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Influence of mouthrinses on 24- / 48-h bacterial biofilm formation *in situ*

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To my beloved parents and brothers

Meinen geliebten Eltern und Brüdenr

Abbreviations

ACP	Amorphous calcium phosphate
ADA	American Dental Association
AM	Acetoxymethyl
AmF	Amine fluoride
CFDA	Carboxyfluorescein diacetate
CLSM	Confocal laser scanning microscopy
CPC	Cetylpyridinium chloride
CPP	Casein phosphopeptides
d	Day
DAPI	4´, 6-diamidino-2-phenylindole
EB	Ethidium bromide
e.g.	*exempli gratia*, for example
etc.	et cetera
FAp	Fluorapatite
FDA	Fluoresceine diacetate
FISH	Fluorescence *in situ* hybridization
h	Hour
HA	Hydroxyapatite
HMDS	Hexamethyldisilazane
i.e.	*id est,* in other words
LDS	Live / Dead staining
max.	Maximum
mg	Milligram
min	Minute
min.	Minimum
ml	Millilitre
mm	Millimetre
mm^2	Square millimetre
nm	Nanometre
n.s.	Not significant

pH	Potential of hydrogen
PI	Propidium iodide
pKa	Acid dissociation constant
ppm	Parts per million
rpm	Rounds per minute
s	Second
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
S. sanguis	*Streptococcus sanguis*
S. oralis	*Streptococcus oralis*
SEM	Scanning electron microscopy
SnF_2	Stannous fluoride
TEM	Transmission electron microscopy
wt%	Weight percent
y	Year
µl	Microlitre
µm	Micrometre

Content

1 Abstract

1.1 English

Influence of mouthrinses on 24- / 48-h bacterial biofilm formation *in situ*

Objectives: The advance of nanotechnology promotes bio-inspired strategies in biofilm management. The aim of this *in situ* study was to investigate the efficacy of a commercially available mouthrinse containing nanobiomaterials (zinc-carbonate hydroxyapatite microlusters) in dentinal biofilm management.

Materials and Methods: The *in situ* biofilim formation was carried out on bovine dentin mounted in the individual upper jaw minisplint for 24 h and 48 h (n = 5 subjects). The samples were rinsed twice a day (20 ml, 30 s) with a 'new' mouthrinse (BioRepair tooth and mouth rinsing) or two conventional mouthrinses (elmex Kariesschutz tooth rinsing (Elmex Kariesschutz) and Chlorhexamed Forte (CHX)), respectively. The group without rinsing served as a control group. After each oral exposure time, the dentinal biofilm were visualized and semi-quantified with the following microscopic methods: *Bac*Light^{TM} viabiltiy assay, scanning (SEM) and transmission electron microscopy (TEM). In addition, the 24-h biofilm on the fractured surface was examined by SEM-analysis. The nanoparticles in the BioRepair solution were also investigated by TEM.

Results: With all methods, the biofilm formation revealed a high interindividual variability. As visualized and semi-quantified by *Bac*Light^{TM} viabiltiy assay and SEM, the bacterial colonization was reduced following rinsing with all three tested mouthrinses in each experimental period (24 h or 48 h), as compared to the control group. In addition, the *Bac*Light^{TM} viabiltiy assay showed more dead than live cells in the CHX group and in the BioRepair group. The amounts of live and dead bacteria on the 24- / 48-h biofilms were comparable after rinsing with Elmex Kariesschutz. Compared to the controls, a similar

amount of adherent dead microorganisms and an evident decline in the volume of vital bacteria could be detected on the Elmex Kariesschutz-treated samples. The fractured surface evaluated by SEM and the ultra-thin sections examined by TEM revealed the bacterial colonization / infiltration at the micro- or even nano-scale. Additionally, the TEM-investigation demonstrated the adsorption of potential nanoparticles in the BioRepair-treated biofilms.

Conclusion: The present study confirms the well-accepted antibacterial performance of chlorhexidine and the moderate biofilm inhibitory capacity of the fluoridated mouthrinse (elmex Kariesschutz tooth rinsing). In addition, the 'new' mouthrinse containing biomimetic hydroxyapatite crystals is demonstrated to be effective in the short-term biofilm control, due to antiadherent and antibacterial effects. However, the long-term clinical efficacy needs to be further studied.

1.2 German

Der Einfluß verschiedener Mundspülungen auf die 24- / 48-h *in situ* Biofilmbildung

Fragestellung: Die Entwicklung der Nanotechnologie fördert bio- inspirierte Strategien im Biofilm- Management, welche z.B. auf Mundspülungen, in denen Nanomaterialien enthalten sind, basieren. Das Ziel der vorliegenden *In-situ*-Studie war es, die Biofilmbildung auf Dentin unter dem Einfluss der auf dem Markt erhältlichen BioRepair- Mundspülung (enthält: Zink-Carbonat- Hydroxylapatit Mikrocluster) zu untersuchen.

Material und Methoden: Die Biofilmbildung erfolgte *in situ* auf Rinderdentin mithilfe individueller Oberkiefer- Tiefziehschienen über Zeiträume von 24 h und 48 h (n = 5 Probanden). Die Proben wurden zweimal täglich (20 ml, 30 s) mit der BioRepair Zahn- und Mundspülung oder zwei herkömmlichen Mundspülungen (elmex Kariesschutz Zahnspülung (Elmex Kariesschutz) and Chlorhexamed Forte (CHX)) gespült, wobei eine Gruppe ohne Spülung als Kontrollgruppe diente. Nach intraoraler Exposition wurden die Proben mit folgenden mikroskopischen Methoden auf bakterielle Biofilme untersucht: Anfärben von vitalen und avitalen Bakterien mit SYTO 9 und Propidiumiodid (*Bac*Light™ viabiltiy assay), rasterelektronenmikroskopische Untersuchung (REM) und transmissionselektronenmikroskopische Untersuchung (TEM). Zusätzlich dazu wurden Bruchpräparate von den 24- h Biofilm- Proben mittels REM analysiert. Letztlich wurden die im BioRepair enthaltenen Nanopartikel mittels TEM untersucht.

Ergebnisse: Bei allen Methoden unterlag die Pellikel- / Biofilmbildung einer hohen interindividuellen Variabilität. *Bac*Light™ Färbung und REM zeigten, dass die bakterielle Besiedlung nach Spülung mit verschiedenen Mundspülungen in jedem Versuchszeitraum (24 h oder 48 h) im Vergleich zur Kontrollgruppe reduziert war. Darüber hinaus zeigte die *Bac*Light™ Färbung mehr avitale als vitale Bakterien sowohl in der CHX Gruppe und als auch in der BioRepair Gruppe. Eine vergleichbare Menge an vitalen und avitalen Zellen im 24- / 48-h Biofilm wurde nach Anwendung mit elmex Kariesschutz Zahnspülung erzielt. Im Vergleich zur Kontrollgruppe wurde nach Anwendung der elmex Kariesschutz Zahnspülung eine deutliche Ver-

ringerung der Anzahl lebender Bakterien nachgewiesen, wohingegen die Anzahl adhärenter avitaler Bakterien vergleichbar blieb. Die bakterielle Besiedlung / Infiltration in die Dentintubuli wurde mit Hilfe von Bruchpräparaten und Ultradünnschnitten im Mikro- und Nanomaßstab dargestellt. Mittels TEM- Analysen konnten potenzielle Nanopartikel auf den mit BioRepair Zahn- und Mundspülung behandelten Biofilmen nachgewiesen werden.

Schlussfolgerung: Die vorliegende Studie bestätigt die allgemein anerkannte antibakterielle Wirkung von Chlorhexidin. Die inhibitorische Kapazität der fluoridhaltigen Mundspülung (elmex Kariesschutz Zahnspülung) hinsichtlich der bakteriellen Biofilmbildung wurde verifiziert. Darüber hinaus wurde für die Mundspülung mit biomimetischen Hydroxylapatit- Aggregaten sowohl eine antiadhärente als auch eine antibakterielle Wirkung nachgewiesen. Deren langfristige klinische Wirksamkeit muss allerdings weiter untersucht und bestätigt werden.

2 Introduction

During the past 50 years, the prevalence of the biofilm associated oral diseases (caries and periodontal diseases) has been significantly declined, which is mainly credited to the increasing mass awareness of the value of oral health and the oral prophylaxis like tooth brushing and the application of fluoride [Longbottom et al., 2009; Wolff and Larson, 2009]. However, these largely preventable diseases are still common and remain a public health problem for a considerable proportion of the world's population [Burt, 1998; Särner et al., 2012]. Thus, there is still a need for effective prevention strategies.

The mechanical oral hygiene approach and chemical plaque control have established as the mass prophylactic approaches. As an effective delivery of chemically active agents for oral biofilm management [Stoodley et al., 2008], mouth rinsing has progressively gained its place in the oral prophylaxis and serves as an addition to the daily mechanical oral hygiene approach [Jardim et al., 2009; Särner et al., 2012]. Today, home-use oral care products available in the marketplace offer a great variety of options. The best products available to prevent dental plaque formation are chlorhexidine, amine fluoride / stannous fluoride (AmF / SnF_2), or triclosan-copolymer. Chlorhexidine can even decrease already accumulated plaque [FDI Commission, 2002a].

Nonetheless, biofilm management is still insufficient despite the tremendous efforts in promoting oral hygiene and fluoridation. Recently, fast growing nanotechnology has facilitated development of bioinspired prophylactic products that are supposed for the prevention of biofilm-associated diseases [Cochrane et al., 2010; Cochrane and Reynolds, 2012; Cross et al., 2007; Hannig et al., 2013a; Hannig and Hannig, 2010b]. For example, casein phosphopeptides (CPP) stabilized amorphous calcium phosphate (ACP) has been proved to be effective with respect to remineralization and antibacterial properties in numerous *in vitro*, *in vivo* and *in situ* studies [Rahiotis and Vougiouklakis, 2007; Rahiotis et al., 2008; Reynolds, 2008]. However, one drawback is that it does not mimic the tooth structure. Other biomimetic approaches are based on hydroxyapatite (HA)-nanocrystals. These particles mimic the size of natural dentinal HA (20 nm) or enamel apatite (100 nm), sometimes aggregated as clusters [Roveri et al., 2008], with different forms such as spheroidal or needle-like shape, and have shown remineralizing as well as antimicrobial effects under *in*

vitro and *in vivo* conditions [Hannig et al., 2013a; Poggio et al., 2010; Tschoppe et al., 2011; Venegas et al., 2006]. Nevertheless, little information about the latest prophylactic products based on nanotechnology is available. Some related questions remain unanswered, such as the magnitude of antimicrobial efficacy and substantivity under *in vivo* conditions.

Although initial bacterial adhesion of healthy teeth occurs mainly on the enamel surface, the colonization and penetration of dentinal surfaces exposed to the oral fluids have at least the same relevance for the pathogenesis and progress of dental caries, especially for the cervical area of the teeth and root surface caries in the elderly. Thereby, the histological and micromorphological differences of dentin have to be taken into consideration.

Thus, the general aim of the present study was to investigate the potential of a 'new' commercially available mouthrinse containing zinc-carbonate hydroxyapatite nanoparticles on the *in situ* dentinal biofilm management, as compared to two other classic mouthrinses (chlorhexidine solution and fluoridated mouthwash).

2.1 Review of literature

2.1.1 Dentin

Dental dentin makes up the body of teeth, which is covered by enamel on the crown and cementum on the root, and surrounds the pulp tissues. Compared with enamel, dentin is less mineralized and brittle. By weight, dentin consists of 70% of inorganic substance, 20% of organic material (collagenous and non-collagenous proteins), and 10% of water [Hellwig et al., 2007]. At the nanometre scale, dentin has hierarchical structures and surface features similar to enamel. The three-dimensional dentinal matrix is mainly composed of type I collagen fibrils and reinforced by dentinal HA crystallites, which are characterized by smaller dimension than in enamel and show a length of 20 nm, a width of 18 to 20 nm and a thickness of 3.5 nm [Hellwig et al., 2007]. The large surface-to-volume ratio of the crystallites leads to a high reactivity of dentinal HA. From a morphological point of view, the dentin is densely perforated by dentinal tubules, the diameter of which is 0.5-1.2 μm and the density of which is 10.000-25.000 tubules/mm^2. At the enamel-dentin junction which is 3.1-3.5 mm away from the pulp, the average tubule diameter is 0.8 μm and the average density is 19.000 tubules/mm^2 [Garberoglio and Brännström, 1976]. Within the dentinal tubules, the dentinal fluid and organic structural elements, such as non-calcified collagen fibrils, exist in addition to the odontoblast process [Baume, 1980; La Fleche et al., 1985]. This ‘porous’ structure results in high dentin permeability [Thomas, 1985].

2.1.2 Pellicle

2.1.2.1 Formation of the pellicle

The ‘pellicle’ is defined as a thin layer deposited on the enamel surface and is composed of salivary proteins and other macromolecules from the oral environment [Hannig and Joiner, 2006]. Pellicle formation is a highly selective and dynamic process, determined by various factors, such as formation time, formation site, physiochemical properties of the supporting surface, and adsorbed molecules [Hannig and Joiner, 2006; Siqueira et al., 2012].

The formation of pellicle proceeds in two stages. The first stage starts with the initial protein adsorption process a few seconds after exposure to the oral environment [Hannig and Hannig, 2009]. A couple of minutes later, a proteinaceous coating is formed with a thickness of between 10 nm and 20 nm, which remains stable for approximately 30 min [Hannig, 1999].

The initial adsorption process is generally conducted by electrostatic interactions between the negatively charged tooth surface and the corresponding positively charged groups of salivary proteins, which are mainly referred to as 'pellicle precursors'. Other forces, such as van der Waals interactions and hydrophobic interactions, also contribute to the attachment of the proteins to the dental surface [Hannig and Joiner, 2006].

The second stage is the maturation process that is characterized by continuous adsorption of biopolymers from saliva to the pellicle-coated surface *via* protein-protein interactions. In a period from 30 min to 90 min, a rapid protein adsorption leads to a plateau at a thickness ranging from 100 nm to 500 nm. Such a dramatically rapid pellicle development is presumably ascribed to the adsorption of protein aggregations / supramolecular pellicle precursors and the local supply of salivary biopolymers [Hannig, 1999]. The 'supramolecular pellicle precursors' are referred to as micelle-like globules and heterotypic complexes, which play a key role in the pellicle development and preventing proteolytic degradation [Hannig and Joiner, 2006].

Host enzymes and microbial enzymes render the initial pellicle at a low proteolytic activity resulting in the pellicle's internal maturation [Hannig and Joiner, 2006; Siqueira et al., 2012]. Peptide fragments generated from salivary proteolysis during adsorption to the tooth surface and comprising the body of pellicle facilitate the pellicle's external maturation [Siqueira et al., 2012].

2.1.2.2 Ultrastructure of the pellicle layer

The ultrastructral appearance of the pellicle has been mainly demonstrated by means of the conventional scanning (SEM) and transmission electron microscopy (TEM) [Brecx et al., 1983; Hannig, 1997, 1999]. At the micro-scale, the pellicle displays a spongy mesh-work, consisting of a tight electron dense basal layer and a loosely arranged outer layer [Hannig and Joiner, 2006]. The basal layer is a continuously and homogeneously structured fine-granular electron dense layer with a thickness of 10-20 nm, which has already been detected in the 1-min *in situ* pellicle [Hannig, 1999]. The outer layer varies in thickness and structure dependent to formation time, intraoral location, and individual, subject-related differences [Hannig and Joiner, 2006]. The 'maturation' results in morphological changes with time. The 'young' pellicle (2 h) displays an uneven and incomplete organic coating of the tooth surface, while the 'mature' pellicle (several hours or days) displays more compact layers of granular

ultrastructural appearance. The site of pellicle formation, the supply of salivary proteins, and environmental factors, such as shearing forces, contribute to site-dependent ultrastructural differences. In self-cleansing sites, less pronounced pellicle formation with a thickness between 30 and 80 nm was detected, as compared to a thickness up to 2 μm in the proximal areas [Tinanoff et al., 1976].

The 2-h old pellicle formed on buccal sites contains both granular and globular components and has various thicknesses between 100 nm and 500 nm [Hannig, 1999]. By contrast, the 2-h old palatally formed pellicle contains only granular components and has various thicknesses between 20 nm and 80 nm. After 24 h, a dense and homogenous layer with a granular appearance is found above the dense basal layer. The thickness of pellicle on buccal sites increases to between 1.000 nm and 1.300 nm, while the thickness of pellicle on palatal sites increases to between 100 nm and 200 nm within 24 h [Hannig, 1999].

2.1.2.3 Function of the pellicle

The pellicle layer is very important, both physiologically as well as pathophysiologically, in the oral cavity. In general, the pellicle plays an important role in the lubrication of tooth surfaces, modulation of de- / remineralization process and bacterial adherence.

Due to its lubricating properties, the pellicle reduces frictions between antagonistic teeth and between teeth and mucosa to make mastication and speech more comfortable [Aguirre et al., 1989; Hannig and Joiner, 2006].

The protective properties of this proteinaceous coating against demineralization have also been revealed *in vitro* [Zahradnik et al., 1976] and *in vivo* [Hannig et al., 2004; Hannig et al., 2003], based on its function as diffusion barrier and semi-permeable membrane [Hannig et al., 2003; Hannig and Joiner, 2006]. On the one hand, the selective permeability of this porous mesh-like structure is able to prevent demineralization from microbial acids and erosive challenges, due to modification of the acid diffusion. On the other hand, the pellicle layer acts as a reservoir for remineralizing ions and functions as a medium for ion exchange (calcium and phosphate). Even a 3-min *in situ* pellicle provides a comparable protective effect on the enamel surface against acid attack as a 2-h *in vivo* pellicle [Hannig et al., 2004]. The protective effect of the pellicle layer against demineralization is well accepted. However, it is not able to completely hinder acid attack, the accompanying morphological changes and mineral loss of the tooth surface [Hannig et al., 2003; Siqueira et al., 2012].

Numerous studies demonstrate that oral microorganisms do not adhere directly to the tooth surface but to the pellicle layer, which serves as the interface between enamel / dentin and oral fluids [Hannig and Joiner, 2006; Sönju and Rölla, 1973]. In turn, the initial microbial biofilm formation is governed by the already formed pellicle [Hannig et al., 2007]. Many proteins of the pellicle function as receptors for specific adherence of pioneer bacteria [Whittaker et al., 1996]. At a later stage, the microbial co-adhesion and attachment to glucans in the biofilm matrix lead to further bacterial adherence and biofilm maturation [Hannig and Joiner, 2006].

2.1.3 Microbial biofilm (dental plaque)

2.1.3.1 Demacration between pellicle and plaque

Initial biofilm formation (pellicle formation) cannot be hindered, but its accumulation does not cause any harm, if the surface coating on the teeth is devoid of bacteria. When one or two layers of bacteria are present on the tooth surface in a healthy individual, the host immune system can effectively manage these microorganisms. Only the mature microbial biofilm leads to caries and gingivitis [Marsh, 2005; Moore and Moore, 1994; Wolff and Larson, 2009]. For the purpose of better understanding the biofilm management strategies, the difference among the initial pellicle, the multilayered plaque, and the mature plaque should be kept in mind.

As described above, pellicle formation is referred to as an instantaneous adsorption of salivary proteins on the tooth surface [Hannig and Hannig, 2009; Hannig and Joiner, 2006]. Microorganisms colonize the pellicle layer and become a microbial biofilm (dental plaque), defined as three-dimensional communities of microorganisms irreversibly attached to a supporting surface or to each other and embedded in an exo-polysaccharide matrix [Costerton, 1995; Marsh, 1999, 2005]. Even within 10 s, first adherent microorganisms are present on the initial pellicle layer [Rönström et al., 1977]. However, this incipient pellicle with randomly distributed single bacteria as well as bacterial aggregates can not be viewed as biofilm, because of the absence of the extracellular matrix [Hannig et al., 2007]. In fact, it is difficult to make a clear demarcation between pellicle and plaque under the *in vivo* conditions.

2.1.3.2 Formation of the microbial biofilm

The microbial biofilm formation is a complex process, involving more than 700 bacterial species [Foster and Kolenbrander, 2004; Kolenbrander et al., 2002; Paster et al., 2001]. It is generally mediated by the pellicle layer and influenced by various factors, such as surface free energy and surface roughness [Hannig and Hannig, 2009; Quirynen et al., 1990].

The stages of biofilm development were described by Marsh and Bradshaw [Marsh and Bradshaw, 1995] as follows:

1. the formation of a conditioning film (acquired pellicle) on the tooth surface;
2. a non-specific reversible phase involving physicochemical interactions between salivary bacteria and the acquired enamel pellicle;
3. short-range, specific, stereo-chemical molecular interactions between primary bacterial colonizers and host receptor molecules in the acquired pellicle;
4. the attachment of secondary colonizers to the primary colonizers (co-aggregation);
5. the development of horizontal and vertical stratification, increased bacterial succession and matrix formation; and
6. the growth and formation of a climax community.

At the initial stage, bacteria present in the oral cavity are passively transported by saliva and then reversibly attached to the pellicle-coated surface via long-range physico-chemical forces (van der Waals force) [Hannig and Joiner, 2006]. The primary colonizers, streptococci including *S. mitis* (*Streptococcus mitis*), *S. sanguis* (*Streptococcus sanguis*) and *S. oralis* (*Streptococcus oralis*) [Cassels et al., 1995], favor surface irregularities [Hannig and Hannig, 2009; McConnell et al., 2010]. When they approach close to the pellicle, short-range stereochemical interactions (receptor-adhesin) lead to irreversible adhesion [Hannig and Joiner, 2006]. Similarly, specific interbacterial adhesion-receptor interactions create co-aggregation of colonizers with early colonizers. Hence, bacterial aggregates not only manifest itself in diverse forms of cellular association (corn-cobs and rosettes), but also might be ascribed to the spatial and functional organization in plaque [Marsh, 2003]. Therefore, the *in vivo* biofilm demonstrates a strong resistance to external challenges. Afterwards, bacterial proliferation plays a key role for further biofilm growth over time (*e.g.* 24 h later) [Brecx et al., 1983]. Bacteria mount to sessile colonies, which provide suitable ecological environments and facilitate additional bacterial colonization. Left undisturbed, the micro-colonies continue to proliferate and become confluent, characterized by bacterial interactions (co-aggregation and quorum sensing) and diverse bacterial populations [Hannig and Joiner, 2006;

Kolenbrander et al., 2006]. A bacterial shift might occur in the mature plaque depending on the microbial community [Marsh, 1994]. In smooth and proximal surfaces, the initial colonizers are displaced by gram-positive, facultative, anaerobic, and acid-tolerant species. Consequently, a complex microflora, predominantly gram-negative obligatory anaerobic bacteria, makes up the plaque composition in the gingival crevice.

