same trend, but level of significance was lower ($p=0.011$, mod. LSD). This

exhibits that storage medium plays an important role [233], especially in long-term storage studies, and results from different studies should be interpreted on that base. Moreover, since storage in artificial saliva resembles the clinical situation, it should be preffered.

6.3 Discussion of the null hypotheses (H_0)

Null hypothesis 1: Experimental or industrial addition of CHX in the adhesives is not able to inhibit secondary caries formation around Class V composite restorations.

Null hypothesis 1 was partially accepted. Regarding total demineralisation values, null hypothesis was accepted since CHX adhesives in enamel exhibited worse behaviour in enamel margins and same as the control group (CTRL) in dentine ($p=0.03$, ANOVA) (Table 9, page 65 and Table 10, page 66). Regarding marginal gap formation, null hypothesis was partially accepted, as DENT ($p=0.009$, mod. LSD), PRIM ($p=0.007$, mod. LSD) and BOND ($p=0.007$, mod. LSD) managed to eliminate gap formation in enamel after biological loading, especially in terms of marginal gap depth. In terms of marginal quality assessment, the null hypothesis was partially accepted, as the percentage of formed gaps in enamel after caries model for DENT, were significantly less ($p<0.05$, ANOVA) compared to all other testedgroups (Table 12, page 69).

Null hypothesis 2: It is not possible for the CHX adhesives to have bond strength values at baseline, after 6- and 12-month storage, same to the control group (CTRL).

Null hypothesis 2 was partially accepted for baseline, since DENT demonstrated no significant difference in immediate μTBS compared to the CTRL ($p>0.05$, ANOVA) (Tables 15, page 72 and Table 16, page 73) and showed significantly less adhesive fractures compared to PRIM ($p=0.022$, Mann-Whitney) and PEAK ($p=0.002$, Mann-Whitney) (Figure 30, 31, page 75). Null hypothesis was not accepted for 6- or 12-month storage.

Null hypothesis 3: CHX adhesives cannot maintain their bond strength after 6- and 12-months storage compared to baseline.

Null hypothesis 3 was partially accepted, since PRIM demonstrated no significant difference after 12-month storage in saliva ($p>0.05$, ANOVA). All other groups failed to maintain their immediate μTBS throughout 6- and 12-month storage. (Table 15, page 72 and Table 16, page 73). Null hypothesis was also partially accepted regarding comparison between the adhesives. DENT exhibited significantly better values compared to all other adhesives ($p<0.05$, ANOVA) at 12-month storage in water and DENT ($p=0.002$, mod. LSD), PRIM ($p=0.000$, mod. LSD), BOND ($p=0.000$, mod. LSD) showed better μTBS values at 12-month storage in saliva, compared to the CTRL (Table 16, page 73). CHX adhesives failed to present higher bond strength compared to the CTRL after 6 months storage.

Null hypothesis 4: There is no difference between artificial saliva and distilled water as storage media in 6- and 12-month storage.

Null hypothesis 4was partially accepted, as PRIM ($p=0.000$, mod. LSD), BOND ($p=0.000$, mod. LSD) and PEAK ($p=0.000$, mod. LSD), demonstrated significant differences between the storage media at 12 months. No significant difference was evident at 6-month storage (Table 17, page 77).

Null hypothesis 5: CHX adhesives cannot withstand bond strength reduction after biological loading with *S. mutans*.

Null hypothesis 5 was partially accepted. CHX adhesives, as well as CTRL,could not withstand the biological degradation andexhibited significantly lower μTBS values after the 10-day biological loading ($p<0.05$, ANOVA) (Table 14, page page 71). However, DENT ($p=0.000$, mod. LSD), PRIM ($p=0.008$, mod. LSD) and PEAK ($p=0.001$, mod. LSD) exhibited significantly higher μTBS after caries model compared to the CTRL after caries model (Table 14, page 71).

6.4 Future directions

This dissertation set out to investigate the role of CHX in improving composite restorations maintainance in terms of antibacterial and antiproteolytic protection. According to the results, 0.1% or 0.2% CHX in adhesives did not provide any antibacterial effect, regarding secondary caries. 0.1% CHX in the primer managed to withstand bond strength degradation after 12 months, however the adhesive's bonding performance at baseline was damaged. Further research should be pointed towards testing the antibacterial and antiproteolytic effect of higher CHX concentrations in the adhesives, in a form of delivery that would provide controlled release and would not harm the material's properties. Nanocapsules loaded with CHX could be an option. Self-life of CHX-adhesives is also a questionable aspect and was not evaluated in the present study, as CHX was freshly mixed with the experimental adhesives before each application. Since only total-etch adhesives were examined, research should be further expanded to comparison between CHX total-etch and CHX self-etch systems. Moreover, comparison of CHX-adhesives with adhesives loaded with other potential antibacterials and/or enzyme inhibitors under standardized parameters, could be suggested as a natural direction for future research. Last but not least, CHX adhesives should be clinically tested with randomized controlled trials in order to confirm their clinical significance in the long-term maintance of restorations.

6.5 Conclusions

According to the results of the present study and within its limitations it can be concluded that:

- Despite the fact that none of the CHX adhesives inhibited secondary caries formation, 2% CHX dentine pre-treatment managed to limit marginal gap formation in enamel compared to the other adhesive protocols in the study.
- Neither experimental, nor industrial addition of CHX in the adhesive procedure could provide protection against secondary caries in dentine.
- It is preferred to add an extra step in the adhesive procedure by separately pre-treating dentine with 2% CHX, than admixing CHX into adhesives. When CHX was admixed with the primer or with the bonding agent in 0.1% concentration or was 0.2% industrially added, bond strength values at baseline dereriorated.
- CHX adhesives did not manage to protect dentine bond from bacteriaduring biological loading with *S. mutans*. However, when 2% CHX was used as dentine pre-treatment, added in primer or industrially added in adhesive, loss of adhesion was less extended.
- Addition of 0.1% CHX in the primer managed to inhibit bond strength degradation after 12 months storage in saliva.
- Regarding comparison between the storage media, 12-month storage in artificial saliva exhibited more favorable μTBS and differed significantly from water storage. That effect was not evident at 6 months storage.

In general, 2% CHX application as dentine pre-treatment performed better regarding its inhibition of demineralisation at enamel margins – but not at dentine margins –, did not interfere with the baseline bond strength of the 3-step total-etch adhesive to dentine and limited loss of adhesion due to biological loading. 0.1% CHX in primer was also able to limit loss of adhesion after the cariogenic challenge and protected the adhesive bond from further hydrolytic and enzymatic degradation up to 12 months *in vitro*. All other CHX adhesives failed to protect adhesive restorations against bacteria or endogenous enzymes.

7. Summary – Zusammenfassung

Summary

Objective

Aim of the study was to investigate the antibacterial and antiproteolytic effect of chlorhexidine (CHX) when added in the adhesive procedure, at baseline, after biological loading and after 6- or 12-month storage in distilled water or in artificial saliva.

Materials & Methods

The study consisted of two parts: biological loading of restorations in a bacterial caries model (n=60 teeth) and μTBS at baseline, after biological loading and after storage (n=2979 sticks). Following groups were formed: i) Control group (CTRL), 3-step adhesive Adper Scotchbond™ Multipurpose, 3M ESPE, ii) 2% CHX as dentine pre-treatment (DENT), iii) 0.1% CHX in primer (PRIM), iv) 0.1% CHX in bonding (BOND), v) 2-step adhesive Peak® Universal Bond with 0.2% CHX (PEAK). PRIM and BOND were fabricated on the basis of the 3-step adhesive used. Class V composite restorations were loaded in caries model with *S. mutans* according to a 10-day biological protocol. Demineralisation was evaluated with fluorescence microscopy and marginal analysis was performed with SEM. For μTBS (Syndicad TC-550), dentine-composite sticks with 0.46 mm^2 adhesive interface were fabricated and tested at baseline, after 2-day caries model, after 6- and 12-month storage in distilled water or artificial saliva. Fracture mode analysis followed and exemplary sticks were evaluated under SEM.