2.1.3.3 Biofilm thickness

Limited information about the biofilm thickness or its measurement has been revealed. From a technical preparation point of view, dehydrated plaque reveals about half of its original thickness [Auschill et al., 2001]. In comparison to the true, native and wet biofilms, the dried biofilms after exposure to fixative provide, however, much more brilliant pictures and more details. Thus, recently collected data are based on the one hand on the dehydrated biofilms using TEM and on the other hand on confocal laser scanning microscopy (CLSM) performed on native biofilms [Auschill et al., 2001; Hannig, 1999; Hannig and Joiner, 2006; Netuschil et al., 1998].

By means of TEM, dental biofilm height ranges from 7 μm to 14 μm 24 h after plaque collection on plastic films *in vivo* [Brecx et al., 1985; Brecx et al., 1981; Brecx et al., 1983], which is consistent with a height of 8 μm measured by CLSM [Netuschil et al., 1998]. The CLSM investigation also revealed a thickness of up to 35 μm after 48 h [Netuschil et al., 1998] and 15-30 μm after five days [Auschill et al., 2001].

2.1.3.4 Bacterial vitality

Bacterial vitality has been documented to be influenced by the host. Several host enzymes (*e.g.* amylase) and enzymes derived from sessile bacteria (*e.g.* glycosyltransferase) promote the microbial attachment [Hannig and Joiner, 2006]. Antimicrobial components in saliva (*e.g.* lysozyme) can hydrolyze the bacterial cell wall to cause cell damage or even death [Van Nieuw Amerongen et al., 2004]. As a result, a balance could be kept in such a stable microbial community.

The microbial vitality seems to increase over time and become individual-independent [Weiger et al., 1995; Zaura-Arite et al., 2001]. Sparse biofilms are mainly consisted of dead bacteria with young biofilms showing low vitality [Auschill et al., 2001; Netuschil et al.,

1998; Zaura-Arite et al., 2001]. Only 20% of the bacteria are vital one hour after prophylaxis compared with 70% vital bacteria one day after cleaning [Weiger et al., 1995]. In an investigation of the 48-h *in vivo* undisturbed biofilm by the Live / Dead staining (LDS) technology, the mean vitality values range from 64% to77% [Arweiler et al., 2004], which is in line with the findings of two studies by cultivating dental plaque bacteria [Gordon et al., 1971; Manganiello et al., 1977].

The bacterial vitality varies spatially throughout the biofilm. Arweiler *et al.* [Arweiler et al., 2004] proposed two vitality patterns. The first pattern represented the most active cells in the central part of plaque according to previous studies [Auschill et al., 2001; Netuschil et al., 1998]. Living plaque microorganisms are embedded in or located on top of dead cells. The second pattern reveals the high vitality in the initial layer nearest to the substrate surface and decreasing vitality towards the external aspects of the biofilm. These differences are assumed to be ascribed to the variable inherent antibacterial properties of saliva.

The microbial vitality can act as a parameter to assess the antibacterial efficacy of an active agent by the comparison of vitality values before and after application of the respective agent.

2.1.4 Biofilm management

2.1.4.1 Overview

It is well documented that the accumulation of dental plaque is the major etiology of dental caries [Marsh, 2005; Moore and Moore, 1994; Wolff and Larson, 2009]. In a review by Brecx [Brecx, 1997], the ‘specific plaque hypothesis’ was proposed to interpret the pathogenesis of caries, based on the idea that plaque is dominated by specific pathogenic microorganisms. Also, gingivitis is well explained by the ‘unspecific plaque hypothesis’ with emphasize on the essential role of plaque mass [Brecx, 1997]. Thus, oral biofilm management is strongly required [Baehni and Takeuchi, 2003; Longbottom et al., 2009] to inhibit and suppress supragingival and marginal plaque accumulation and, thereby, promote dental hard and soft tissue’s health.

Nowadays, home-use oral care products in the market offer a great variety of options. Mouthrinses are increasingly used, especially as supplements to daily mechanical oral hygiene approaches (*e.g.* tooth brushing) [Jardim et al., 2009; Särner et al., 2012]. The bulk of plaque is reduced mechanically with only disorganized and thin dental plaque remaining,

which can be further reduced chemically [Brecx, 1997]. Mouthrinses are well recognized as a routine oral hygiene measure. In some situations with the unavailability and comprisment of mechanical approaches, mouthrinses even replace the mechanical plaque control, such as for orthodontic patients, the elderly with disabilities, and in patients after periodontal surgery, *etc.* [Eley, 1999].

The active agents, which are delivered to the oral cavity, should target at biofilm formation without disrupting the biological equilibrium. In the last decades, the development of active chemicals has gone through several generations according to the substantivity [Brecx, 1997]. Only provided with substantivity, which is defined as the duration of action of a substance *in situ*, the chemical approach is considered to be effective in the *in vivo* situations [Netuschil et al., 2003]. First generation antimicrobial agents possess only antimicrobial activity with no substantivity (only minutes). In this group, there are certain antibiotics, essential oil phenolic compounds, quaternary ammonium compounds (cetylpyridinium chloride (CPC)), fluorides including monofluorophosphate and sodium fluoride, plant alkaloids and oxidizing agents. Bacteriostatic or bactericidal agents with some degrees of substantivity are recognized as the second-generation. Such compounds remain active *in situ* for hours, including bisbiguanides (*e.g.* chlorhexidine), AmF / SnF_2 mouthrinse, and triclosan when associated with a copolymer of polyvinyl methyl ether and maleic acid copolymer. The third-generation agents possess mild antimicrobial activity but also other anti-plaque activities that may take the form of bacterial adhesion prevention by disrupting existing deposits or properties that block specific inter-genic and intra-genic interactions between oral species involving the aminoalcohols (octapinol and decapinol). From a clinical point of view, second-generation antimicrobial agents are the agents of choice nowadays.

The main mechanism of active agents involves killing or suppressing microbial growth. However, it is insufficient to exhibit positive clinical performance results with antimicrobial properties alone. According to clinical effects on the basis of their modes of action, the active ingredients were categorized into five groups in a review by Netuschil *et al.* as follows [Brecx, 1997; Netuschil et al., 2003]. First, antimicrobial agents are characterized by the chemicals that have a bacteriostatic or bactericidal effect *in vitro* but fail to exhibit a proven efficacy *in vivo* against plaque alone. Second, the agents that have only been shown to reduce the plaque quantity (the numbers of plaque microorganisms) and / or plaque quality (the portion of vital plaque bacteria), which might be (in)sufficient to influence gingivitis and / or caries, are considered as plaque-reducing agents with examples of Sanguinarine, CPC,

Listerine. Third, anti-plaque agents possess a more pronounced effect on plaque and thereby, a benefit against gingivitis and / or caries. Representative agents in this category involve chlorhexidine, AmF / SnF_2, Delmopinol. Forth, the antigingivitis agents target at gingival inflammation without necessarily influencing bacterial plaque. The fifth is aimed to act against periodontitis. It is important to stress that the simple elimination of microbes within the mouth may not produce the best outcome for dental patients, because of disrupting the natural ecology in the oral cavity. Therefore, the efficient agent should control the levels of bacteria within the mouth to achieve a level of plaque that is constituent with optimal oral health. Based on this notion, anti-plaque agents are the optimal option because of their efficacy in biofilm control and primary prevention purpose [Baehni and Takeuchi, 2003]. The mechanisms are summarized as follows [Baehni and Takeuchi, 2003]:

1. Interference with the adhesion of oral bacteria to surfaces and prevention of biofilm formation,

2. Interference with co-aggregation mechanisms or impact on bacterial vitality which thereby prevent further growth of colonies,

3. Removal or disruption of existing biofilm.

Numerous active ingredients, such as chlorhexidine, hexetidine, delmopinol, AmF / SnF_2, triclosan, phenolic compounds, may inhibit biofilm development, maturation and bacterial metabolism. Among the active ingredients delivered by mouthrinses, chlorhexidine is the most efficient antiplaque agent [Adams and Addy, 1994; Brecx, 1997] and has been identified as the gold standard [Jones, 1997].

In spite of the success achieved by the above mentioned chemicals in promoting oral health, new compounds are still being sought. The tremendous progress in nanotechnoglogy allows the introduction of new biological and biomimetic measures in preventive dentistry [Hannig et al., 2012a; Hannig and Hannig, 2010b]. Such approaches are based on the hypothesis proposed by Hannig [Hannig and Hannig, 2010a] that ‘the abraded nano-crystallites or nano-particles represent some kind of physiological oral health care or biofilm management’. In order to optimize biofilm management and remineralization processes at the nano- and micro-scale, nanostructured materials had been synthesized to mimic the size of natural HA crystallites, the smallest building unit of tooth hard tissues. The size-specific effects of the particles could explain the principle behind this bioinspired strategy, *i.e.* nanoparticles with a comparable dimension to natural HA-crystallites fit to submicrometer-sized tooth surface

defects and are effective in repair processes [Hannig and Hannig, 2010b, 2013d]. In this approach, the tooth works as a 'self-healing material' due to application of the synthesized HA. Additionally, the nano-sized biomimetic material acts as anti-plaque and antimcirobial agent, effecting microbial adhesion and bacterial biofilm formation [Hannig et al., 2013a]. As nano-particles are synthesized to mimic the natural tooth structure, this novel strategy is expected to keep the microbial ecology equilibrium and, thus, to be more effective than traditional approaches. Recently, several mouthrinses containing nano-sized bioinspired particles have been released to the market. Two representative examples are CPP-ACP nano-complexes and zinc-carbonated HA crystallites, which will be discussed in detail in the following chapters.

2.1.4.2 Chlorhexidine

Chlorhexidine is a dicationic bisguanide (1,6-di(4-chlorophenyl-diguanido)hexane) that has been widely used as an antiplaque additive for mouthrinses for over 40 years [Brecx, 1997; Gjermo, 1974]. Due to its antibacterial efficacy, substantivity, and low mammalian toxicity in the oral cavity, chlorhexidine has been accepted as an effective treatment for gingivitis by the American Dental Association (ADA) [Van Strydonck et al., 2012] and is the gold standard for chemical plaque control [Jones, 1997]. Therefore, chlorhexidine is selected as a positive control with respect to assessing the antimicrobial ability of new products. The anti-plaque mode of action of chlorhexidine is well understood as an immediate bactericidal action during the application time and a subsequent bacteriostatic action of chlorhexidine immobilized at the biofilm-coated tooth surface [Jenkins et al., 1988]. First and foremost, a close look on the antimicrobial mechanisms is necessary. The degree of such efficacy depends on the concentration. At high concentration, chlorhexidine has a broad spectrum of bactericidal activity encompassing gram-positive and gram-negative bacteria as well as yeasts, with a potential effect on gram-positive bacteria [Hennessey, 1973]. Among them, *S. mutans* (*Streptococcus mutans*) is in particular sensitive, whereas *S. sangunis* (*Streptococcus sangunis*), an early colonizer of the biofilm, is relatively resistant [Emilson, 1994]. Chlorhexidine acts on the bacterial cell wall and interacts with the phospholipids of the inner membrane, which results in the increase of membrane permeability [Jones, 1997]. After the attack, the osmotic equilibrium is disrupted and as a result, cytoplasmic membrane is extruded. Vesicles are formed and the cytoplasm precipitates [Brecx and Theilade, 1984; Davies, 1973]. In this approach, chlorhexidine is bactericidal and causes irreparable damage

to cell membrane. At low concentration, chlorhexidine is bacteriostatic [Thomas, 1985] and only interferes with the sugar transport and acid production of the cariogenic species. It should be kept in mind that chlorhexidine is gradually diluted in saliva over time [Brecx, 1997]. Usually, it was applied in the concentration of between 0.1% and 0.2%. Also, dose, calculated by frequency, concentration, rinsing time, and rinsing volume, directly link to the efficacy of chlorhexidine mouthrinse in plaque inhibition [Jenkins et al., 1994]. The optimal dose of chlorhexidine is generally considered to be approximately 20 mg each at twice per day. Numerous clinical studies have already confirmed its anti-plaque, anti-gingivitis and biofilm inhibitory efficacy [Löe and Schiott, 1970; Löe et al., 1972].

In spite of its potent anti-plaque property, chlorhexidine cannot be recommended for long-term usage and should be carefully monitored due to the undesirable side effects, such as staining on teeth [Hannig and Joiner, 2006; Van Strydonck et al., 2012] and tongue [Jenkins et al., 1994], perturbation of the taste [Flötra et al., 1971], and desquamation of the oral mucosa [Emilson, 1994]. Thus, chlorhexidine does not suit patients suffering from mucositis [Foote et al., 1994; Vissink et al., 2010].

The above descriptive side effects are being well known to the profession. However, chlorhexidine has gained its place in preventive dentistry and is recommended for short-term or medium-term application. Chlorhexidine is advised subjecting to high caries risk and being used as an adjunct to mechanical oral hygiene in initial periodontal therapy [Addy, 1986]. In particular, chlorhexidine is suitable in populations, when the conventional mechanical oral hygiene procedures are lacking or compromised, such as elderly, people with disabilities, patients after radiation and periodontal surgery [Sanz et al., 1989], and orthodontic patients [Addy, 1986]. Still, the impact of long-term usage remains questionable.

2.1.4.3 Fluoride

Fluoride was introduced into dental medicine over 70 years ago and is currently recognized as the main contributor of the marked decrease in caries prevalence worldwide [Buzalaf et al., 2011; Rosin-Grget and Lincir, 2001]. Fluoridation involves systematic fluoridation measures (fluoridated water) as well as topical fluoride application (fluoride-containing toothpaste, mouthrinse and varnish). The topical affects are currently assumed to be of great relevance to the observed significant caries prevention, which is accomplished in two ways [Buzalaf et al., 2011; Rosin-Grget and Lincir, 2001; Van Loveren, 2001]: modulation of de- /

üremineralization processes on the one hand and inhibitory impact on microbial metabolism on the other hand. The former is viewed as the main anti-caries mode of action. Fluoride mainly presents constantly at a low concentration (sub-ppm range) in the oral fluids and retains on the tooth substance / biofilm as CaF_2 or actually the CaF_2-like material [Cruz and Rölla, 1991]. CaF_2 has been verified in a bulk of studies as pH-controlled fluoride and calcium reservoir [Buzalaf et al., 2011; ten Cate, 1997]. Upon acid challenge, fluoride, released from CaF_2, adsorbs to the dental surface to hinder demineralization. When pH is re-established, a shift toward remineralization occurs. Fluoride incorporates into the dental surface to produce a more acid-resistant fluoride modified hydroxylapatite (Fluorhydroxyapatite, $Ca_{10}(PO_4)_6F_2$) [Buzalaf et al., 2011]. On the other hand, the trace of fluoride in the oral environment make it supersaturated with fluorhydroxyapatite, which could in turn speed up the process of remineralization. The new tooth surface after fluoride treatment, generated from the cyclic dissolution and reprecipitation, renders the tooth a resistance to further acidic attack and subsequent lesion formation [Buzalaf et al., 2011].

Fluoride retained in biofilm not only participates in the de- / re-mineralization process but also impacts the microbial metabolism. Even at a sub-millimolar concentration, the enrichment of *S. mutans* is inhibited in the presence of fluoride [Bradshaw et al., 2002]. Concentrations of fluoride in plaque can reach the millimolar range [Gaugler and Bruton, 1982], sufficient to render fluoride bioacitive effects on the biofilm microorganisms. As suggested by abundant investigations, fluoride exhibits multi-fold antibacterial effects. Under acidic conditions, fluoride diffuses easily into the bacterial cell in the form of HF (a weak acid, pKa 3.15), due to the higher permeability of HF to cell membrane. As a result of a higher internal pH of cells, HF dissociates in H^+ and F^- in the cytoplasm [Rosin-Grget and Lincir, 2001; ten Cate, 1999], leading to an accumulation of fluoride and protons in the cell, a direct inhibitory effect of the intracellular fluoride on glycolytic enzyme activity (enolase) results in the decrease in acid production from metabolic process. Furthermore, fluoride brings the excreted H^+ back into the cell and thus, inhibits the cell membrane-associated proton-extruding adenosine triphosphatases (H^+-ATPases), which is associated with the generation of proton gradients. The accumulation of protons in the cell acidifies the cytoplasm, resulting in the inhibition of the entire glycosis, phosphotransferase sugar transport system, intracellular polysaccharide formation as well as decreased acid-tolerance of *S. mutans* [Buzalaf et al., 2011; Koo, 2008]. Therefore, the above mentioned mechanisms provide optimal inhibitory effects of fluoride against bacterial colonization, in accordance

with promising results from *in vitro* studies [Bradshaw et al., 2002; Shani et al., 2000]. However, the *in vivo* implication is still not clear [Buzalaf et al., 2011; Koo, 2008]. Clinical studies seem to indicate that fluoride possesses a limited antimicrobial effect [Buzalaf et al., 2011], dependent on the concentrations of fluoride applied and the associated antibacterial ions [Van Loveren, 2001], especially Sn^{2+} and amine. However, high concentrations of fluoride are needed for the effective impact on metabolic activity of bacteria, and surpass markedly the concentration which can reduce the solubility of apatite [Rosin-Grget and Lincir, 2001]. To date, there is still no consensus that the observed anti-glycolytic property of fluoride can contribute to its promising performance in caries control.

Dental fluorosis due to excessive intake of fluoride during the tooth development is the only proven side effect of fluoride application in dentistry. Thus, the fluoridated products are not advised for preschool children [Stephen, 1994]. However, fluoride has obtained recognition as an important tool in preventive dentistry by ADA. Topical at-home fluoride application, such as tooth pastes or mouthrinses, remain a cost-effective preventive and regular prophylactic approach [Stephen, 1994] and are encouraged to the public population, especially to the pregnant [Buzalaf et al., 2011] and people on a high sugar diet [Bradshaw et al., 2002].

2.1.4.4 Sodium fluoride (NaF)

In series of reviews on chemical plaque control, NaF is classified as the first-generation antimicrobial agent, revealing minor antimicromial capacity and no substantivity [Brecx, 1997; Netuschil et al., 2003]. Nevertheless, NaF has given decisive indication that a fluoride solution will be of great relevance to caries prevention, as compared to the organic fluoride solution (amine fluoride) and other inorganic fluoride salts (stannous-, zinc-, ferricfluoride) when used alone in solution [Buzalaf et al., 2011]. A significant reduction in incidence of caries was demonstrated with a 2-y extended use of sodium fluoride in toothpastes and mouthrinses [McGrath et al., 2009]. It is the preferred formulation for fluoride mouth rinsing as a public prophylactic program [FDI Commission, 2002b; Stephen, 1994].

2.1.4.5 Amine Fluoride (AmF)

AmF was first developed at the University of Zürich in the beginning of the 1950s. It is present in 4 forms: Ethanolamin-hydrofluorid, Olaflur, Oleaflur and Dectaflur. Among them, Olaflur ($C_{27}H_{60}F_2N_2O_3$,N,N,N'-tris(2-hydroxyethyl)-N'-octadecylpropane-1.3-diamine

dihydrofluoride) is most commonly used in dentistry. Its chemical structure is illustrated in Fig. 1.

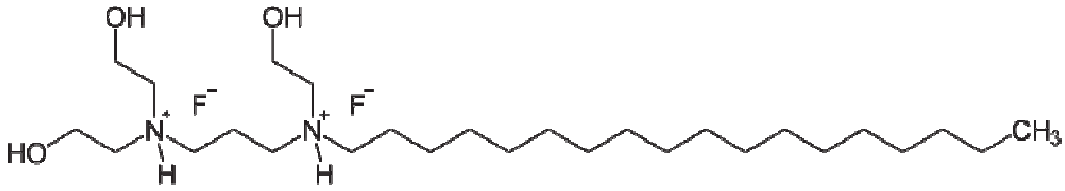


Fig. 1: Chemical structure of Olaflur.

Due to its surfactant property, AmF acts as carrier for the labile fluoride and thus, exhibits substantial anti-caries activities [van Strijp et al., 1999]. It exerts potential protection against acid attack and acts as an anti-erosive agent [Yu et al., 2010]. In the 1980s, an *in vitro* study [Kay and Wilson, 1988] indicated that a wide range of plaque microorganisms, especially gram-positive strains, were sensitive to the amine fluorides. Such antibacterial activity was confirmed to prevent the growth of cariogenic bacteria [Schmid et al., 1984] as well as periodontal pathogens [Bullock et al., 1989]. The amine group is the main factor responsible for this antimicrobial property [Shani et al., 2000; Shani et al., 1996]. The mode of action could be explored as follows. Due to the double charged amine cation and its surface activity, a high absorption but low desorption of amine fluoride to saliva-coated HA makes it possible to keep such moiety at a high concentration in plaque [Shani et al., 2000]. Similarly, the strong electrostatic interaction between the amine cation and the negatively charged cell surface also leads to a rapid and stable attachment of this active compound to bacteria [Embleton et al., 1998; Shani et al., 2000]. The above mentioned mechanisms facilitate the anti-glycolytic activity of amine groups on immobilized bacteria [Shani et al., 2000; Van Loveren, 2001], resulting in decreased acid production [Schneider and Mühlemann, 1974]. Recently, a three layer structure model of fluoridated enamel containing CaF_2, $Ca(OH)_2$ and fluoroapatite (FAp) was proposed with the purpose to summarize the overall activity of AmF [Gerth et al., 2007; Müller et al., 2010]. The surface CaF_2 layer was found to act as the fluoride reservoir. In the middle layer, $Ca(OH)_2$ functioned to prevent against bacteria and demineralization. This layer is covered by an acid resistant FAp.

All the above described activities of fluoride are based on data from the *in vitro* studies. Under *in vivo* conditions, the use of this agent alone does not seem to be effective in reducing plaque or gingivitis [Paraskevas et al., 2005]. In addition, surprisingly the anti-adhesive

performance on plaque has not been proven *in vitro* as well as *in vivo* [Embleton et al., 1998, 2001; Weiger et al., 1998]. In spite of its mild activities on the microorganisms, the long-term use of amine fluoride containing products, especially gels, are effective to control the caries progress [Brambilla et al., 1999; Brambilla et al., 1997; López et al., 2012].

2.1.4.6 Amine fluoride / stannous fluoride (AmF / SnF_2)

Stannous fluoride (SnF_2) exerts anti-plaque and antigingivitis properties due to the stannous ion [Yu et al., 2010]. When delivered in solution, stannous fluoride is not active, as compared to that present in toothpaste and gel. This discrepancy is relevant for the instability of stannous fluoride, as a result of the rapid oxidation and hydrolysis of stannous ions [Paraskevas et al., 2005]. Thus, stannous fluoride is used in combination with amine fluoride in mouthrinse formulations, as the former substance could stabilize the instable stannous cation. This regime is commercially available as Meridol® in the market. A bulk of *in vitro*, *in vivo* and clinical studies have identified the antiplaque and anti-caries properties of the combination of amine fluoride and stannous fluoride [Etemadzadeh et al., 1989; Shapiro et al., 2002], derived from the synergistic modes of action from all three functional groups: the amine group, fluoride and stannous ions. A long-term application of this combination is recommended to public population, particularly to the erlderly [Meurman et al., 2009].

2.1.4.7 CPP-ACP

The CPP-ACP nanocomplex, which was patented by the University of Melbourne, Australia, and the Victorian Diary Industry Authority, Abbotsford, Australia, is a nano-sized bioinspired calcium phosphate particle and commonly delivered in formulations as mouthrinse, chewing gum, dental cream *etc.* [Azarpazhooh and Limeback, 2008; Reynolds et al., 2008]. It contains two components: CPP and ACP. Because of its generally accepted molecular formula: $Ca_3(PO_4)_2$-nH_2O, ACP could be described as a tricalcium phosphate and is expected to play a core role in the remineralization process. However, the past systems with the attempt to deliver calcium and phosphate ions have experienced troubles with the solubility of these ions at neutral pH ranges. Such problem has already been overcome with utilization of a CPP stabilized system. CPP containing the cluster sequence of –Ser (P)-Ser (P)-Ser (P)-Glu-Glu from Casein could stabilize calcium and phosphate ions into nanoclusters without the capacity to perform nucleation and crystallization [Cross et al., 2005]. Thus, a large number of calcium

phosphate is able to be retained at the tooth surface by binding the phosphopeptide to the biofilm-tooth surface interface.

The bioactive agent CPP-ACP has been already proven to be efficacious in caries prevention in short-term as well as long-term studies [Yengopal and Mickenautsch, 2009] on the basis of its pronounced performance in inhibition of demineralization and promotion of remineralization in enamel [Cai et al., 2007; Morgan et al., 2008] and dentin carious lesion [Rahiotis and Vougiouklakis, 2007]. This potential has been explained by the high affinity of CPP-ACP (diameter of 2.12 nm) to the surfaces of bacterial cells, the components of intercellular plaque matrix and already adsorbed salivary macromolecules [Cross et al., 2007; Reynolds et al., 2008; Rose, 2000a, b, c]. After its incorporation into the biofilm, CPP-ACP serves as a reservoir of calcium [Cross et al., 2007]. During acidic attack, such a bioactive agent is able to buffer plaque acid [Rahiotis and Vougiouklakis, 2007]. In addition, CPP stabilizes and localizes the calcium and phosphate ionized from the adsorbed ACP and, in consequence, maintains a supersaturated mineral environment, promoting remineralization [Reynolds et al., 2003], and producing a new mineral of higher resistance to acid [Cai et al., 2007]. Moreover, an inhibitory effect of CPP-ACP on microbial biofilm formation has also been demonstrated [Rahiotis et al., 2008; Rose, 2000a, b, c]. The mechanism involved is the competing action between the attached particles and bacteria for calcium, resulting in a decrease in the amount for calcium binding sites. In this approach, bacterial receptors are blocked and therewith, CPP-ACP exerts an inhibitory effect on microbial coaggregation and bacterial adhesion to the pellicle, *i.e.* delay of biofilm maturation.

Numerous studies have suggested that CPP-ACP could be utilized to combat dental caries and dentin hypersensitivity [Azarpazhooh and Limeback, 2008]. However, investigations on the long-term clinical effectiveness of oral products containing CPP-ACP are still needed [Yengopal and Mickenautsch, 2009].

2.1.4.8 Nanosized HA particles and the derivatives

Biomimetic strategies at the nanolevel are known as a promising approach in preventive dentistry without unpredictable adverse effects [Hannig and Hannig, 2010a; Hannig and Hannig, 2013d]. Hydroxyapatite nanoparticles and derivatives have been synthesized to mimic the natural HA. In theory, these particles are believed to provide excellent biological properties, including biocompatibility, lack of toxicity, fewer immunological and inflammtory

responses [Hannig and Hannig, 2013d]. Many preparations have been developed based on these properties. However, most of them are in the experimental stage with few commercially ready products. Oral hygiene products containing zinc-carbonated HA nanoparticles are 'new' products released to the market [Hannig et al., 2013a; Poggio et al., 2010; Tschoppe et al., 2011].

Biomimetic HA nanoparticles might work in a similar way as CPP-ACP. However, unlike CPP-ACP, these apatite nanoparticles truly mimic the nanostructure of either natural dentinal hydroxylapatite (20 nm, spehroidal-like shape) or enamel hydroxylapatite (100 nm, needle-like shape) and these possess extraordinary physicochemical properties as a result of their large surface-to-volume ratio [Roveri et al., 2008]. Thus, the potential for modulation of the de- / remineralisation and effects on biofilm management are expected.

The nanosized apatite particles can fit with nano-scaled defects caused either by caries or erosive acid challenges. In addition, they serve as a slow-release depot at the tooth surface or in the pellicle layer, facilitating remineralization. The potential effect of HA nanocrystales on remineralization of the initial caries lesions has been demonstrated by *in vitro* studies [Poggio et al., 2010; Tschoppe et al., 2011]. Recently, Tschoppe *et al*. [Tschoppe et al., 2011] and Huang *et al*. [Huang et al., 2011] revealed the excellent performance of an experimental 10 wt% nanosized HA aqueous slurry or a 20 wt% clustered zinc carbonate-nano HA containing dentifrice on the remineralzation of incipient enamel caries lesions under *in vitro* conditions. Interestingly, Tschoppe *et al*. [Tschoppe et al., 2011] also revealed a higher remineralizing effect of nano-HA on dentin by applying a nano-HA containing toothpaste compared to an amine fluoride toothpaste. In addition, a suppressing effect of a 10% nano-HA dentifrice on demineralization was demonstrated not only in demineralized enamel but also on sound enamel over a 28-d period in an *in situ* study [Najibfard et al., 2011]. Based on the above described data, the nanosized HA might be also applied for the treatment of eroded tooth surfaces. After 10-min treatment of eroded enamel in aqueous slurries containing nano-HA (clusters of dimensions ranging from 0.5-3.0 μm), a coating of mineral deposites, which is less crystalline than the native enamel apatite, was revealed covering the etched enamel surface and filling the etched enamel micro spaces [Roveri et al., 2008]. More recently, an *in vitro* study also demonstrated the effectiveness on preventing enamel demineralization caused by soft drink consumption [Poggio et al., 2010]. However, the *in vivo* evidences for the repairing effects on erosion are inadequate, yet [Hannig and Hannig, 2013d].

The nanosized HA particles could be adorbed to the membrane of the planktonic bacteria as well as to bacteria adherent on the tooth surface [Venegas et al., 2006], resulting in an interference with bacterial adhesion. In addition, the nanoparticles could be integrated in the pellicle layer, leading to an alteration on the nano-level in the physicochemical characteristics of the pellicle. Thereby, a modulation of biofilm formation and subsequent bacterial colonization is conceivable [Hannig and Hannig, 2010b, 2013d]. In an animal model, the capacity of nano-HA (needle-like or spheroidal HA) to promote the remineralization of artifical caries lesions and to modify bacterial accumulation at the tooth surface was confirmed [Lu et al., 2007]. At the same time, the inhibitory effect of spherical nano-HA on the bacterial adherence and subsequent colonization was revealed under *in vitro* conditions [Meng et al., 2007]. An experimental dentifrice containing nanosized HA provided a decreased adherence / accumulation of bacteria to bovine enamel in a laboratory experiment [Park et al., 2007]. In addition, Arakawa *et al.* [Arakawa et al., 2010] reported that the *in vivo* application of a 38% nano HA spherulites containing toothpaste significantly reduced the *S.mutans* levels in saliva over a 4-week post-treatment evaluation period. In a recent study, a comparable *in vitro* antimicrobial effect of clustered zinc-carbonate HA nanoparticles in a mouthrinse preparation (BioRepair Zahn-und Mundspülung) on *S. mutans* was demonstrated [Hannig et al., 2013a]. In the same study under *in situ* conditions, the application of both, the commercially available mouthwash and the pure zinc-carbonate HA nanoparticle clusters in saline solution, showed a suppressed initial biofilm formation as well as bacterial adherence by means of diaminophenolindol (DAPI) staining and BacLight^{TM} viability staining [Hannig et al., 2013a].

Based on the numerous investigations conducted on enamel, biomimetic HA nanoparticles seem to be promising for preventive dentistry. However, little information on their efficacy on dentin is available. Thus, further research is necessary.

2.2 Aim of this work

Nanotechnology has been considered to provide a new approach for dental prophylaxis. On the one hand, biomimetic apatite nanocrystals in toothpaste formulations have been demonstrated to be effective in terms of promoting remineralization and preventing demineralization *in vitro* [Poggio et al., 2010; Tschoppe et al., 2011]. On the other hand, an *in situ* investigation revealed that a mouthrinse based on apatite microclusters reduces bacterial colonization on enamel specimens [Hannig et al., 2013a]. However, the potential capacity of such microclusters in mouthrinse preparations to influence biofilm formation on dentinal surfaces has not been tested until now. Moreover, a systematic comparison among the customary rinses containing hydroxyapatite microclusters, fluoride and other chemical substances is lacking. Therefore, this study based on *in situ* experiments is aimed to investigate the potential impact of a mouthrinse with zinc-carbonate hydroxyapatite nanoparticles on the management of biofilm formation taking place on dentinal specimens. In detail, the study addresses the following questions:

1. How do the mouthrinses work related to the antibacterial and antiadherent properties under *in situ* conditions?
2. What morphological alterations in the *in situ* biofilm are caused by the application of the mouthrinses?

3 Materials and Methods

3.1 Subjects

Five healthy volunteers, aged between 24 and 31 years, who are departmental staffs of the Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University, participated in this study. An experienced dentist conducted visual oral examination and found no signs of caries, periodontal pathology or salivary dysfunctions. In other words, the subjects had a physiological salivary flow rate, a minimum of 28 evaluable permanent teeth or sufficient dental fillings, no bleeding on gentle probing and the periodontal pocket depths were between 1 mm and 3 mm. All subjects had been on their regular daily diet with no excessive consumption of polyphenolic beverages and foods and had no history of smoking or alcohol. In addition, they had not taken any antibiotic medicine in the past six months. Prior to the experiments, informed written consent about participation in this study was obtained from all volunteers. The study protocol had been checked and approved by the Medical Ethics Committee of the Medical Association of Saarland, Germany (# 18/10).

3.2 Production of splints

Individual intraoral fixtures for mounting dentinal specimens were manufactured for all subjects in the form of acrylic appliances in the first and second quadrants of the upper jaw.

The impression of the maxilla was taken with alginate impression material (Blueprint, Dentsply DeTrey GmbH, Konstanz, Germany) to produce an elastic mould. Afterwards, a hard plaster model was fabricated, where the individual appliances (‘minisplints’) were constructed. The minisplints were made of Duran® (Scheu-dental GmbH, Iserlohn, Germany) with a thickness of between approximately 0.5 mm and 0.7 mm, covering the molar and premolar teeth on the left and right upper jaw, and extending 3 mm beyond the buccal / palatal marginal sulcus. In order to better stabilize the mounted specimens, minisplints were provided with small perforations.

3.3 Production of specimens

3.3.1 Extraction and preparation of bovine teeth

Non-damaged bovine permanent lower incisors of 2-y old cattle were used in this study. The crowns of the freshly extracted bovine teeth were separated from the root at the cementum-enamel junction under water cooling using a diamond cutting disc (Schott Diamantwerkzeuge GmbH, Stadtoldendorf, Germany), which was conducted with a saw (Conrad Apparatebau Clausthal GmbH, Clausthal-Zellerfeld, Germany). Then the roots and pulp tissues were carefully discarded. From each crown, samples with a surface area size of 4 x 4 mm^2 (for *Bac*Light™ viability assay and for SEM) and 2 x 2 mm^2 (for TEM) were cut from the middle third of the labial surfaces in order to standardize the test pieces. The samples were then stored in 0.1% thymol solution (pharmacy of the Saarland University Hospital, Homburg, Germany) at 4 °C.

3.3.2 Production of dentinal specimens

To expose the sound dentin, the enamel was ground away under permanent water cooling by a polishing machine (Buehler, Düsseldorf, Germany), using silicon carbide grinding paper (P600-P2.500, FEPA-P, waterproof silicon carbide paper, Buehler, Düsseldorf, Germany). Then the dentinal surface was ground plan-parallelly and polished by wet grinding with abrasive paper down to P-grit size of '4.000' (according to Federation of European Producers of Abrasives (FEPA) standard, mean grain size = 5 µm). The samples were 1 mm thick. Subsequently, a light-microscopic examination (Motic Deutschland GmbH, Wetzlar, Germany) with 10-fold magnification was performed on the surface area of each dentinal specimen. Samples with any microscopically visible discolorations, demineralization or surface inhomogeneity were discarded and excluded from the study.

According to Hannig *et al.* [Hannig et al., 2005], pre-treatment with NaOCl and disinfection with 70% ethanol was adopted. Polished dentinal specimens (n = 240) were firstly cleaned using a 3% NaOCl (Hedinger, Stuttgart, Germany) solution for 30 s to remove any residues from the polishing procedure. Next, the specimens were washed five times in distilled water (Ecotainer; B. Braun Melsungen AG, Melsungen, Germany), followed by disinfection in 70% ethanol (Hedinger, Stuttgart, Germany) for 20 min and finished with another five washes in

double distilled water. Before intraoral exposure, rehydration of specimens took place by storage in distilled water for 24 h.

3.4 Fixing / mounting of dentinal specimens

Specimens were fixed on the maxillary minisplints at a defined position by means of a thin layer of polyvinyl-siloxane impression material (President light-body, Coltene, Altstätten, Switzerland). The specimens were placed on buccal sites of the left and right upper 1st molar (16, 26) and upper 2nd molar (17, 27) (Fig. 2). The margin was completely covered by the impression material to ensure a save fixation with only the surfaces exposed to the oral environment. The buccally mounted specimens should not be directly in contact to the ductal orifice of the parotid gland.

Fig. 2: Splint with mounted specimens.

3.5 Biofilm formation

A total of 240 dentin specimens were randomly assigned into four experimental groups (n = 60 each) based on the applied mouthwashes. They were classified as follows: control group (with no mouthwash), BioRepair group (BioRepair mouth and tooth rinsing solution, Dr. Wolff, Bielefeld, Germany), Elmex Kariesschutz group (elmex® Kariesschutz Zahnspülung, GABA, Lörrach, Germany), CHX group (Chlorhexamed® Forte, GlaxoSmithKline, Bühl, Germany). The latter served as a positive control. The principal compositions of each mouthrinse products are listed in Table 1. In each group, each subject wore six dentinal specimens for 24 h, and another six specimens for 48 h.

To avoid carry-over interfering effects, a washout phase to eliminate antimicrobial substances was carried out between each of the eight test cycles of each study section [Newcombe et al.,

1995]. Also, the 3 products were distributed to each subject in a different order, which based on a Latin square Design [Newcombe, 1992].

The splints with mounted dentinal specimens were exposed to the oral environment for periods of 24 h and 48 h. The splints with 6 dentinal samples were inserted into the oral cavity at 8:00 a.m., 30 min after individual toothbrushing without toothpaste. The samples were rinsed *in situ* twice a day with the respective mouth rinsing solution (20 ml) for 30 s, according to the instruction of the manufacturers. The first rinse occurred after oral exposure for 3 min, while the second rinse took place at 8:00 p.m.. In the case of the 48-h trial, the third rinse was conducted at 8:00 a.m. on day 2 and the fourth 12 h later. In the control group, the specimens were not treated with any mouthrinse. During the intraoral exposure period, the subjects were instructed to refrain from any consumption of diatery products and drinks. Splints were only removed and stored in 100% humidity environment during meals and daily toothbrushing. After each experimental period (24 h or 48 h), 6 dentinal specimens were quickly removed from the splints and thoroughly rinsed with sterile water for 5 s to remove non-adherent bacteria and residual saliva, followed by the *Bac*Light^{TM} viability assay, SEM- and TEM-analyses, each with two samples. The detailed experimental design of specimen exposure to oral environment is summarized in the flowchart of Fig. 3.

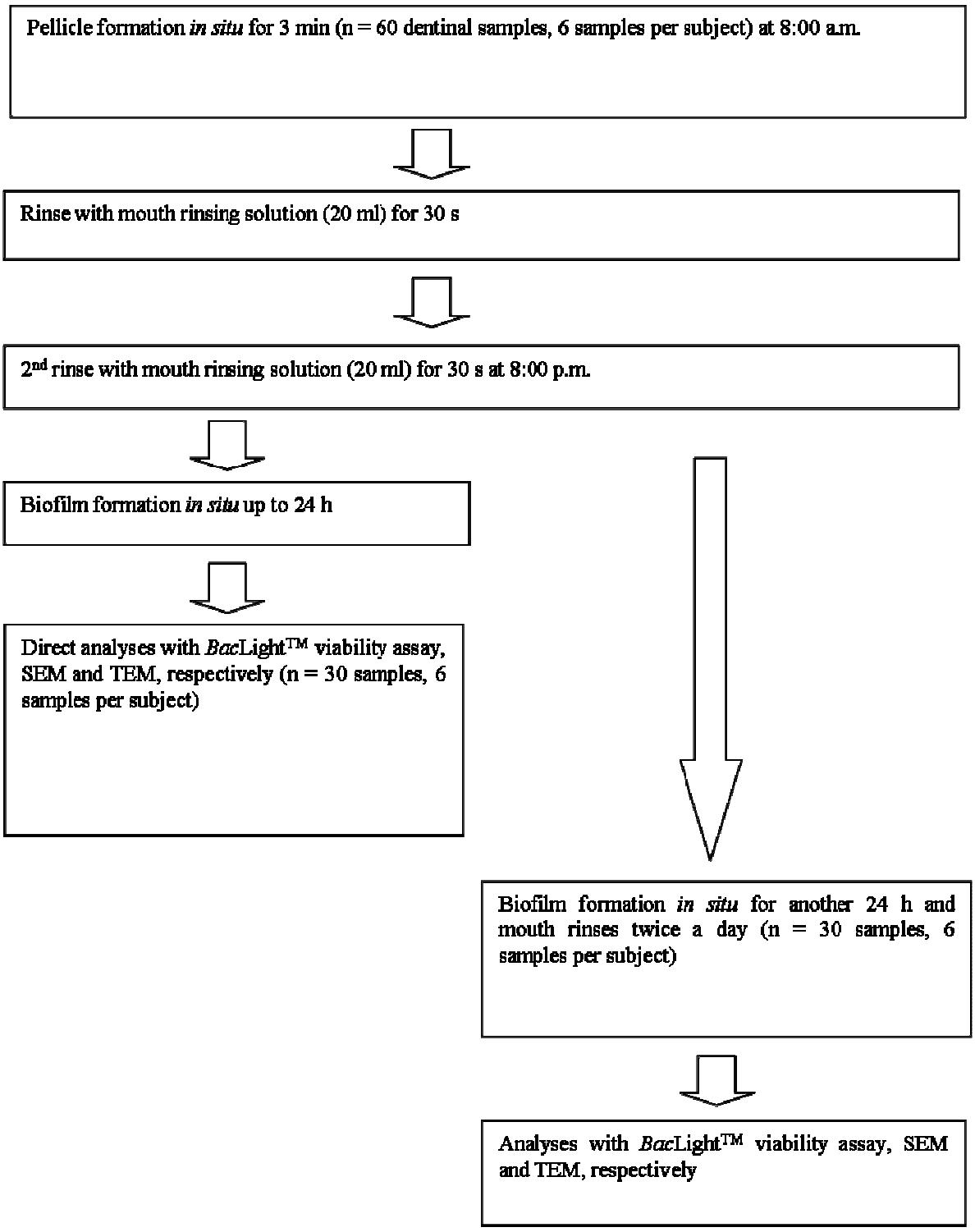


Fig. 3: Flowchart of the *in situ* experiments and subsequent analyses.

Table 1 Principal compositions of rinsing agents used in this study

Mouthrinse	Code	Main ingredients	Percentage
BioRepair mouth and tooth rinsing solution (Dr. Wolff, Bielefeld, Germany)	BioRepair	Water	>50
		Sorbitol	<25
		Alcohol denat.	4
		Glycerin	<5
		Xylitol	<5
		Cellulose gum	<5
		Zinc PCA	<1
		Zinc hydroxyapatite	<1
		Aroma	<1
		Sodium lauryl sulphate	<1
		Silica	<1
		Ricinus communis seed oil	<1
		Ammonium-acryloyldimethyltaurate / VP-copolymer	<1
		Sodium saccharin	<1
		Sodium benzoate	<1
elmex® Kariesschutz Zahnspülung (GABA, Lörrach, Germany)	Elmex Kariesschutz	Water PEG-40 Hydrogenated Castor Oil Olafluor (amine fluoride) Sodium fluoride Aroma Potassium Acesulfame Polyaminopropyl Biguanide Hydrochloric Acid	Fluoride content:250 ppm
Chlorhexamed® Forte (GlaxoSmithKline, Bühl, Germany)	CHX	Chlorhexidine gluconate Water	0.2%

3.6 *Bac*Light™ viability assay

3.6.1 Mechanism of *Bac*Light™ viability assay

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Art. No. L7012, Invitrogen, Molecular probes, Eugene, Oregon, USA) was used to distinguish and quantify live and dead bacteria in bacterial population, based on the membrane integrity of cells. Cells with a compromised membrane that are considered to be dead were stained red, whereas cells with an intact membrane were stained green. Two dyes, SYTO 9 green-fluorescent nucleic acid stain and propidium iodide ($C_{27}H_{34}I_2N_4$, PI), the red-fluorescent nucleic acid stain, were utilized. These two dyes differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stains all bacteria in a population, whereas PI penetrates only bacteria with compromised membranes. When both dyes are used, PI reduces the SYTO 9 dye fluorescence. A clear and reliable distinction of the bacterial viability can be easily achieved with an appropriate mixture of SYTO 9 and propidium iodide stains. The total number of adherent bacteria and the correlation between the viable (green) and damaged (rot) bacteria can hence be evaluated [Boulos et al., 1999].