Results

None of the CHX adhesives inhibited secondary caries formation but 2% CHX dentine pre-treatment (DENT) managed to limit marginal gap formation in enamel ($p<0.05$). Bond strength of CHX adhesives at baseline was worse compared to the CTRL ($p<0.05$) and decreased significantly after caries model ($p<0.05$). Only PRIM exhibited same μTBS values after 12-month storage in saliva, compared to baseline.

Conclusions

2% as dentine pre-treatment inhibited marginal degradation at enamel margins, but no CHX adhesive protected composite restorations against secondary caries. 0.1% CHX in primer protected bond strength degradation after 12 months in artificial saliva, exhibiting a potential antiproteolytic effect.

Zusammenfassung

Zielsetzung

Das Ziel dieser Dissertation war die Untersuchung von antibakteriellen und antiproteolytischen Effekten des Chlorhexidins als Zusatz während verschiedener Schritte der Adhäsivtechnik zur Baseline, nach mikrobiologischer Belastung mit S. mutans, sowie nach 6- bzw. 12-monatiger Lagerung in destilliertem Wasser oder künstlichem Speichel.

Material und Methode

Die vorliegende Studie besteht aus 2 Teilen, auf der einen Seite die Untersuchung der restaurierten Zähne nach mikrobiologischer Belastung im Karies Modell (n=60 Zähne) auf der anderen Seite die Bestimmung von Haftwerten mittels Mikrozugversuch zur Baseline, nach mikrobiologischer Belastung sowie nach Lagerung (n=2979 Stäbchen). Es wurden folgende Gruppen gebildet: i) Kontrollgruppe (CTRL), 3-Schritt Adhäsiv Adper Scotchbond Multipurpose™, 3M ESPE, ii) 2% CHX als Dentin-Vorbehandlung (DENT), iii) 0.01 % CHX im Primer (PRIM), iv) 0.01 % CHX im Adhäsiv (BOND), v) 2-Schritt Adhäsiv Peak® Universal Bond mit 0.2 % CHX, Ultradent (PEAK). PRIM and BOND wurden auf der Basis des gebräuchlichen 3-Schritt Adhäsivs hergestellt. Klasse V-Komposit-Restaurationen wurden im Kariesmodell mit *S. mutans* gemäß Protokoll über 10 Tage belastet. Die Auswertung der Demineralisationen erfolgte fluoreszenzmikroskopisch, die Randspaltananlyse mittels REM. Die Haftwerte wurden im µ-Zuggerät TC 550 (Syndicad) ermittelt. Dazu wurden Dentin-Komposit-Stäbchen mit einem Adhäsiv-Interface von 0.46 mm² hergestellt, die zur Baseline, nach 2-tägiger Belastung im Kariesmodell sowie nach 6- und 12-monatiger Lagerung in destilliertem Wasser oder künstlichem Speichel dem µ-Zugversuch unterzogen wurden. Es erfolgte eine Analyse des Frakturmodus und eine exemplarische Beurteilung mittels REM.

Ergebnisse

Keines der CHX-Adhäsive hemmte die Bildung von Sekundärkaries, jedoch konnte die Dentinvorbehandlung mit 2 %-igem CHX (DENT) die Randspaltbildung im Schmelz begrenzen ($p<0,05$). Die Haftwerte der CHX Adhäsive waren schlechter im Vergleich zu denen der Kontrollgruppe (CTRL) ($p<0,05$). Nur die Gruppe PRIM nach 12-monatiger

Lagerung in künstlichem Speichel zeigte die gleichen Haftwerte verglichen mit den Werten der Baseline.

Schlussfolgerung

2% CHX verhindert als Dentin-Vorbehandlung die Randspaltbildung, jedoch schützte kein CHX-haltiges Adhäsiv die Restauration vor Sekundärkaries. 0.01 % CHX im Primer schützte vor einer Verminderung der Haftwerte nach 12-monatiger Lagerung in künstlichem Speichel, vermutlich durch einen antiproteolytischen Effekt.

8. Literature

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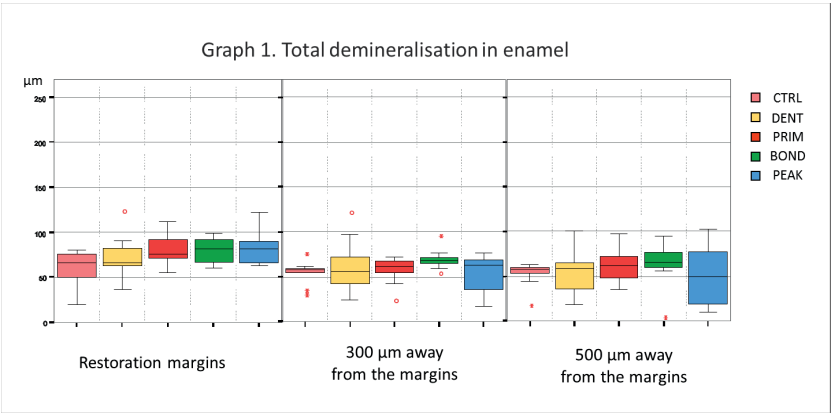
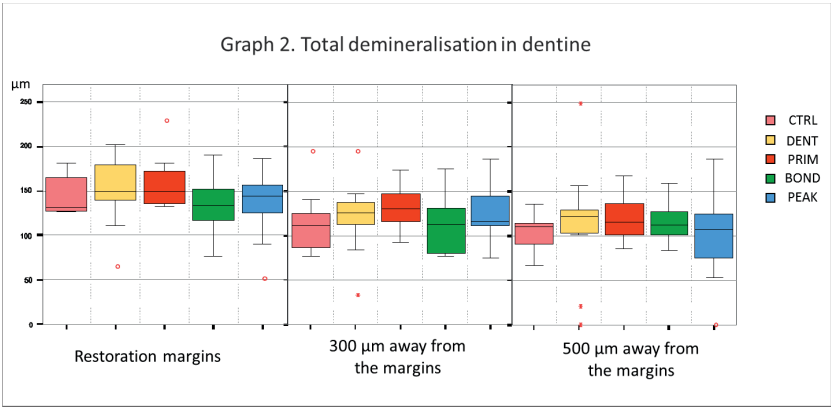
9. Appendix