3.6.2 Preparation of staining solution

The staining solution was prepared with 1 ml 0.9% saline solution (B. Braun Melsungen AG, Melsungen, Germany), 1µl SYTO 9 and 1µl PI. In the following, the staining solution was thoroughly vortexed.

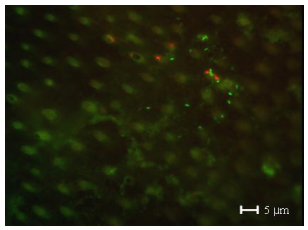
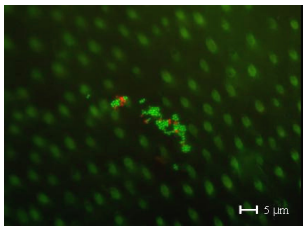
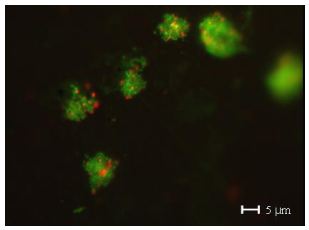
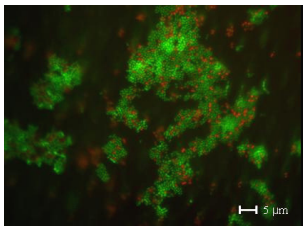
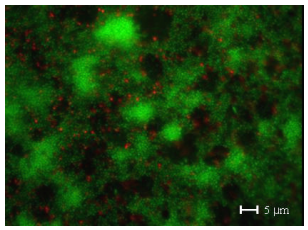
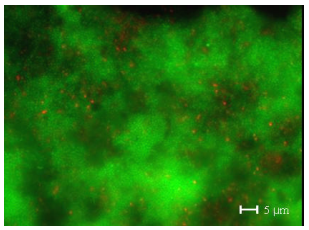
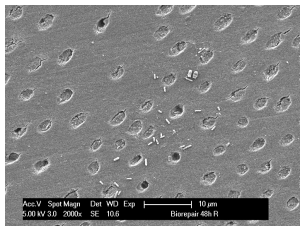
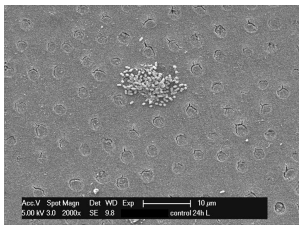
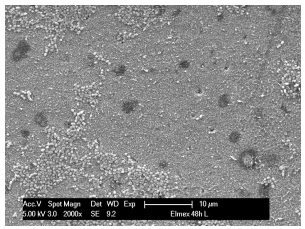
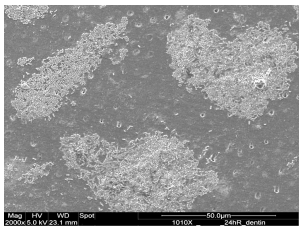
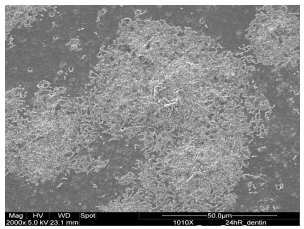
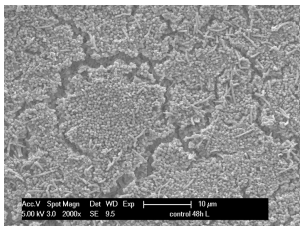
3.6.3 Staining of samples

After the oral exposure time, the biofilm-covered samples were carefully removed from the splint, gently washed in saline and then processed without delay for vital fluorescence staining. This was carried out at room temperature in a 6-well-plate, in which the samples were washed in sterile 0.9% saline solution, covered with 0.1 ml staining solution and then were wrapped with aluminium foil to protect from light for 10 min. Afterwards, samples were washed 3 times in saline solution to remove the staining solution. As soon as samples were air dried, they are glued to a specimen glass slide with double-faced tape (Leit-Tabs, Art. Nr. G3347, Plano, Wetzlar, Germany) and the surface was mounted in *Bac*Light oil.

3.6.4 Fluorescence microscopic examination and semi-quantification of *Bac*Light™ viability assay

The mounted samples were investigated in a fluorescence microscope (Leica DMRB, Leica Mikroskopie & SystemeGmbH, Wetzlar, Germany) at 1.000-fold-magnification. Representative images in nine randomized microscopic ocular grid fields per sample were taken and then analysed with Axiovision 4.8 (Axiocam, Zeiss MicroImaging GmbH, Göttingen, Germany). The amount of adherent bacteria in the biofilm observed was scored as follows (Table 2).

Table 2 Score scale for estimating amount of bioiflm formation detected with *Bac*Light™ viability assay and SEM

Score 1	Score 2	Score 3	Score 4	Score 5	Score 6
Separately distributed single bacteria	Mono-layered bacterial chain or isolated bacterial agglomerates (n < 100 bacteria)	Insular bacterial agglomerates (n < 1.000 bacteria)	(Mono-layered) bacterial colony, which reaches approximately 50% of the surface area; or large multi-layered agglomerates	Bacterial colony, which reaches more than 50% of the surface area	Multi-layered bacterial biofilm over the whole surface (more than 90% of the surface)
					
					

3.7 Fixation of the *in situ* biofilm

After intraoral exposure, the biofilm-covered dentinal samples considered for SEM and TEM analyses were directly washed gently with sterile water and fixed in 2.5% glutardialdehyde solution, which consisted of 2.5% glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany), 1.5% formaldehyde (Science Services, München, Germany) in phosphate buffer (pH 7.4, PAA Laboratories GmbH, Pasching, Austria), for 2 h at 4°C. Then, the samples were washed 5 times in phosphate buffered saline (pH 7.4) for 10 min each and stored in the last buffer solution at 4 °C.

3.8 Scanning electron microscopy (SEM)

3.8.1 Preparation of dentinal samples for SEM analysis

The samples with fixed biofilm were dehydrated in an ascending series of ethanol from 50% to 100% and then dried in hexamethyldisilazane (HMDS, $[(CH_3)_3Si]_2NH$) solution (98.5%, ABCR GmbH & Co. KG, Karlsruhe, Germany) overnight. After critical point drying, specimens were sputtered and coated by a thin layer of gold-palladium (SC 7640 High Resolution Sputter Coater, Quorum Technologies Ltd., U.K.).

In order to determine biofilm structures on fractured specimens as a function of depth, samples after 24-h oral exposure were firstly selected, fractured into halves and prepared for SEM analysis.

3.8.2 SEM analysis

Dentinal specimens were examined by a scanning electron microscope XL 30 ESEM FEG (FEI, Eindhoven, The Netherlands) operating at 5 kV under a magnification ranging from 2.000- to 20.000-fold to investigate the surface and fractured surface with the purpose of a thorough description of bacterial adherence. The settings for the scanning electron microscope, such as tilt angle, spot size, scanning mode, were kept constant for all samples.

3.8.3 Semi-quantification of SEM analysis

Surface was scanned consecutively through each sample. The scale of bacterial population was evaluated in 3 randomized micrographs at a 2.000-fold-magnification and scored as shown in Table 2.

3.9 Transmission electron microscopy (TEM)

3.9.1 Post fixation and dehydration

For visualization of organic structures, samples were contrasted with 2% osmium tetroxide (O_2SO_4) in phosphate buffer for 2 h. After incubation with the osmium acid solution, the specimens were washed five times in phosphate buffer and dehydrated in increasing concentrations of ethanol [Hannig and Balz, 1999].

3.9.2 Embedding

The specimens were firstly incubated twice in propylenoxide (SERVA Electrophoresis GmbH, Heidelberg, Germany) for 20 min each, followed by a incubation in a mixture of Araldite M (Agar Scientific Ltd., Stansted, England) and propylenoxide (1:1) overnight. Then, the samples were transferred into new Araldite M overnight. Subsequently, the specimens were placed in the silicone form (Agar Scientific Ltd., Stansted, England) and hardened for 48 h at 65°C.

3.9.3 Production of ultrathin-sections

Ultrathin-sections (about 50-80 nm) of the biofilm samples were cut in series with an ultramicrotome (Ultracut E, Reichert, Bensheim, Germany), using a diamond knife (Microstar 45°, Plano GmbH, Wetzlar, Germany). The ultrathin sections were mounted on Pioloform-F coated cooper grids (Plano GmbH, Wetzlar, Germany) and contrasted with uranylacetate and lead citrate.

3.9.4 TEM analysis

The characteristic of the biofilm structure under influence of different mouthrinses was investigated under a magnification varied from 6.800- to 180.000-fold by a TECNAI 12 (FEI, Eindhoven, The Netherlands).

Besides the biofilm, the nanoparticles in the BioRepair solution were also investigated by TEM. The BioRepair solution was centrifuged at 4.000 rpm for 30 min. Then, the supernatant was diluted with distilled water in proportion of 1:100. In the following, 1 µl of the diluted solution was air dried on a Pioloform-F coated copper grid for 24 h and directly evaluated by TEM.

3.10 Statistics

The biofilm scores derived from *Bac*Light™ viability assay and SEM analyses were evaluated using the Kruskal-Wallis test and the Mann-Whitney U test. The Kruskal-Wallis test was to check whether the rinses have any effect at the exposure times of 24 h or 48 h respectively, while the Mann-Whitney U test was adopted for pair-wise comparison with adjustment by Bonferroni correction.

In addition, the Wilcoxon test was used to estimate whether or not a significant difference occurred between the respective biofilm scores of live / dead microorganisms.

In Kruskal-Wallis test and Wilcoxon test, the level of statistical significance was set at $p < 0.05$. As Bonferroni correction was conducted prior to the Mann-Whitney U test, the level of statistical significance was determined at $p < 0.0083$. The statistics were calculated using the SPSS 18 software package (SPSS Inc., Chicago, IL., USA)

4 Results

All subjects completed the study with no side effects. The semi-quantification of bacterial biofilm formation and bacterial vitality using *Bac*Light™ viability assay allows the investigation of the biofilm quantity without or with adoption of mouthrinses. In addition, SEM (surface and fractured specimens), together with TEM, is able to strikingly inllustrate the biofilm formation under influence with different mouthwashes.

4.1 *Bac*Light™ viability assay

4.1.1 Visualization of adherent bacteria in the *in situ* biofilm

*Bac*Light™ viability assay allowed the vital and non-vital bacteria in the *in situ* biofilm to be presented simultaneously and to be exactly distinguishable (Fig. 4). The detected bacteria were mostly cocci. Rods and some epithelial cells were also observed (Fig. 5 and Fig. 6). Rods appeared mostly on control samples and on specimens after application of Elmex Kariesschutz. Additionally, *Bac*Light™ staining visualization revealed the arrangement of microorganisms, such as single bacteria, mono-layered chain, and accumulated colonies, *etc.* (Fig. 7). As shown in figures below (Fig. 4, Fig. 5, Fig. 6 and Fig. 7), the dentinal surface with tubules was clearly visible. Hence, an estimation of whether bacteria adhered within or around the tubules could be reached. An overview of bacterial adherence detected using *Bac*Light™ viability assay was shown in Fig. 8.

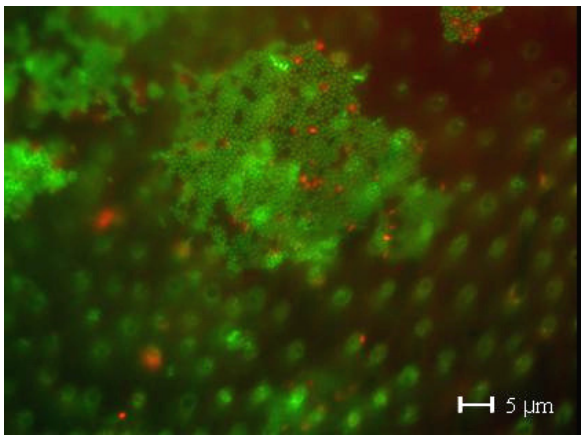


Fig. 4: *Bac*Light™ viability assay: Clear differentiation of vital (live = green) and non-vital (dead = red) bacteria. Control samples exposed at the buccal site in upper jaw for 24 h. Green: live; Red: dead. Original magnification: 1.000-fold.

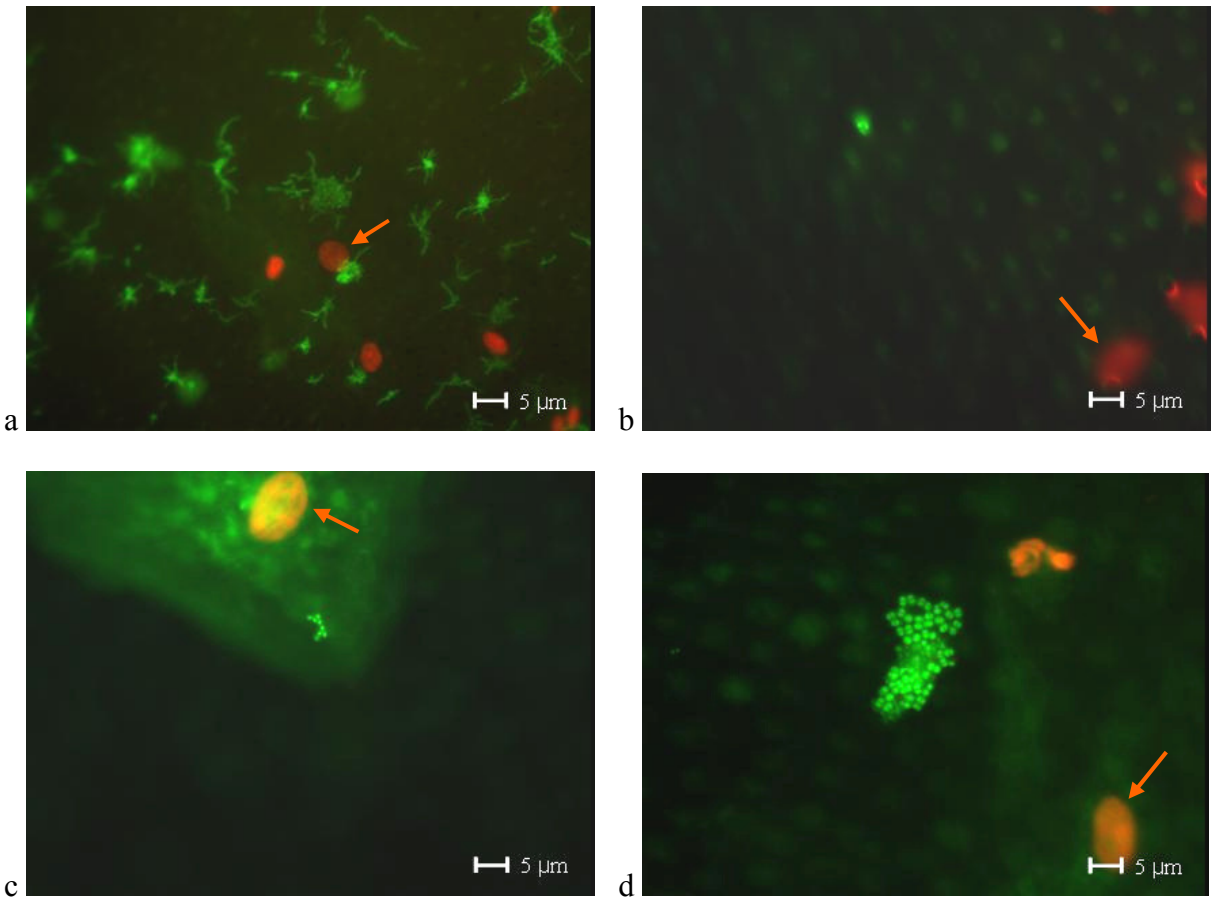


Fig. 5: *Bac*Light™ viability assay: Epithelial cells detected in the 24-h biofilm. Epithelial cells were not taken into account with respect to the quantification of adherent bacteria. The arrows show the nucleus of the epithelial cell. (a) Control group; (b) BioRepair group; (c) Elmex Kariesschutz group; (d) CHX group. Green: live; Red: dead. Original magnification: 1.000-fold.

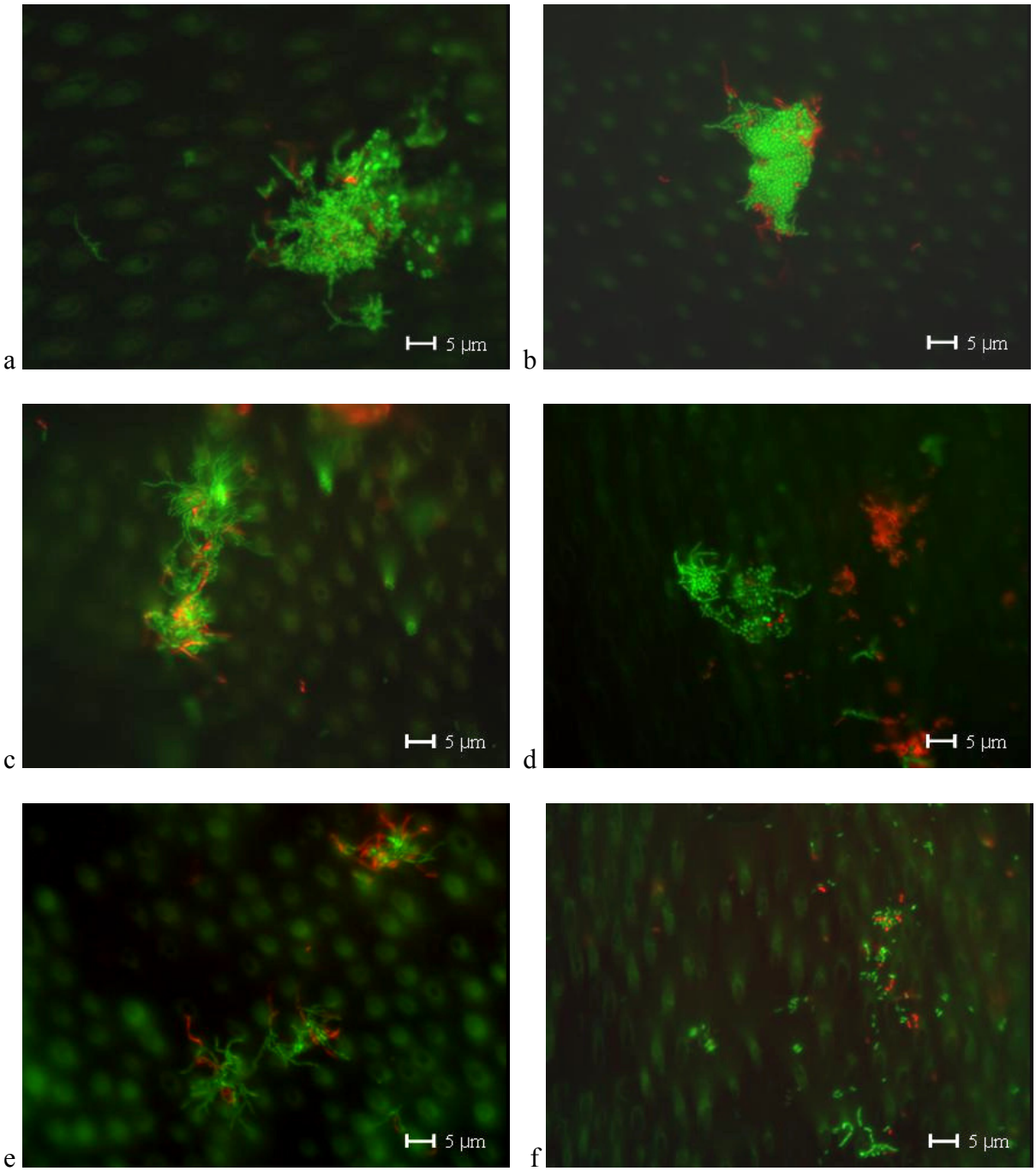


Fig. 6: *Bac*Light ™ viability assay: cocci as well as rods in the 48-h *in situ* biofilm without rinsing (a, c and e) and after treatment with Elmex Kariesschutz (b, d and f). Green: live; Red: dead. Original magnification: 1.000-fold.

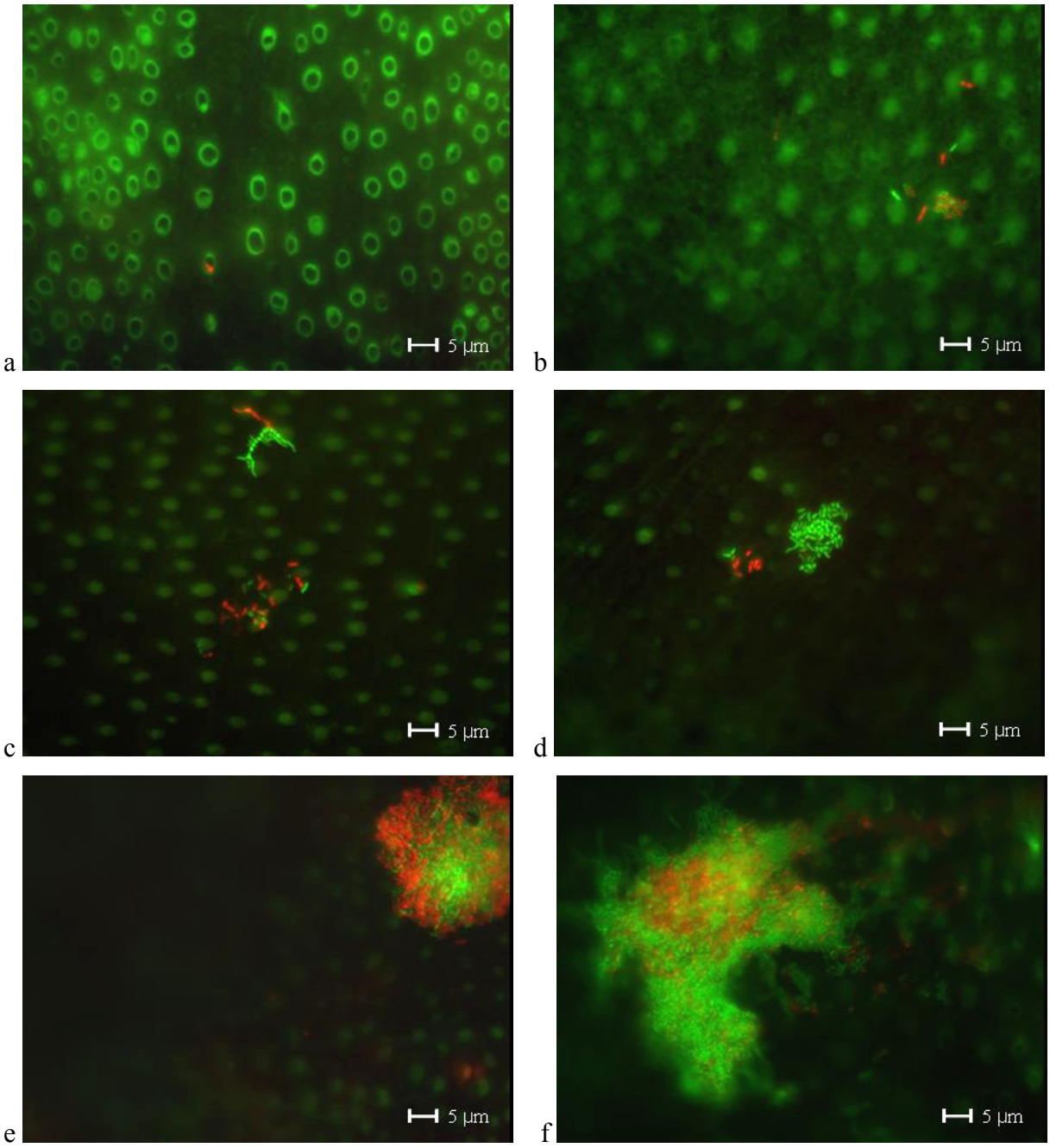


Fig. 7: *Bac*Light™ viability assay: Visualization of different arrangements of bacteria in the 48-h biofilm. Single bacteria, mono-layered chains or bacterial colonies were observed. (a) CHX group; (b and d) BioRepair group; (c and e) Elmex Kariesschutz group; (f) Control group. Green: live; Red: dead. Original magnification: 1.000-fold.

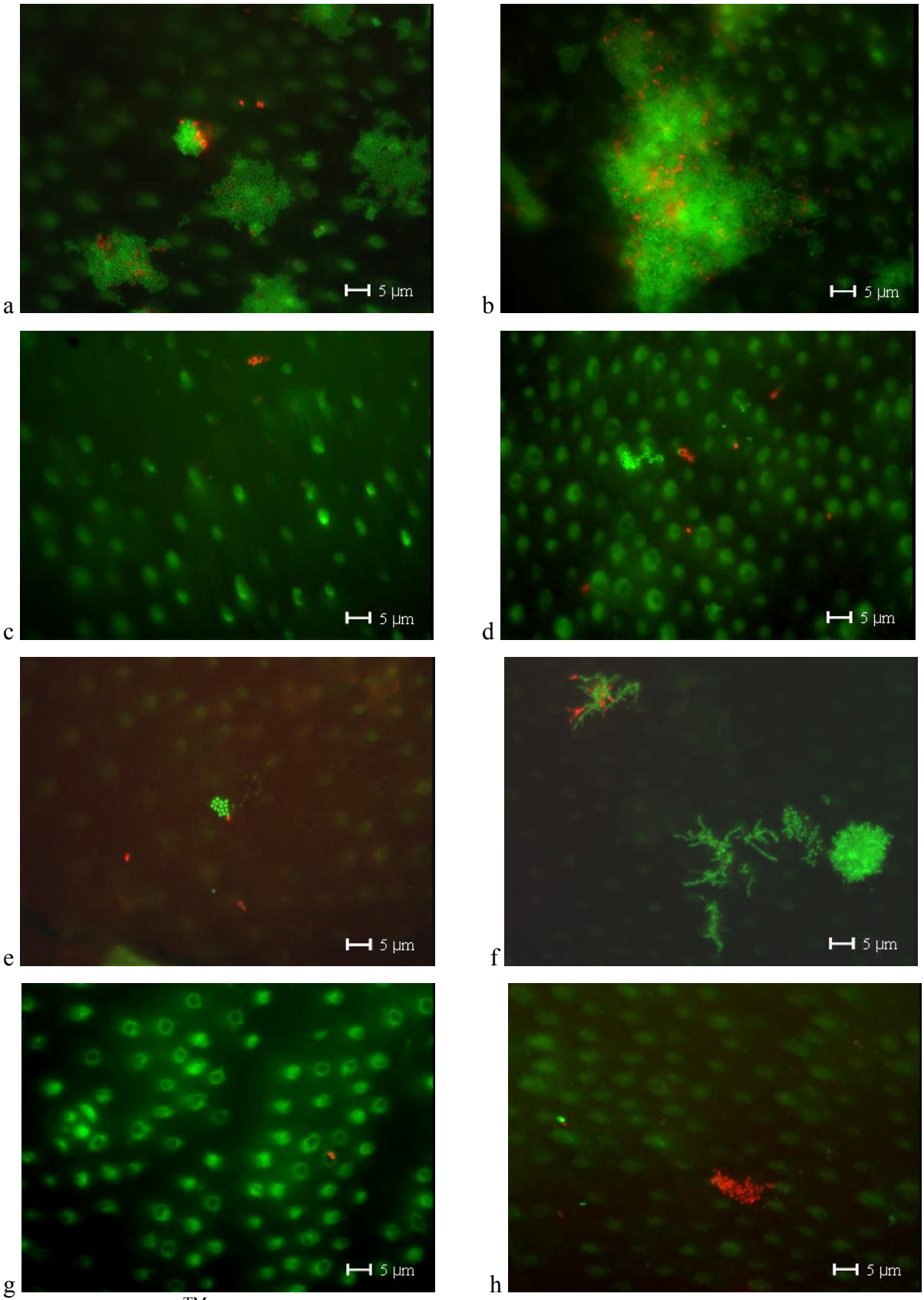


Fig. 8: *Bac*Light ™ viability assay: an overview of biofilm formation without rinsing and after rinsing with different mouthrinses in each experimental period. (a and b) Control group; (c and d) BioRepair group; (e and f) Elmex Kariesschutz group; (g and h) CHX group. (a, c, e and g) 24-h biofilm; (b, d, f and h) 48-h biofilm. Green: live; Red: dead. Original magnification: 1.000-fold.

4.1.2 Semi-quantification of adherent bacteria in the *in situ* biofilm

4.1.2.1 Total bacteria

After each exposure, the amount of microorganisms detected by *Bac*Light™ viability assay was evaluated according to the scores defined in Table 2. Medians and range of the biofilm mass from all groups are summarized in Table 3, along with the results of the statistical analyses. Kruskal-Wallis test indicated that mouthwashes had significantly different effects on bacterial growth, regardless of biofilm formation time. An inter-subject variability was also shown.

All three tested mouthwashes inhibited the bacterial biofilm formation as compared with the unrinsed group. Statistical test results also revealed significantly reduced bacterial surface coverage after treatments with BioRepair and CHX in each experimental trial. However, Elmex Kariesschutz showed only a tendency in reducing biofilm formation during the 24-h experiment (Fig. 9 and Fig. 10). Single bacteria rarely adhered to the CHX-treated dentin, whereas an inhibition of bacterial growth to a lesser extent was observed on samples following rinsing with Elmex Kariesschutz.

Bacterial biofilms grew with increasing oral exposure time. The control group had the largest increase of median scores from 2 after 24-h exposure to 4 after 48-h exposure at the end of the intraoral exposure. Despite the slight increase at the endpoint (48 h), the bacterial amount on the Elmex Kariesschutz- and CHX-treated samples remained at the same level. In the BioRepair group, it was observed that the 48-h biofilm stayed stable without an apparent bacterial progression, as compared with 24-h biofilm (Fig. 9 and Fig. 10).

Table 3 Biofilm formation without or following rinsing with different mouthrinses evaluated by *Bac*Light™ viability assay. Exposition of the dentinal specimens at the buccal sites of the upper 1st and 2nd molars for 24 h and 48 h. Median scores of biofilm formation according to **Table 2**. (Median (Min. - Max.)). A significant difference between treatments was shown in each experimental period (Kruskal-Wallis test, $p < 0.001$). Within each experimental period, the use of different superscripts indicated significant difference between groups (Mann-Whitney U test, $p < 0.0083$).

Groups / Exposure time	Control	Elmex Kariesschutz	BioRepair	CHX
24 h	2 (1 - 4) [a]	1.75 (0 - 4) [a]	1 (0 - 2.5) [b]	1 (1 - 2) [b]
48 h	4 (1 - 6) [a]	2 (1 - 6) [b]	1 (0 - 4) [c]	1 (1 - 3) [c]

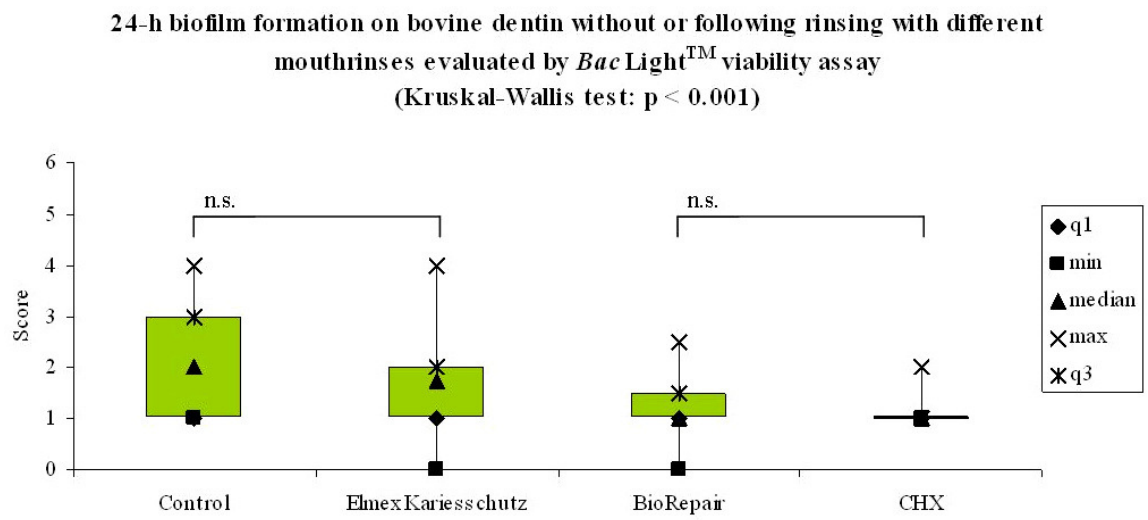


Fig. 9: *Bac*Light™ viability assay for detection of total adherent bacteria without rinsing and after rinsing with different mouthrinses. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentinal samples at buccal sites of the upper 1st and 2nd molars for 24 h. A significant difference was shown between treatments (Kruskal-Wallis test, $p < 0.001$). Compared to controls, bacterial growth on specimens after rinsing with BioRepair and CHX was significantly reduced in 24-h experimental trial (Mann-Whitney U test, $p < 0.0083$). n.s.: not significant.

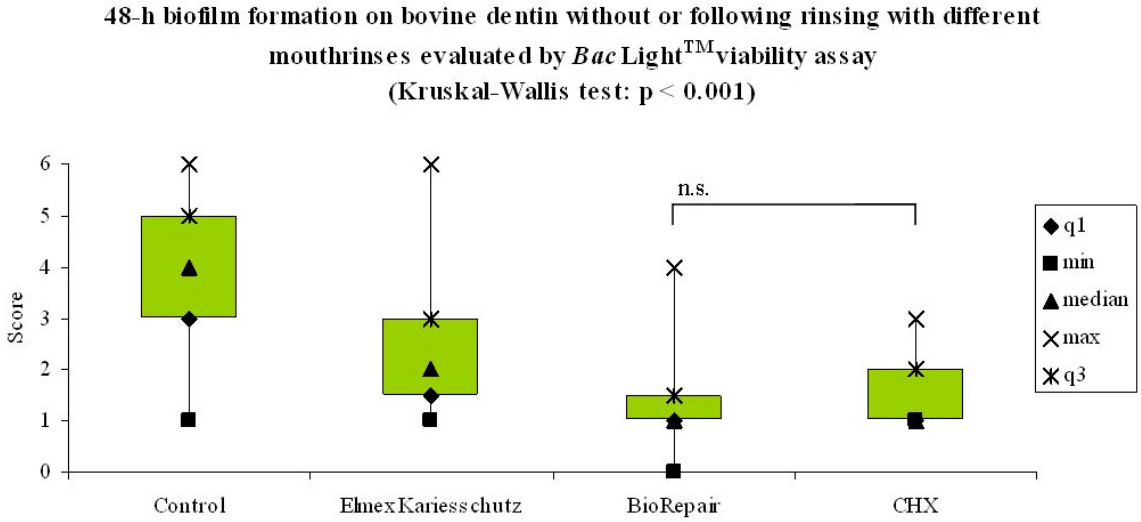


Fig. 10: *Bac*Light™ viability assay for detection of total adherent bacteria without rinsing and after rinsing with different mouthrinses. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentinal samples at buccal sites of the upper 1st and 2nd molars for 48 h. A significant difference was shown between treatments (Kruskal-Wallis test, $p < 0.001$). Compared to controls, bacterial growth on specimens after rinsing with all the three mouthrinses was significantly reduced in 48-h experimental trial (Mann-Whitney U test, $p < 0.0083$). n.s.: not significant.

4.1.2.2 The live / dead bacterial colonization

With *Bac*Light ™ viability assay, it is possible to respectively assess live and dead bacterial adherence in the intra-orally formed biofilm with the purpose of estimating the antibacterial property of the tested products. The evaluation of live and dead bacteria detected is given in Table 4. After each experimental period (24 h and 48 h), the amounts of live and dead cells were similar, when the samples had been rinsed with Elmex Kariesschutz. As compared with the control samples, the Elmex Kariesschutz-treated samples also showed an interesting result of a similar amount of adherent dead microorganisms, whereas an obvious decline in the volume of vital bacteria could be detected (Fig. 11 and Fig. 12). Irrespective of the exposure time, a significant difference with respect to the bacterial vitality was found in the other three groups (Wilcoxon test: $p < 0.05$). More live cells than dead bacteria were detected in the control group. Following rinsing with CHX, almost no bacteria, neither vital cells nor avital cells, were detectable in the CHX-treated biofilms after the first 24-h exposure time. Also, few dead bacteria existed in the 24-h biofilms following rinsing with BioRepair, whereas single live cells were detected. During the 48-h biofilm formation time, a similar behaivor against bacteria was revealed in the BioRepair group and in the CHX group, with establishment of similar vital / avital bacterial amounts.

The bacterial biofilm grew with increasing oral exposure time in all groups (Fig. 11 and Fig. 12). Despite of the slightly elevated bacterial amount in both, vital and avital bacteria, in the 48-h biofilms after rinsing with Elmex Kariesshutz, the proportion (live cells : dead cells) was similar to that of the 24-h biofilm. The vital bacteria on the samples after CHX application increased faster than dead bacteria in the 48-h intraoral exposure time. When BioRepair is used, the stagnation in the vital bacterial growth and a subtle increment in the adherence of avital bacteria could be observed in the 48-h experimental trial (Fig. 11 and Fig. 12).

Table 4 Live / dead bacteria colonization evaluated by *Bac*Light ™ viability assay.Exposition of the dentinal specimens at the buccal sites of the upper 1st and 2nd molars for 24 h and 48 h. Median scores of biofilm formation according to **Table 2**. (Median (Min. - Max.)). For each data pair (*e.g.*, live *vs*. dead bacteria, control, 24-h oral exposure time), the use of different superscripts indicated significant difference (Wilcoxon test, $p < 0.05$).

Groups / Exposure time	Control		Elmex Kariesschutz		BioRepair		CHX	
	live	dead	live	dead	live	dead	live	dead
24 h	2 (0 - 5) [a]	1 (0 - 3) [b]	1.5 (0 - 4) [a]	1 (0 - 3) [a]	0 (0 - 2) [a]	1 (0 - 2) [b]	0 (0 - 2) [a]	1 (0 – 2) [b]
48 h	4 (0 - 6) [a]	1 (1 - 4) [b]	2 (0 - 4) [a]	1.5 (0 - 6) [a]	1 (0 - 3) [a]	1 (0 - 2) [b]	0 (0 - 3) [a]	1 (0 - 3) [b]

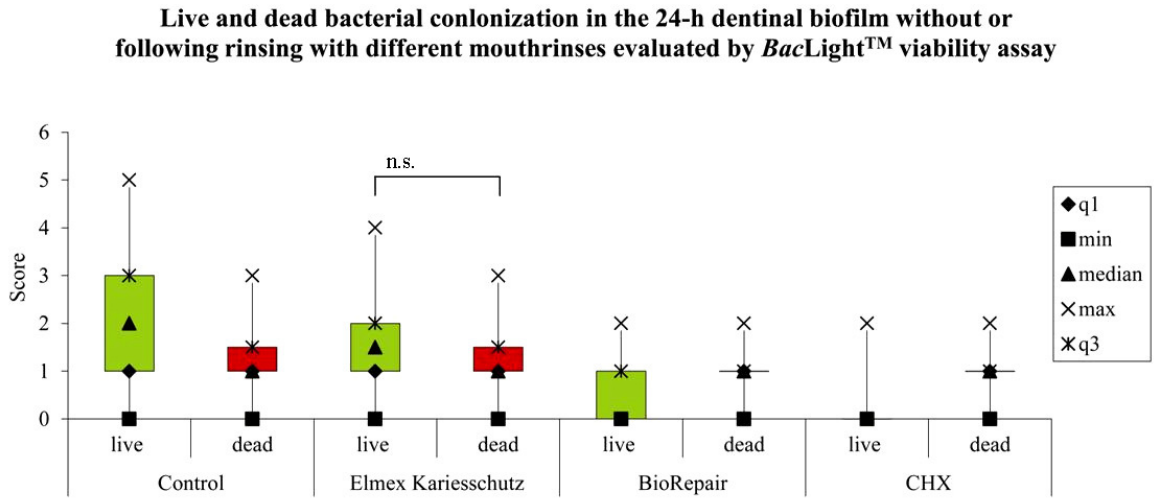


Fig. 11: *Bac*Light™ viability assay for determination of live and dead bacteria in 24-h biofilm without rinsing and after rinsing with different mouthrinses. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentinal samples at buccal sites of the upper 1st and 2nd molars for 24 h. A significant difference between the amounts of live and dead bacteria was found in the controls and after treatment with BioRepair and CHX (Wilcoxon test, $p < 0.05$). n.s.: not significant.

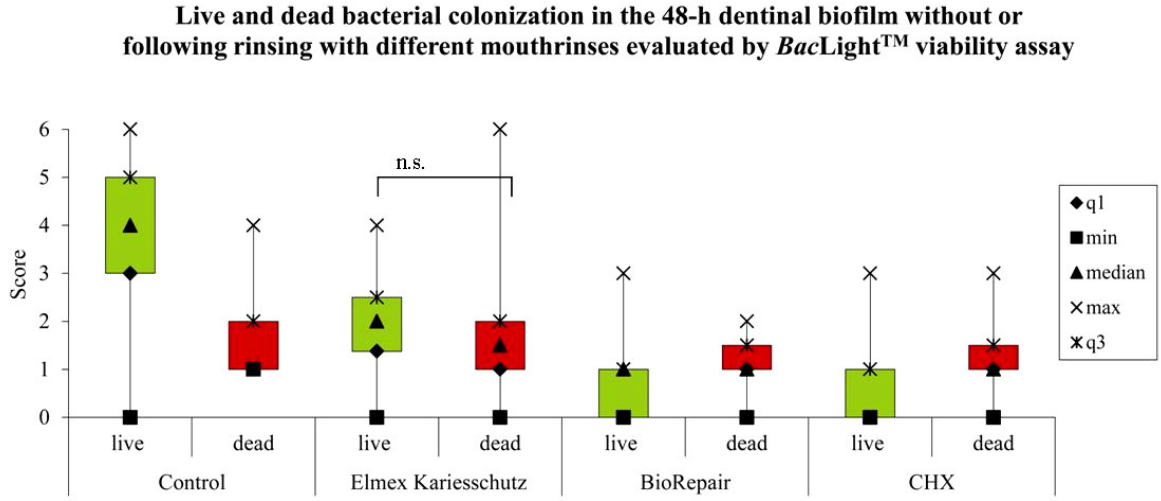


Fig. 12: *Bac*Light™ viability assay for determination of live and dead bacteria in 48-h biofilm without rinsing and after rinsing with different mouthrinses. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentin samples at buccal sites of the upper 1st and 2nd molars for 48 h. A significant difference between the amounts of live and dead bacteria was found in the controls and after treatment with BioRepair and CHX (Wilcoxon test, $p < 0.05$). n.s.: not significant.

4.2 SEM

4.2.1 Morphological appearance of the *in situ* biofilm

SEM confirmed the microbial diversity in the *in situ* biofilm (Fig. 13), which was examined by *Bac*Light™ viability assay (Fig. 6). Bacteria appeared in coccoid or rod-shaped configurations. Fig. 14 shows that a substantial number of bacteria were arranged on the intertubular dentin as individual bacteria, small mono-layered chains, or aggregates. In addition, morphological alterations in biofilm caused by different rinses over periods of both, 24 h and 48 h, were strikingly demonstrated. In spite of the pronounced inter-individual variations observed in morphogenesis of biofilm, a preferential bacterial colonization in and around the tubules was revealed as a characteristic pattern of dentinal biofilm formation. An overview of such comparison is provided in Fig. 14.

In general, biofilm develops with the lapse of time, which was supported by the progressive spread of bacterial surface coverage on the one hand and the more compact pellicle arrangement on the other hand. For example, the undisturbed 24-h *in situ* biofilm was characterized by an irregular surface contour with multi-layered bacterial colonies or even a microbial confluent, covering most of the surface area (Fig. 14 and Fig. 15). At the end of the 48-h period, a multi-layered confluent could be observed on the entire surface with an establishment of bacteria in the form of rods and cocci, which were embedded in a network comprising of heterogeneous pellicle structures appearing as globules, granules and fibrils, too (Fig. 14 and Fig. 15). In such a 48-h biofilm, the microbial interaction was evident. Bacteria interacted with each other or with pellicle structures through fine fibrils, resulting in bacterial co-aggregation and bacterial adhesion. Particles with diameters of 50-100 nm, presumably organic material, were observed at the bacterial cell wall. In addition, cell division was apparently detected. The biofilm formed on unrinsed control samples for a period of both, 24 h and 48 h, manifested itself as a microbial community embedded in a polysaccharide matrix.

Compared with the controls, all the rinsing groups showed a reduction in bacterial adherence to different levels. After 24-h exposure in the oral cavity, it was observed that the bacteria on the surface were almost eliminated in series of micrographs from CHX groups (Fig. 16). After another 24-h oral exposure period, a thicker layer of deposits with globular or amorphous aggregates along with occasional occurrence of bacteria in tubules or at the tubular orifice was established in the 48-h specimens. These results raised the question of whether the

bacteria had already settled down in the tubules to some extent. As such, the investigation on the fractured surface was necessary (see in chapter 4.2.3). As shown in Fig. 16, it can be observed that a reticular pellicle occluded partially the dentinal tubules after CHX rinsings within 48-h oral exposure.