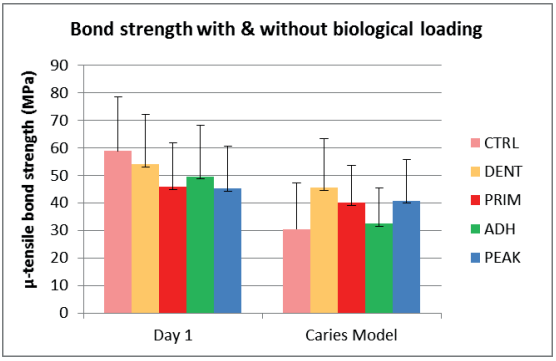
9.1 Appendix I – III

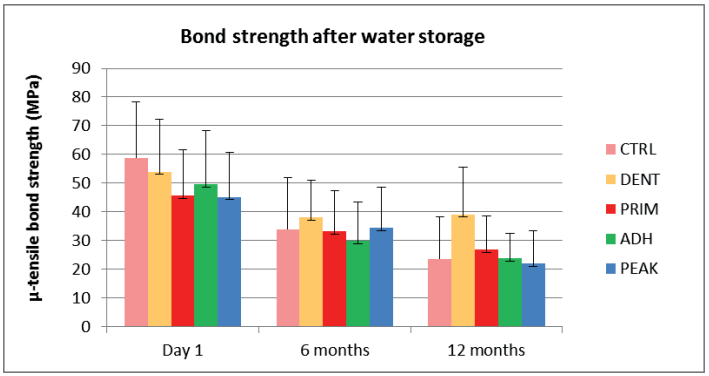


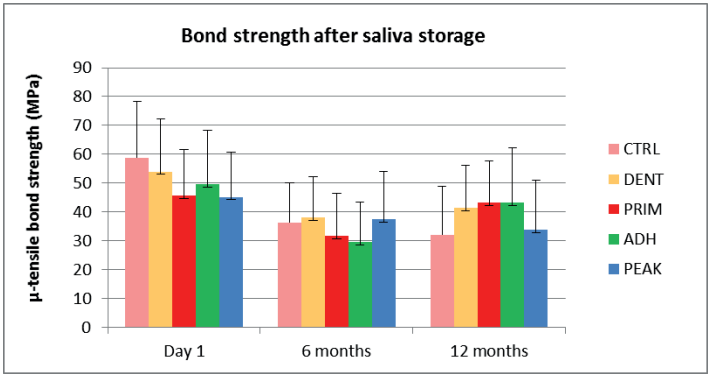
Appendix I. Boxplot of total demineralisation (TOTAL) in enamel after 10 days biological loading in caries model.Total demineralisation (TOTAL) = demineralisation (DEM) + substance loss due to demineralisation (SUB). Evaluation took place at restoration margins, 300 µm and 500µm away from the margins. Values displayed in Table 9.



Appendix II. Boxplot of total demineralisation (TOTAL) in enamel after 10 days biological loading in caries model.Total demineralisation (TOTAL) = demineralisation (DEM) + substance loss due to demineralisation (SUB). Evaluation took place at restoration margins, 300 µm and 500µm away from the margins. Values displayed in Table 10.







Appendix III. µTBSvalues in MPa. Graphs correspond to values in Tables 14-16.

9.2 Abbreviations list

ANOVA	Analysis of variance
B. Cereus	Bacillus Cereus
BHI	Brain heart infusion
BOND	Chlorhexidine in bonding
CC	Cysteine cathepsin
CFU	Colony forming unit
CHX	Chlorhexidine
CTRL	Control
DNA	Deoxyribonucleic acid
DEM	Demineralisation
DENT	Dentine pre-treatment with 2% chlorhexidine
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FDI	Federation Dentaire Internationale
LED	Light emitting diode
L. monocytogenes	Listeria monocytogenes
LP	Light polymerization
LSD	Least significant difference
MMP	Matrix metalloproteinase
MOD	Mesio-occlusal-distal
MPa	Megapascal
mRNA	Messenger ribonucleic acid
rpm	Revolutions per minute
OD	Optical density
P. Aeuruginosa	Pseudomonas aeruginosa
PBS	Phosphate buffered saline
PEAK	Peak® Universal Bond with 0.2% Chlorhexidine
PRIM	Chlorhexidine in primer

SD	Standard deviation
SEM	Scanning electron microscope
S. mutans	Streptococcus mutans
spp.	species
SUB	Substance loss
TIMP	Tissue inhibitor of MMP
TOTAL	Total deminerlisation
µTBS	Microtensile bond strength
^{o}C	Degree of Celcius

9.3 Tables – figures list

	saliva (clear) and nutrition medium (dark) 20 l glass bottles are placed outside the incubator due to limited space (right).	
Figure 11	*S. mutans* (DSM Nr: 20523) culture after 48 h incubation. 1 inoculation loop is taken in order to dilute the bacteria for the overnight culture.	44
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Figure 19	Placement of the stick on the metal plates of the bond strength testing machine	53

9.4 Publications

- Poster presentation, 25th Congress of International Association of Paediatric Dentistry (IAPD), Glasgow, UK (July 2015) – ID: 341
  ‘’Evaluation of secondary caries in a microbial caries model after the addition of chlorhexidine in the adhesive procedure‘‘
  C. Boutsiouki, S. Lücker, S. Amend, K. Tolidis, P. Gerasimou, N. Krämer

- Oral presentation, 42th Panhellenic Paediatric Dentistry Congress, Thessaloniki, Greece (September, 2015) – ID: 10
  ‘’The antibacterial action of chlorhexidine adhesives on their bond strength with dentine’’
  C. Boutsiouki, K. Tolidis, P. Gerasimou, S. Lücker, N. Krämer.