After mouth rinsing with BioRepair twice in the first 24 h, bacteria were present in arrangements of mono-layer chains or tiny aggregates (Fig. 17). The pellicle formed on the BioRepair-treated samples represented a coating with dispersed spherical or amorphous aggregates revealing a diameter of 200 nm. At the end of the experiment (48 h), the globular / amorphous particles observed previously were more evident in a random distribution on the surface. The pellicle with filamentous appearance was clearly shown. Occlusion of tubule orifices to various extents could be accomplished by different forms, such as 'pellicle-bridges', penetration of pellicle materials or even microbial invasion (Fig. 17). At some areas, it is interesting to find a "shedding" pellicle layer surrounding mono-layered bacterial colonies (Fig. 18).

Biofilms formed on the samples following rinsing with Elmex Kariesschutz demonstrated a more heterogeneous complexity of pellicle structure as compared with the other groups. In the first 24-h experimental period, filamentous pellicle and globular pellicle in a dense arrangement were observed (Fig. 19). Also, pellicle represented itself as a network comprising of fibrils connecting the globular agglomerates. In higher magnifications, teeny round-shaped globules with a diameter of 50 nm were evident. After an exposure time of 48 h, a thicker pellicle layer masked the entire dentinal surface completely so that tubules and scratches were masked. Globular particles (150 nm) were distributed randomly on the surface. In spite of a suppressing bacterial growth shown in the Elmex Kariesschutz group, bacterial colonies were detectable after 24 h, and even noticeable after 48 h. The observed bacteria were arranged in insular agglomerates and in small multi-layered colonies (Fig. 19).

In both, BioRepair and Elmex Kariesschutz groups, microbial morphological changes were detected (Fig. 26). With respect to rinsing with BioRepair, it is interesting to observe that bacteria lost their distinct cell wall structure with the cell outlines unrecognizable. In the other group, bacteria attached to the Elmex Kariesschutz-treated samples represented a comparatively smoother cell wall. The specific cell features were not evident.

SEM micrographs shed light on the changes after rinsing with different mouthrinses, however, a further precise statistical analysis was necessary (see in chapter 4.2.2).

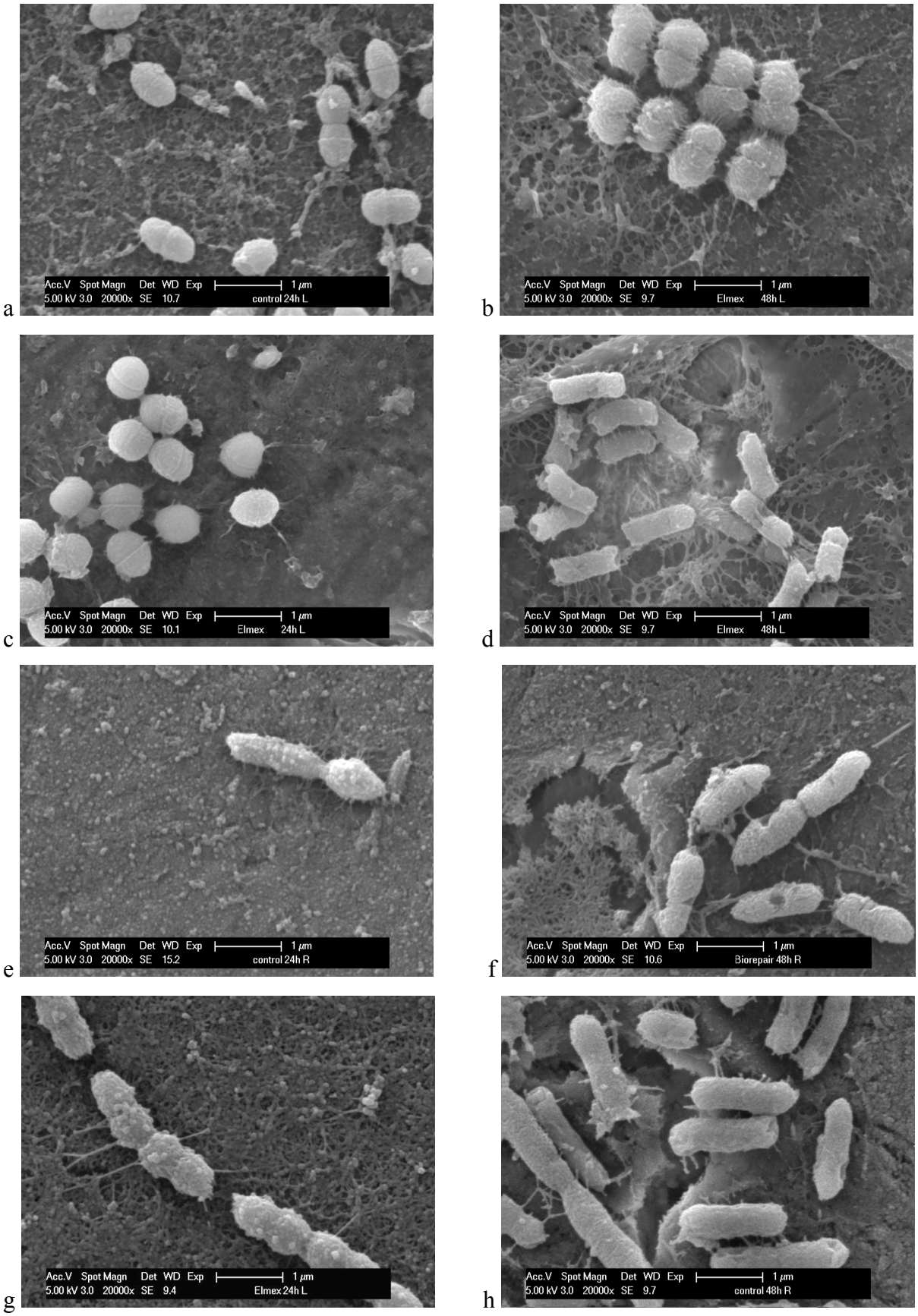


Fig. 13: SEM micrographs: cocci and rods adherent to the *in situ* pellicle. (a, c, e and g) 24-h biofilm; (b, d, f and h) 48-h biofilm; (a, e and h) Control group; (f) BioRepair group; (b, c, d and g) Elmex Kariesschutz group. Original magnification: 20.000-fold.

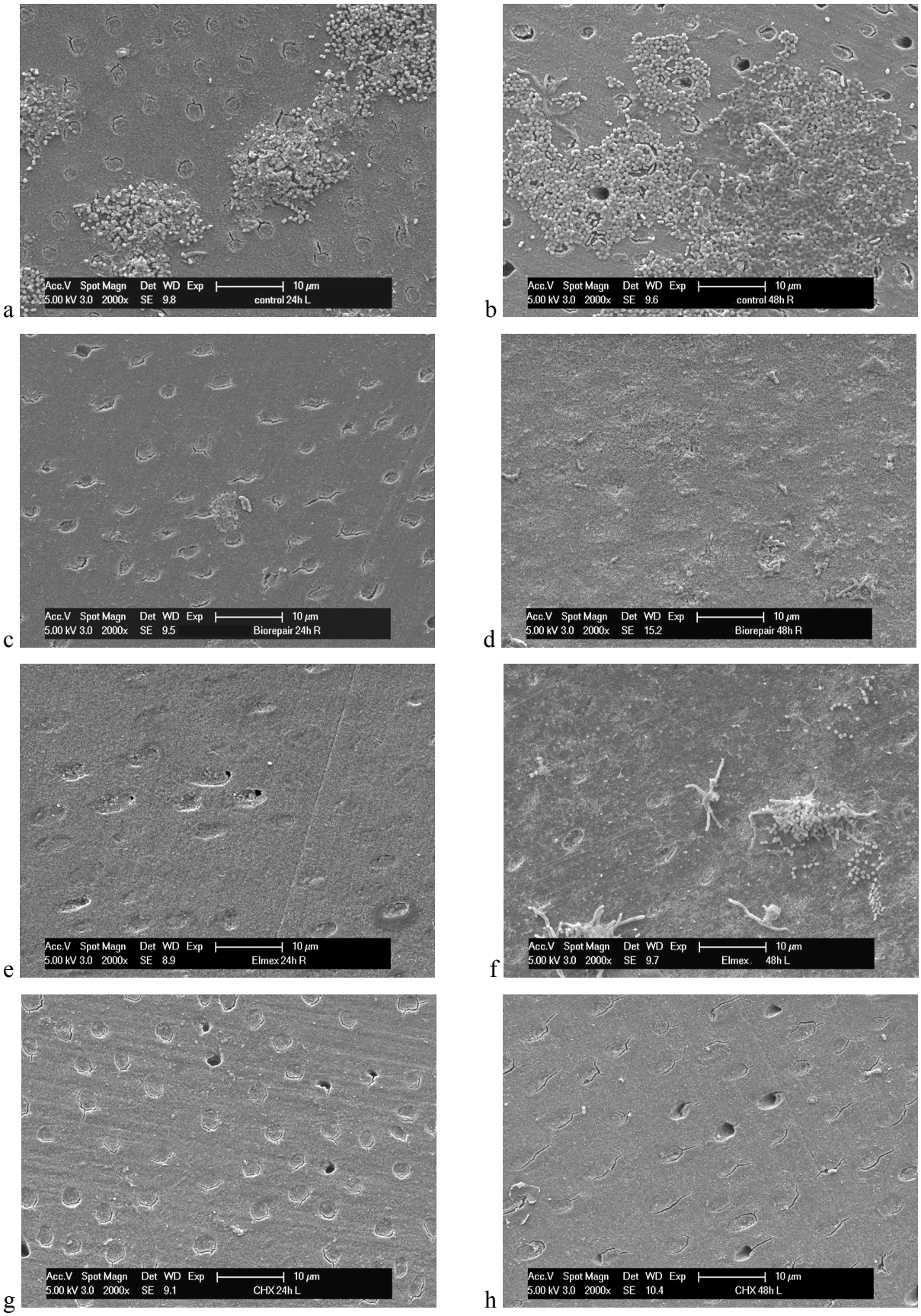


Fig. 14: SEM micrographs: an overview of biofilm formation following application of the different rinses after 24- and 48-h of oral exposures. (a, c, e, g) 24-h biofilm; (b, d, f and h) 48-h biofilm; (a and b) Control group; (c and d) BioRepair group; (e and f) Elmex Kariesschutz group; (g and h) CHX gorup. Original magnification: 2.000-fold.

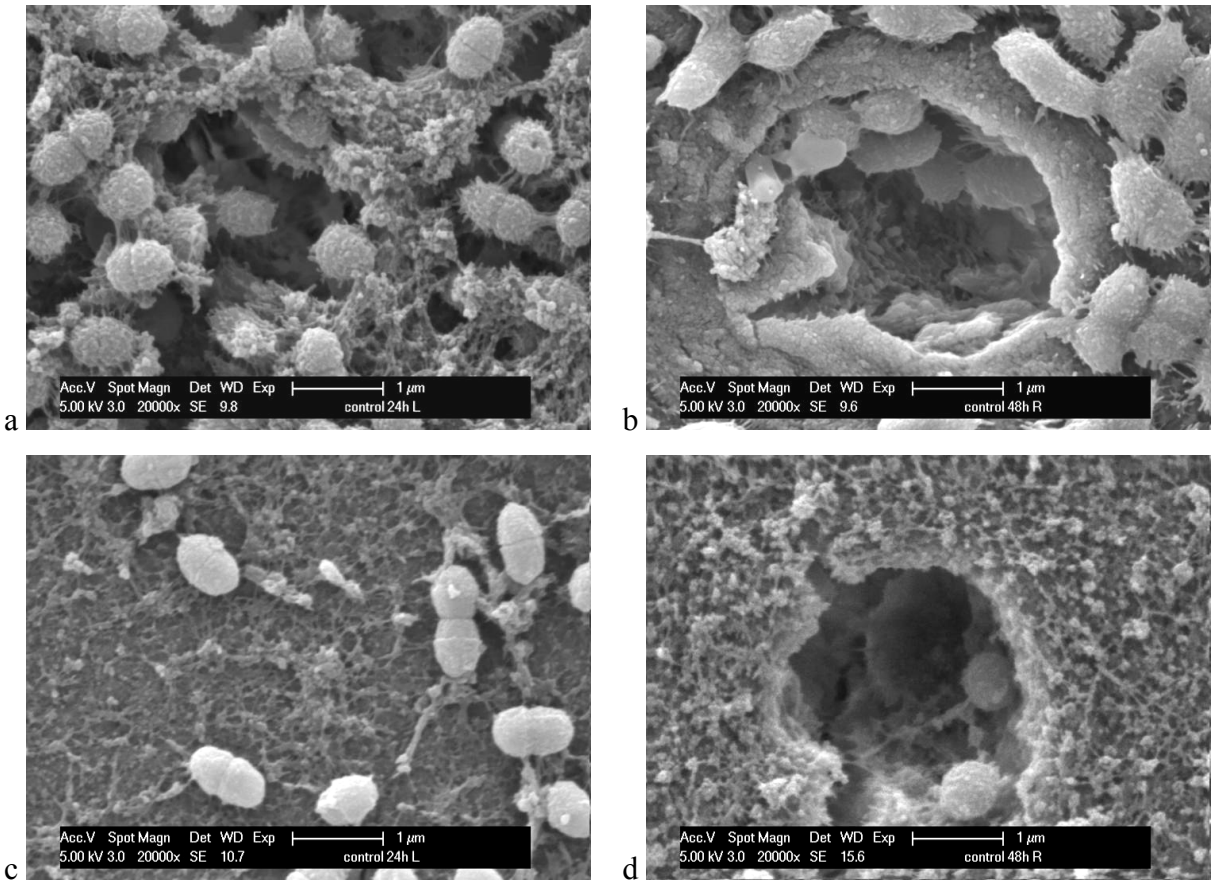


Fig. 15: SEM micrographs: Biofilm on unrinsed samples (Control group). Bacteria are adherent to the pellicle layer and embedded in the matrix as well (a and c). Bacterial adhesion to the tubule wall is evident (b and d). (a and c) 24-h biofilm; (b and d) 48-h biofilm. Original magnification: 20.000-fold.

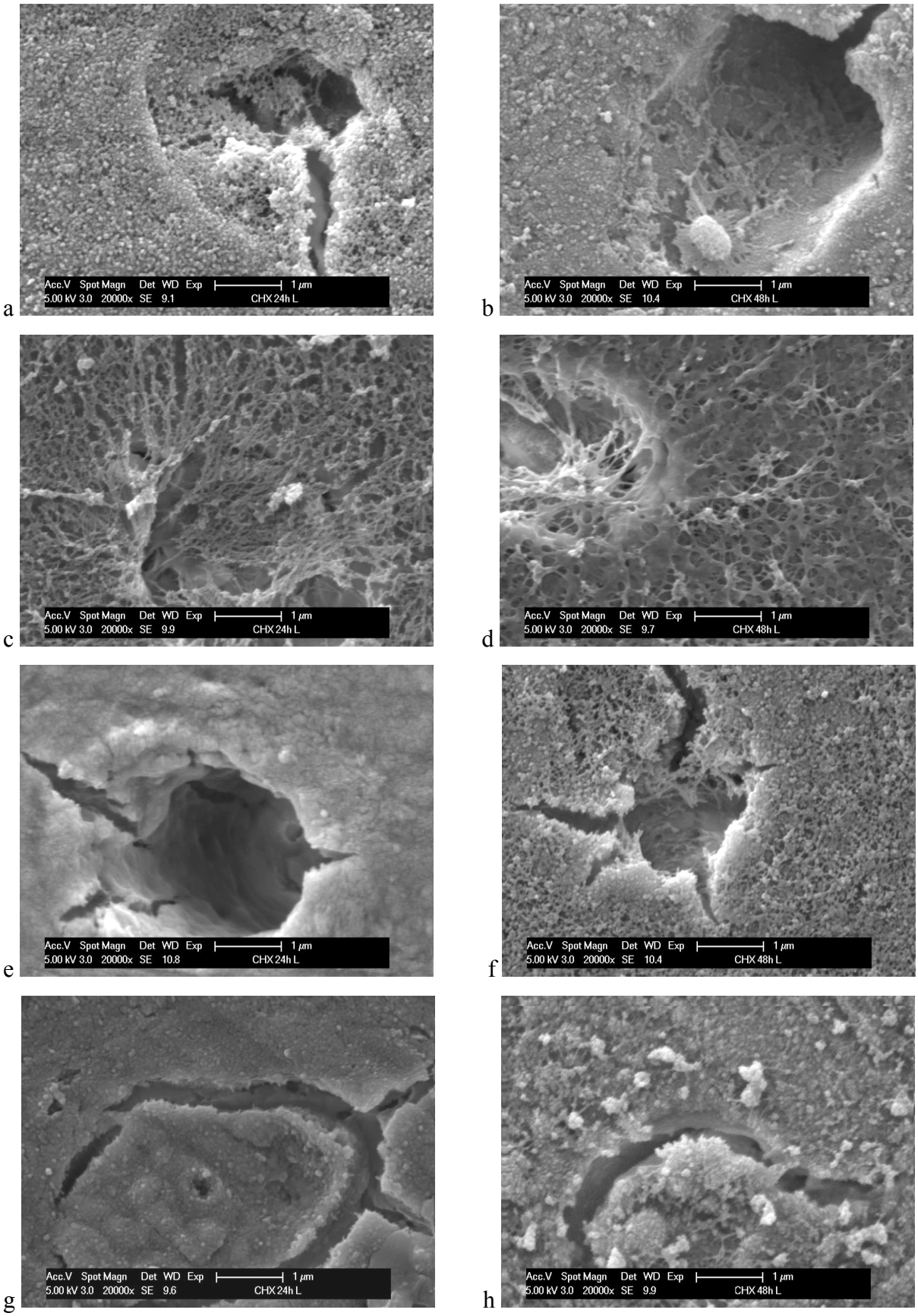


Fig. 16: SEM micrographs: Biofilm formation on samples following CHX rinsing. (a, c, e and g) 24-h biofilm; (b, d, f and h) 48-h biofilm. Original magnification: 20.000-fold.

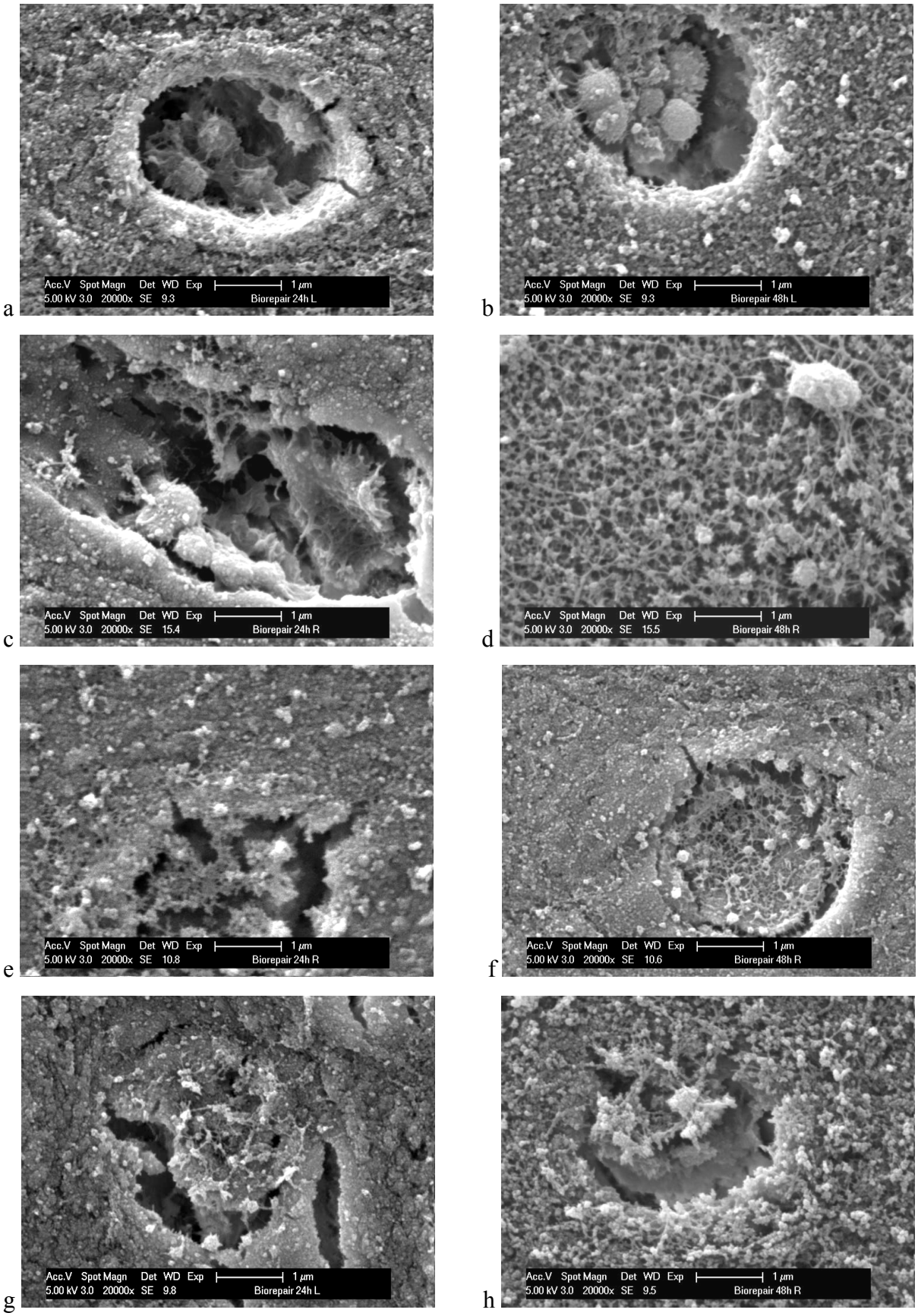


Fig. 17: SEM micrographs: Biofilm on samples following BioRepair rinsing. (a, c, e and g) 24-h biofilm; (b, d, f and h) 48-h biofilm. Original magnification: 20.000-fold.