- 22te Jahrestagung der DGKiZ zum Deutschen Zahnartztetag, Frankfurt, Deutschland (November 2015)– ID: 2080
  „Hat der Chlorhexidin Zusatz in Adhäsiven einen Einfluss auf die Dentinadhäsion?“
  C. Boutsiouki, S. Lücker, I. Heidmann, N. Krämer.

- 95th General Session & Exhibition International Association of Dental Research IADR/AADR/CADR, San Francisco, USA (March 2017) – ID:0766
  ‘’Chlorhexidine in bonding procedure decreases bond degradation after 12 months’’
  C. Boutsiouki, S. Lücker, N. Krämer.

9.5 Prizes – Scholarships

2014

IKYDA 2014 / 12-month project (supported by DAAD, Germany and IKY, Greece)
Title: Prevention of demineralisation by addition of chlorhexidine in the adhesive procedure of composite resins assessed with an artificial mouth model.

Short description: *In vitro* evaluation of the antibacterial effect of chlorhexidine adhesives on the inhibition of secondary caries formation around Class V composite restorations assessed in a bacterial caries model set-up with *S. mutans*.

2015

Funding program Forschungsgemeinschaft Dental e.V. (FGD) – Project Nr. 3/2014

Title: Chlorhexidine addition in bonding procedure: 6- and 12-month effect on μ-tensile bond strength

Short description: μ-tensile bond strength test of chlorhexidine adhesives with dentine after 1 day in distilled water, after biological loading with *S. mutans* and after long-term storage (6 or 12 months) in distilled water or artificial saliva.

Best poster presentation prize
Title: Hat der Chlorhexidin Zusatz in Adhäsiven einen Einfluss auf die Dentinadhäsion?
Jahrestagung der deutschen Gesellschaft für Kinderzahnheilkunde, Frankfurt

Declaration of Honour

„I hereby declare that I have completed this work independently and without inadmissible assistance or theuse of other than the resources quoted. All texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such. In the analyses that I have conducted and to which I refer in this thesis, I have followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice, as well as ethical principles and those governing data protection and animal welfare. I give my assurance that third parties have not received from me, either directly or indirectly, any financial remuneration for work in connection with the content of this doctoral thesis and that the work presented has not been submitted in the same or a similar form to another assessment authority in Germany or elsewhere for the purpose of being awarded a doctorate or another assessment procedure. All material taken from other sources and other persons and used in this thesis or to which direct reference is made has been identified as such. In particular, all those who took part directly and indirectly in the production of this study have been named. I agree to my thesis being subjected to scrutiny by plagiarism detection software or by an internet-based software programme."

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Thanksgiving

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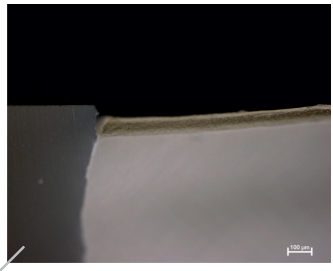

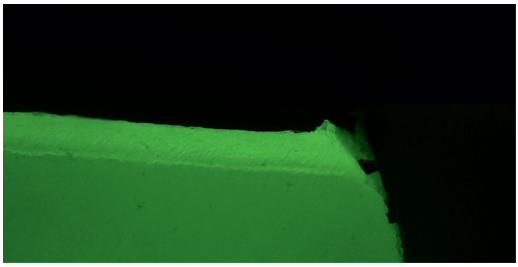

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