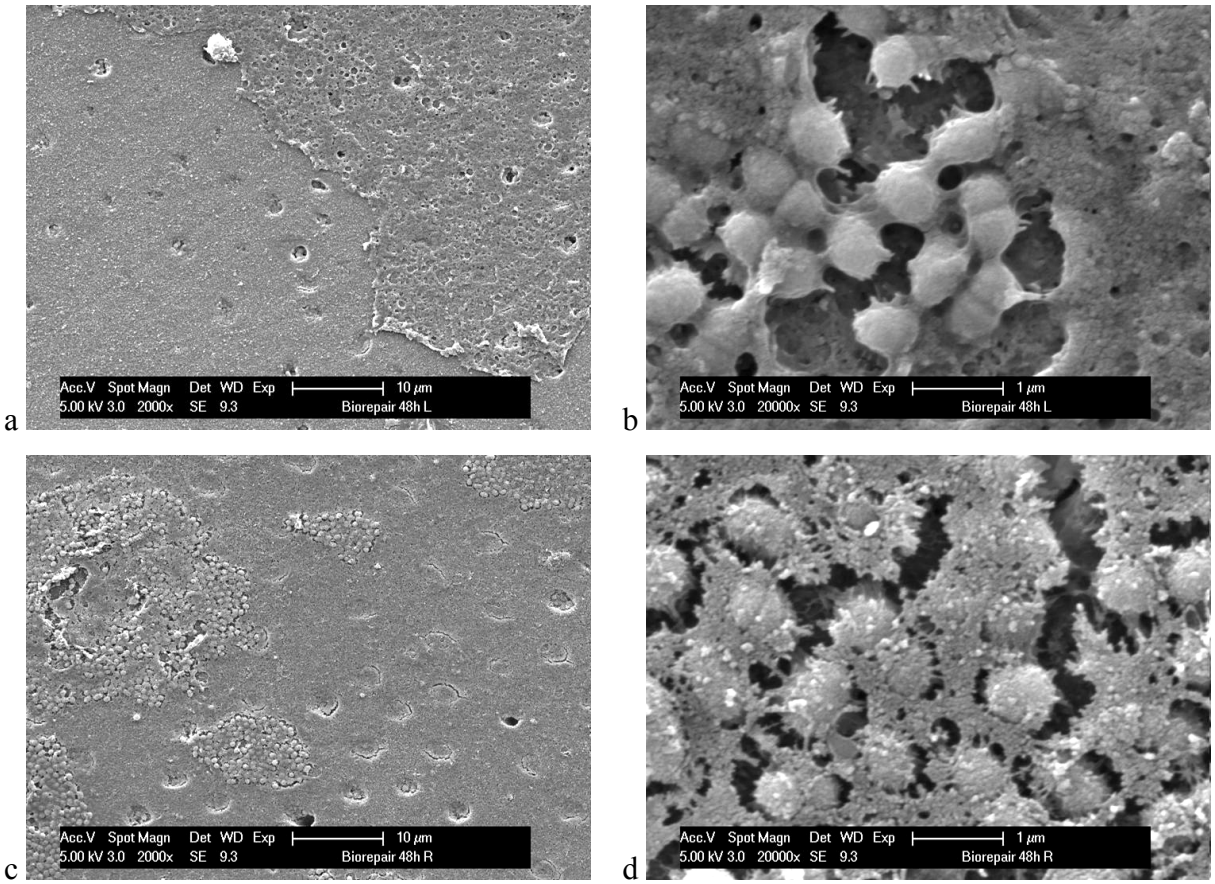


Fig. 18: SEM micrographs: a layer of pellicle with surrounding mono-layered bacterial colonies on the BioRepair-treated samples in the 48-h experimental trial. Original magnification: (a and c) 2.000-fold; (b and d) 20.000-fold.

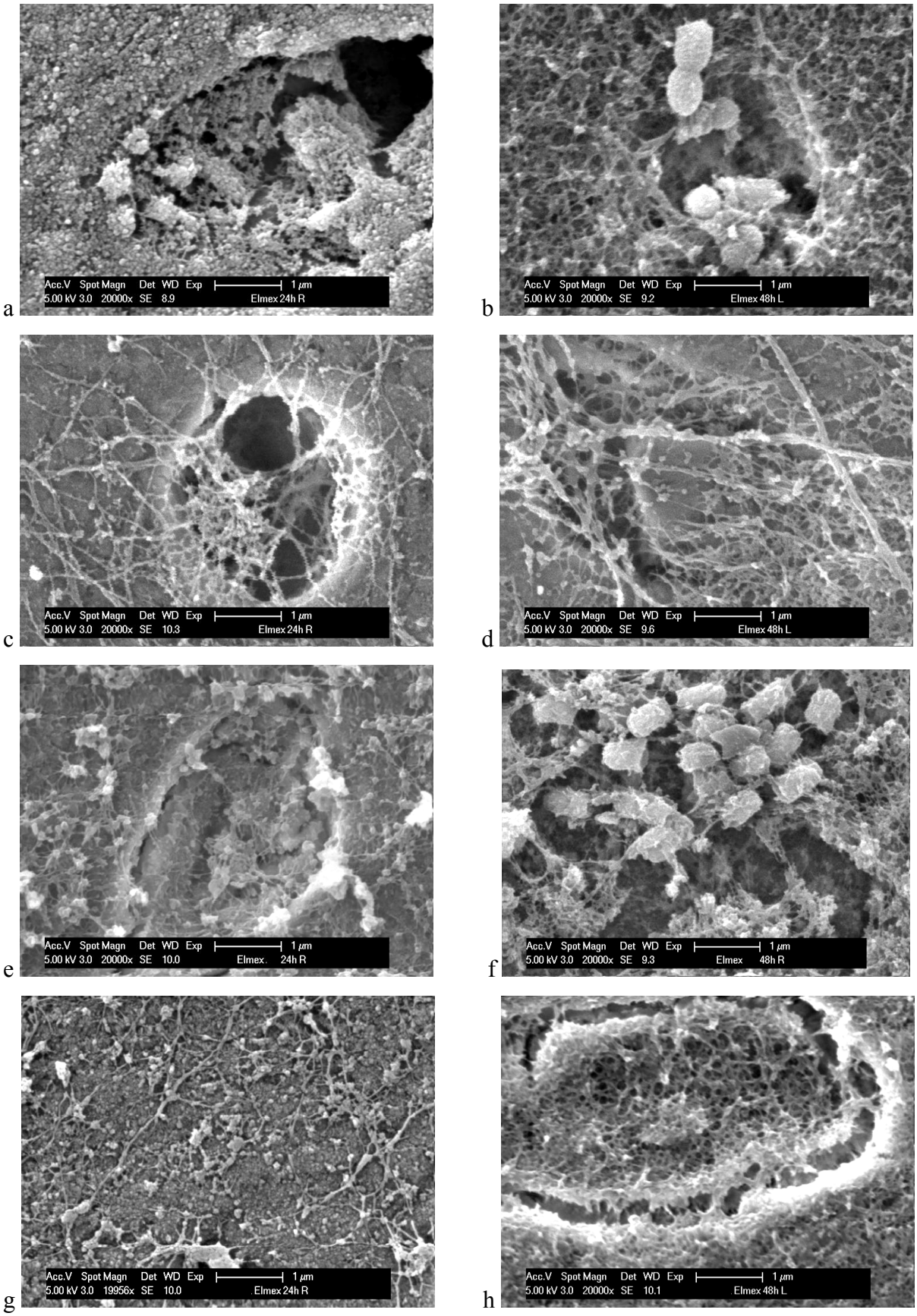


Fig. 19: SEM micrographs: Biofilm formation on samples following rinsing with Elmex Kariesschutz. (a, c, e and g) 24-h biofilm; (b, d, f and h) 48-h biofilm. Original magnification: 20.000-fold.

4.2.2 Semi-quantification of total bacteria in the *in situ* biofilm

After each oral exposure time, 2 dentinal samples (per subject and mouthwash) that were mounted at the buccal sites of 1st and 2nd upper molars, were examined by SEM to asess the amount of adherent bacteria. Kruskal-Wallis test revealed a significant difference among treaments for the experimental periods of 24 h and 48 h. However, a statistical difference among subjects was observed during the 48-h rather than the 24-h biofilm formation time (Fig. 20 and Fig. 21). Thus, an inter-subject variability was suspected.

SEM demonstrated a significantly inhibited bacterial biofilm formation derived from application of all the three tested mouthrinses (Table 5, Fig. 20 and Fig. 21). Single bacteria were rarely observed after 24 h following rinsing with CHX, whereas BioRepair and Elmex Kariesschutz hindered the bacterial growth to a lesser degree. With increasing biofilm formation time, a slight increase in the bacterial adherence could be detected on the CHX- and Elmex Kariesschutz-treated samples. In contrast, the 48-h biofilm in the BioRepair group remained constant, as compared to the 24-h biofilm.

In general, the results derived from SEM were in line with that revealed by *Bac*Light™ viability assay.

Table 5 Biofilm formation without or following rinsing with different mouthrinses evaluated by SEM. Exposition of the dentinal specimens at the buccal sites of the upper 1st and 2nd molars for 24 h and 48 h. Median scores of biofilm formation according to **Table 2**. (Median (Min. – Max.)). A significant difference between treatments was shown in each experimental period (Kruskal-Wallis test, $p < 0.001$). Within each experimental period, the use of different superscripts indicated significant difference between groups (Mann-Whitney U test, $p < 0.0083$).

Groups / Exposure time	Control	Elmex Kariesschutz	BioRepair	CHX
24 h	3 (1 - 6) [a]	1 (0 - 4) [b]	1 (0 - 5) [b]	0 (0 - 1) [c]
48 h	5 (2 - 6) [a]	2.25 (0 - 6) [b]	1 (0 - 5) [b, c]	0 (0 - 6) [c]

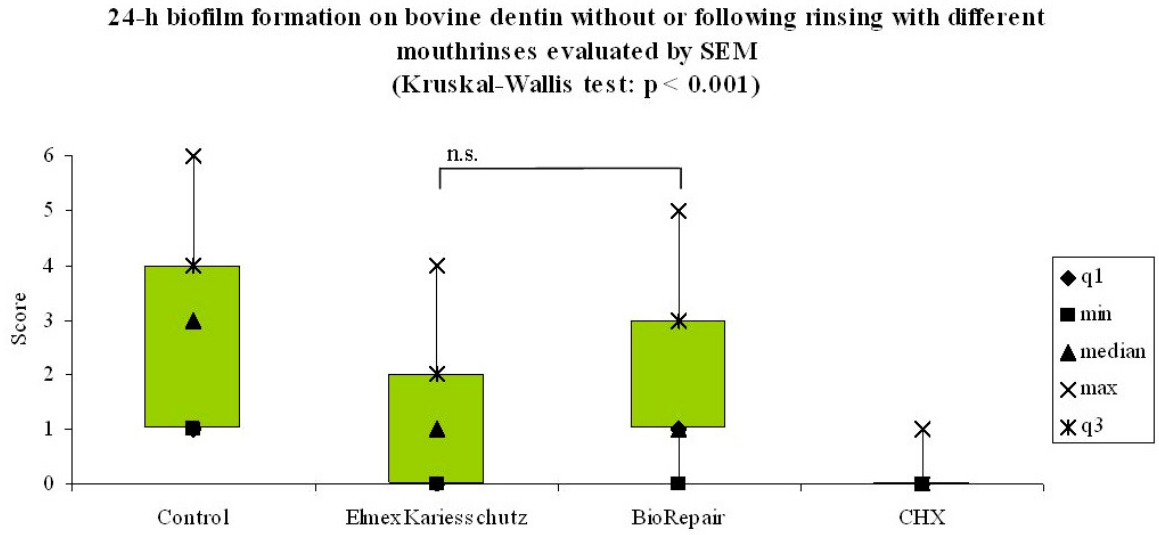


Fig. 20: Total adherent bacteria without rinsing and after rinsing with different mouthrinses evaluated by SEM. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentinal samples at buccal sites of the upper 1st and 2nd molars for 24 h. A significant difference was shown between treatments (Kruskal-Wallis test, $p < 0.001$). Compared to controls, all the three mouthrinses reduced significantly the bacterial growth after 24-h oral exposure time (Mann-Whitney U test, $p < 0.0083$). n.s.: not significant.

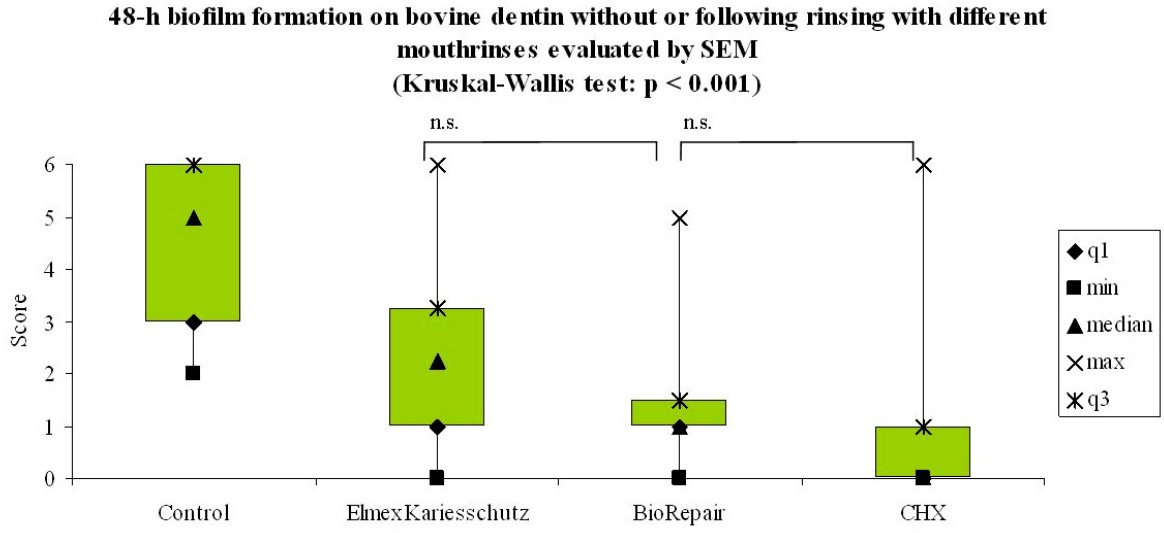


Fig. 21: Total adherent bacteria without rinsing and after rinsing with different mouthrinses evaluated by SEM. Median values of biofilm scores according to **Table 2**, (box-and-whisker plot, median). Exposition of dentinal samples at buccal sites of the upper 1st and 2nd molars for 48 h. A significant difference was shown between treatments (Kruskal-Wallis test, $p < 0.001$). Compared to controls, bacterial growth on specimens following rinsing with all the three mouthrinses was significantly reduced after 48-h oral exposure time (Mann-Whitney U test, $p < 0.0083$). n.s.: not significant.

4.2.3 SEM analysis of fractured specimens

As already stated, the microorganisms can settle down in the tubular lumen to some depth as well. Therefore, an investigation on the fractured specimens' tubules was necessary. As biofilm progressed with time, the samples worn for a period over 24 h and rinsed twice a day were firstly fractured to get a full range of picture of the distribution of bacteria.

On the fractured surface, longitudinally sectioned tubules and intertubular dentin were visible. In agreement with the SEM analysis of the surface, almost all of the tubules on the non-rinsed samples were covered with a biofilm, facilitating the formation of pellicle / microbial tubule infiltration. Single or a couple of cocci were found near the dentinal tubule orifices in almost all samples (Fig. 22).

A few tubules on the CHX-treated samples were open while most of the tubular orifices on the BioRepair- and Elmex Kariesschutz-treated specimens were closed. In the CHX group, it was observed that the agglomerate was located in the tubular lumen with an open tubular orifice. In one instance, following rinse with BioRepair, a pellicle-like material sealed the tubular orifices with a clear lumen. In another instance, the tubules were occluded partially with an infiltrate consisting of aggregates and microorganisms at a depth of approximately 10 µm (Fig. 22). Moreover, some tubules were occluded completely. When treated with Elmex Kariesschutz, the tubules were infiltrated by a filamentous pellicle, distinctly different from the biofilms formed in other groups (Fig. 22).

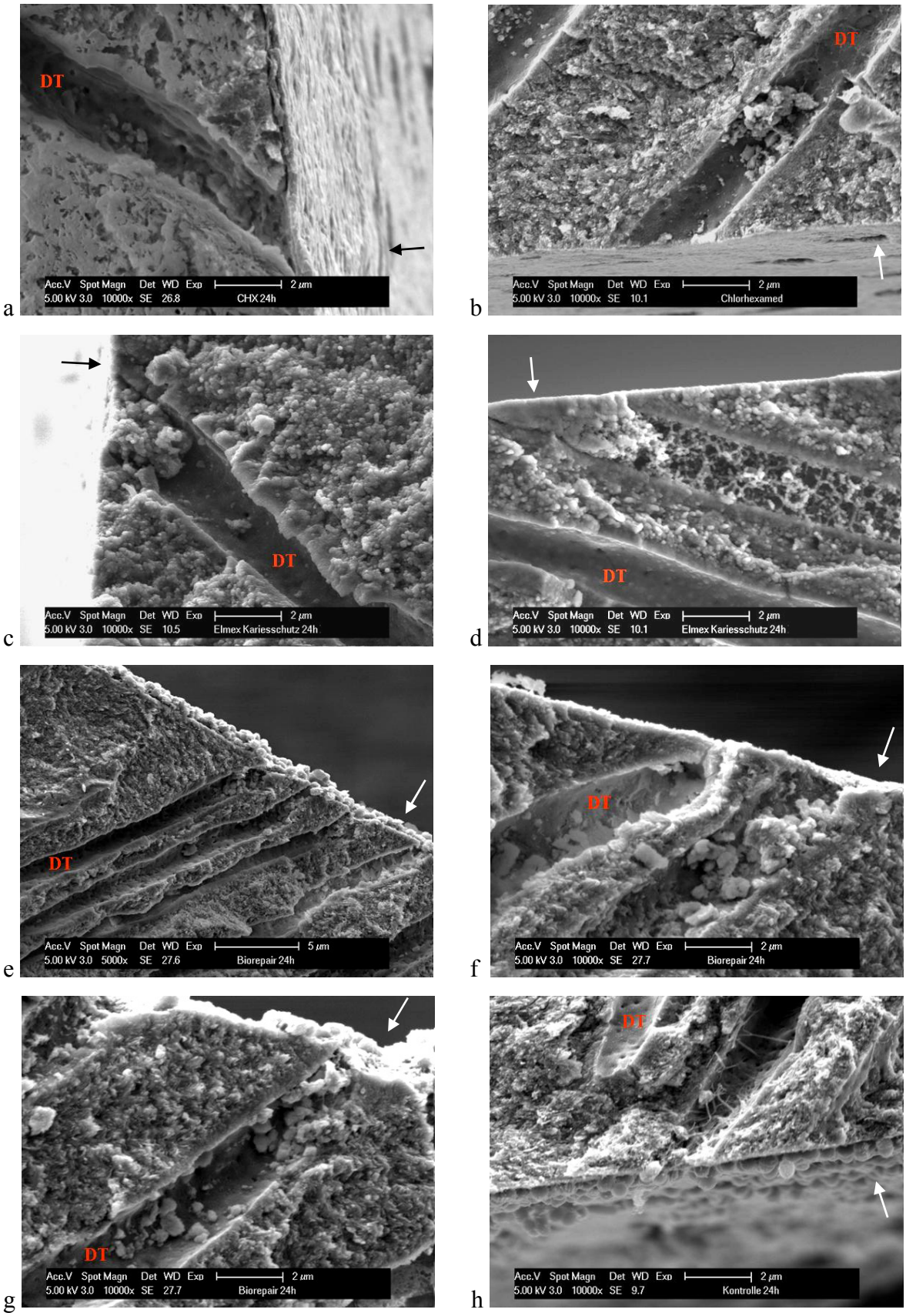


Fig. 22: Scanning electron microscopic micrographs of longitudinally fractured dentinal tubules. 24-h biofilm. (a and b) CHX group; (c and d) Elmex Kariesschutz group; (e, f and g) BioRepair group; (h) Control group. The arrows inidicate the surface of specimens. DT: dentinal tubule. Original magnification: (e): 5.000-fold; (a, b, c, d, f, g and h):10.000-fold.

4.3 TEM

An overview of TEM investigations on dentinal pellicle / biofilm formation is given in Table 6. Apart from enamel pellicle, the basal pellicle layer fails to clearly manifest itself on the dentinal surface. Another feature of the dentinal pellicle is the invasion of the pellicle into the dentinal tubules to various degrees, such as 'pellicle-bridges' over the tubular orifices and a partial or complete occlusion of tubules (Fig. 24).

A series of TEM images indicated an inter-subject variation and a progressive biofilm development in aspects of pellicle texture and microbial growth. As compared to the 24-h pellicle with a loose, variable structured network, the 48-h pellicle was identified as a relatively homogeneous, electron dense layer with globular aggregates and microbial colonies in tubules, lining the board of surface as well as above the outer pellicle layer. Although the thickness of the pellicle showed a subtle increase with the lapse of time, it remained at the same level (Table 6).

In the control group, the 24-h pellicle formed a granular coverage of the entire surface. Left undisturbed for another 24 h, the 48-h pellicle was characterized by a mottled structure with both granular and globular agglomerates on the one hand and adherent bacteria on the other hand. The thickness of the loosely structured layer remained at the same level, whereas it reached 5 µm in the presence of microorganisms (Table 6). The observed bacteria are located throughout the pellicle: in the tubules, adjacent to the dentinal surface, in the pellicle and on the top of pellicle, too. Cell division was evident. In addition, fibrillar structures were detected on most of all the bacterial cell walls. Pellicle in the tubules not only deposits along the tubular wall, but also levels up the tubule. For the more, a bacterial penetration was detected up to 10 µm (Fig. 23).

A pellicle with few bacteria or even devoid of bacteria was observed in the CHX-treated samples, either for an intraoral exposure period of 24 h or a period of 48 h (Fig. 24). In the initial 24 h, the pellicle consisted of a granularly structured and loosely arranged layer with variable thickness, occasionally up to 1.300 nm. As time passing, a heterogeneous structure with appearance of globular agglomerates with diameter of 200 nm and granules was present. The absorbed salivary components filled both, surface irregularities and tubules. It is noted that the CHX-treated pellicle was relativly electron-dense as compared with the control pellicle (Fig. 24).

The pellicle in Elmex Kariesschutz group was characterized by predominant granular aggregates. Even within 24 h, some areas were covered by a homogenous dense layer at a thickness of up to 1.300 nm. After 48-h exposure, it was evident that isolated bacteria / bacterial colonies were embedded in the filamentous pellicle or above the outmoster of the pellicle layer (Table 6 and Fig. 24).

In comparison with Elmex Kariesschutz, a similar but less pronounced pellicle manifested itself after rinsing with BioRepair. However, several peculiar structures produced interesting results. For instance, a number of amorphous and spherical particles at a diameter of 50 nm were detected in tubules, which made it different from the other groups (Fig. 25). In addition, several clusters of particles revealing a nanoapatite-like ultrastructure presented throughout the pellicle: on the surface, in the external pellicle layer, at the entrance of tubules and in the tubules (Fig. 25). It is also interesting to find a bulk of agglomerates composed of such particles above the pellicle layer. Similar to the Elmex Kariesschutz Group, single bacteria colonized on the top of pellicle. After an oral exposure for 48 h, bacterial invasion into tubules reaching 7.5 µm was demonstrated (Table 6).

Except the CHX Group, bacterial adherence in the form of single bacteria or bacterial colonies was easily revealed in all other groups. As compared to the control group, BioRepair and Elmex Kariesschutz groups showed microbial alterations. Bacteria embedded in the 48-h biofilm following rinsing with Elmex Kariesschutz showed an unrecognizable cell outline and blurred cytoplasmic structures (Fig. 26). In the BioRepair group, features on the cell wall such as fibrils, were not evident. Several voids, which were assumed to be occupied by single bacteria previously, were present. It was presumably related to the denaturation process of organisms (Fig. 26).

Table 6 An overview of TEM analysis of the pellicle / biofilm-covered dentin

Oral exposure time	**Groups**	**Thickness (nm)**	**Description of the *in situ* pellicle**
24 h	Control	50-230 (occassionally up to 1.300)	Loosely arranged, granularly structured pellicle layer
	Elmex Kariesschutz	70-1.000 (occasionally up to 1.300)	Filamentous / granular pellicle layer
	BioRepair	70-1.000	Loosely arranged, granularly / globularly structured layer on surface and in tubules; Bacterial colonization on top of pellicle layer
	CHX	70-670 (occasionally up to 1.300)	Continuous, loosely arranged and granularly structured layer; Pellicle deposition in microreliefs and tubules; No detectable bacteria
48 h	Control	70-1.000 (2.000-5.000 in presence of bacterial colonies)	Loosely arranged, fine granular layer / mottled layer with both granular and globular agglomerates; Close-up of tubule with pellicle and microbial penetration into the tubule up to 10 μm; Bacterial colonization on the top of pellicle layer or in tubules.
	Elmex Kariesschutz	170-2.300 (occasionally up to 4.700 in presence of bacterial colonies)	Continuous, homogeneously granular / filamentous structured layer; Bacterial colonization above the pellicle layer; Single bacteria in the pellicle layer; Mono-layered bacteria and multi-layered bacterial colonization
	BioRepair	170-1.000	Continous, loosely and granularly / globularly structured layer on surface and in tubules ; A microbial invasion of tubule up to 7.5 μm
	CHX	70-700	Continuous, loosely arranged, granular / globular structured layer; Pellicle deposition in surface irregulates and tubules; No observed bacteria

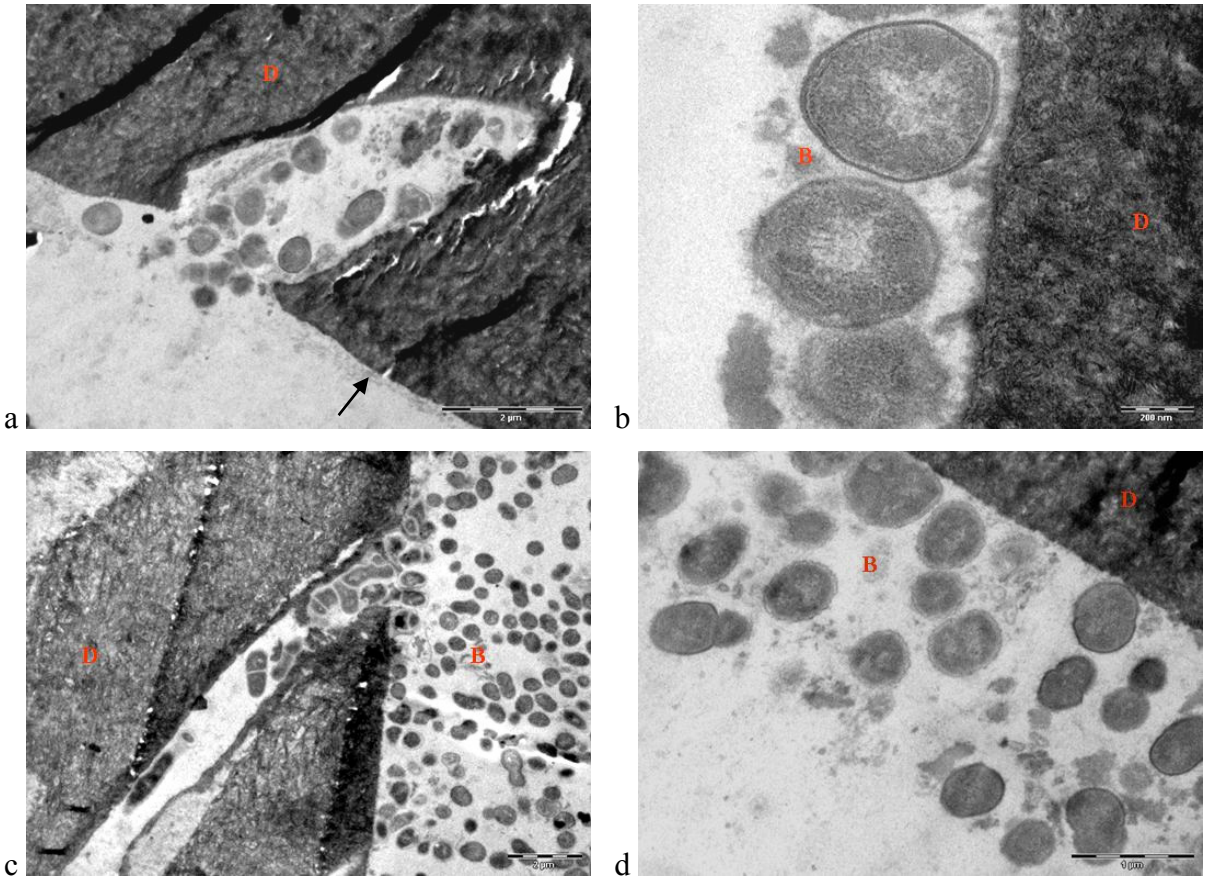


Fig. 23: TEM micrographs: bacterial biofilm on dentinal specimens without rinsing (Control group). Cell division and the morphological features of cells are evident. (a and b) 24-h biofilm; (c and d) 48-h biofilm. The black arrow indicates the biofilm. B: biofilm. D: dentin. Original magnification: (a) 13.000-fold; (b and d) 23.000-fold; (c) 6.800-fold.

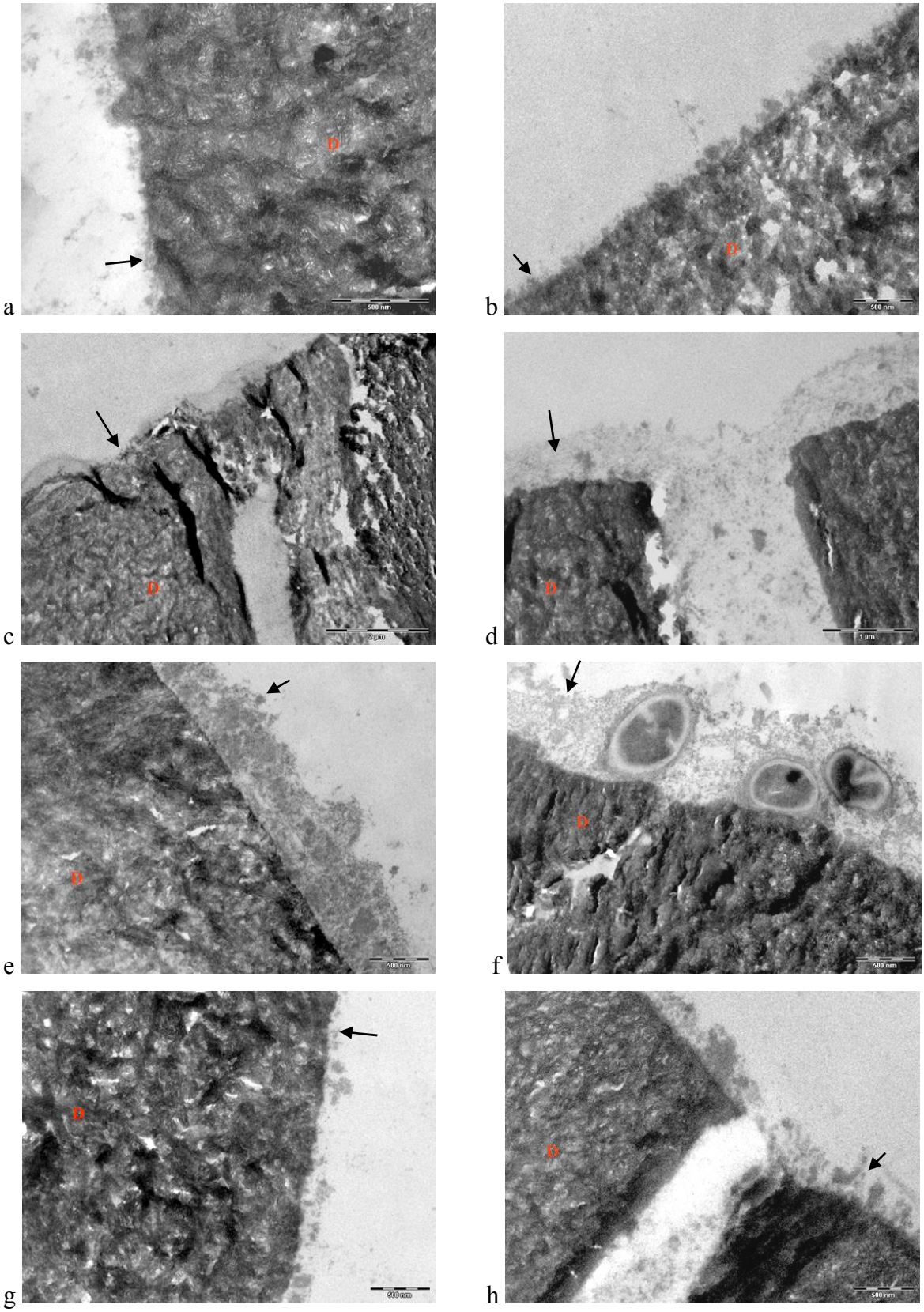


Fig. 24: TEM micrographs: gallery of representative pellicle layers following rinsing with different mouthrinses in each experimental period. (a, e and g) 24-h biofilm; (b, c d, f and h) 48-h biofilm; (a, b, c and d) BioRepair group; (e and f) Elmex Kariesschutz group; (g and h) CHX gorup. The black arrows indicate the biofilm. D: dentin. Original magnification: (a, b, e, f, g and h) 30.000-fold; (c) 13.000-fold; (d) 23.000-fold.

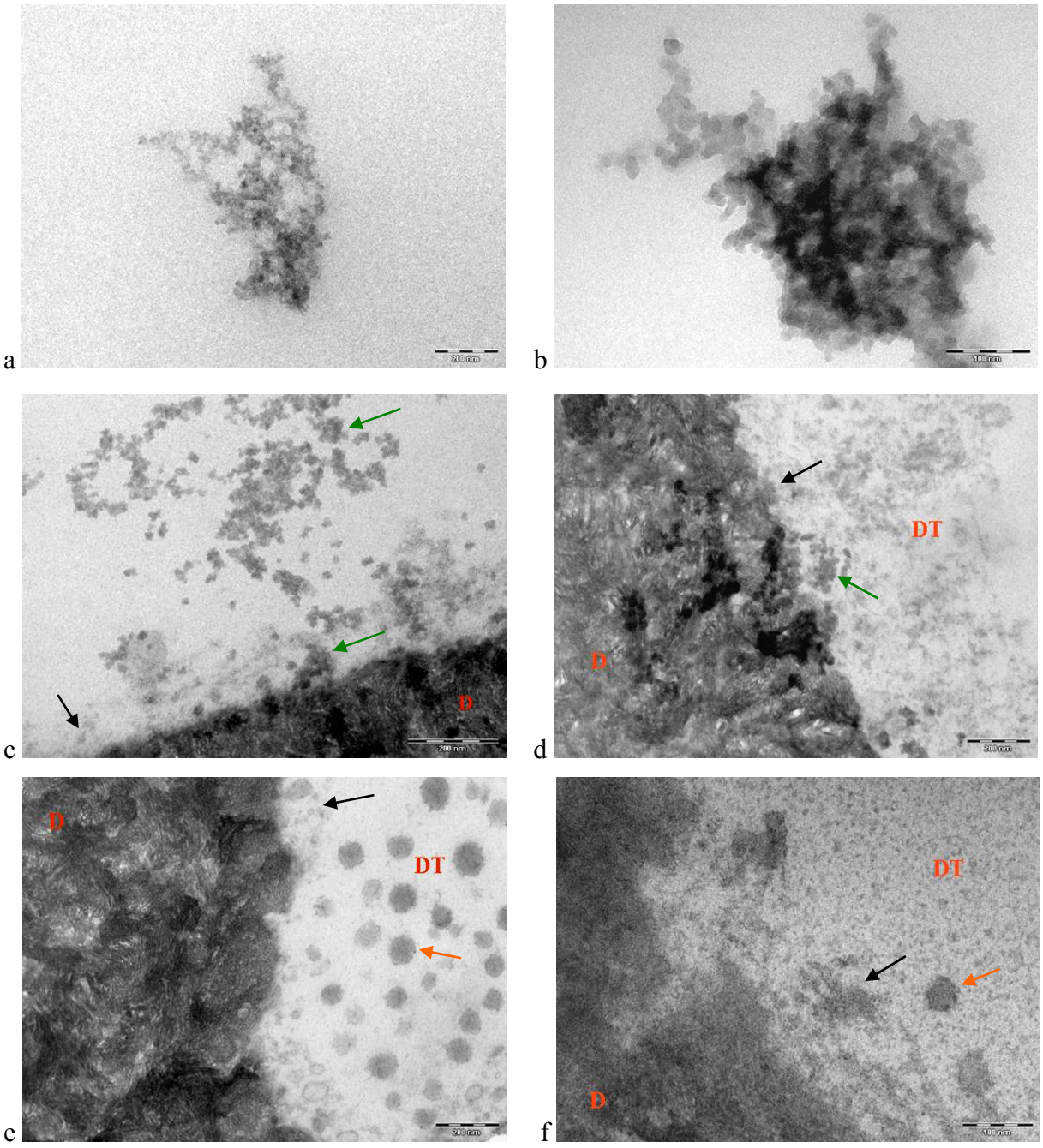


Fig. 25: TEM micrographs: clustered hydroxyapatite particles (200-1.000 nm), consisting of aggregated crystallites (15-20 nm in length), in the BioRepair mouth and tooth rinsing solution (a and b). Clusters of apatite-like particles (green arrows) are present throughout the 48-h biofilm with adoption of BioRepair: on the surface, in the external pellicle layer, at the entrance of tubules and in the tubules and even above the pellicle (c and d). Little globules (orange arrows) were observed in the tubules in 24-h BioRepair-treated biofilm (e and f). The black arrows indicate the biofilm. D: dentin. DT: dentinal tubule. Original magnification: (a, c and e): 68.000-fold; (b):180.000- fold; (d): 98.000-fold; (f) 150.000-fold.

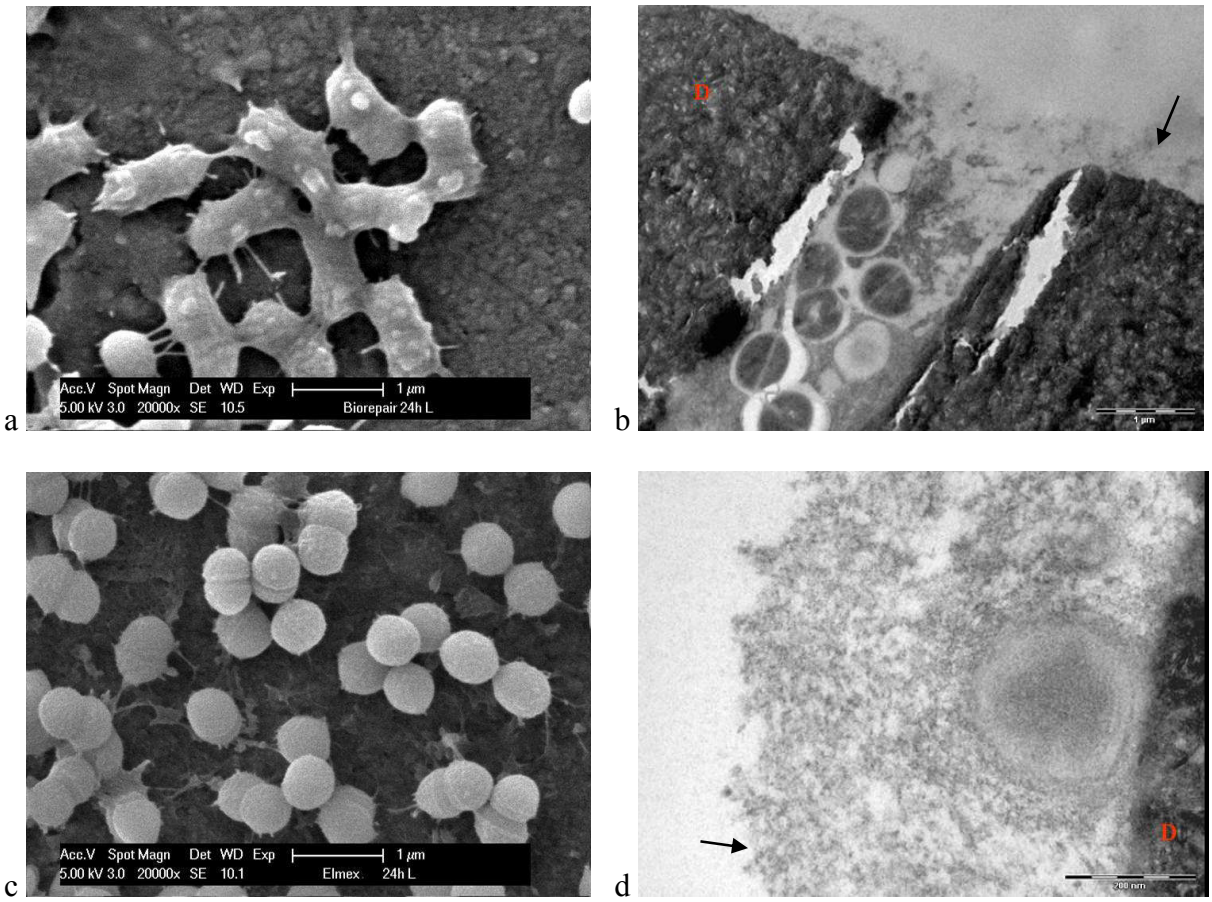


Fig. 26: SEM and TEM micrographs: microbial morphological changes observed with application of BioRepair (a and b) and Elmex Kariesschutz (c and d). The black arrows indicate the biofilm. D: dentin. Original magnification: (a and c) 20.000-fold; (b) 18.500-fold; (d) 120.000-fold.

5 Discussion

5.1 Discussion of Materials and Methods

5.1.1 Adoption of bovine teeth

Both human and bovine teeth are used in studies on biofilm formation as well as biofilm control. However, bovine teeth are gradually considered as an appropriate substitute to human teeth [Jung et al., 2010; Wang et al., 2012; Wegehaupt et al., 2008; Wegehaupt et al., 2010]. First, it is easier to get homogenous, standardized samples and large surface areas from bovine teeth compared to human teeth. Second, bovine samples are much more accessible to meet the huge demand of specimens for dental research. Third, bovine teeth have similar physicochemical characteristics as compared to human dental hard tissues [Nakamichi et al., 1983]. The assumption that the bacterial adherence to bovine samples is comparable to that on human enamel is reasonable [Hannig et al., 2007; Jung et al., 2010; Wegehaupt et al., 2010]. Thus, the investigation of bacterial adherence on bovine teeth is appropriate to offer an insight into the antibacterial strategy of human teeth [Hannig et al., 2013a; Hannig et al., 2013b; Hannig et al., 2013c; Tawakoli et al., 2013]. In the present study, all bovine samples are obtained from 2-y old cattle which are from the same region in consideration of the same environmental factors and the comparable level of tooth mineralization.

In contrast to the broad knowledge of microbial biofilm formation on enamel, little information on dentin is available [Hannig et al., 2013a; Hannig et al., 2013b; Jung et al., 2010]. This can be ascribed to the general mechanisms behind biofilm development and the similar aspect of the dentin and enamel. Therefore, it is reasonable that the abundant information obtained from studies on enamel biofilms can also be applied to dentinal biofilms. However, the characteristic of the dentinal structure gives rise to a different bioadhesion to some extent, for instance, a more pronounced microbial colonization [Nyvad and Fejerskov, 1987a, b]. In a recent *in vivo* study on 30-, 120- and 360-min microbial colonization, the total bacterial count on dentinal samples increased significantly with increasing oral exposure time, whereas that on enamel remained constant [Jung et al., 2010]. These differences might be attributed to bacterial interactions with the collagen matrix of dentin [Love, 2002]. Furthermore, Fagrell *et al.* [Fagrell et al., 2008] found that the bacteria were mainly attached to the odontoblast processes rather than to the tubular walls. However, TEM and SEM

analyses in the present study revealed that the dentinal orifices are free of odontoblast processes. The fractured surfaces examined by SEM showed that odontoblast processes existed in the deeper part of the tubules, and the tubules near to the enamel-dentin junction are free of odontoblast processes.

5.1.2 Experimental design

5.1.2.1 *In situ* biofilm model

Many *in vitro* (also known as laboratory model) and some *in situ* models have been adopted to investigate biofilm formation as well as the impact of chemical agents on biofilm development. Although *in vitro* studies remain a mandatory first step in investigating the mode of action and evaluating the efficacy of active ingredients [Baehni and Takeuchi, 2003; Shani et al., 1996, 1998, 2000], the outcome of *in vitro* experiments has to be decoded carefully. First and foremost, in absence of saliva, a physiological bioadhesion is not possible to be mimicked *in vitro*. As a consequence, bioadhesion processing *in vitro* is distinctly different from *in vivo* conditions [Hannig and Hannig, 2009]. Due to reduced diffusion processes, the *in vivo* / *in situ* biofilm reveals an up to 100 times higher resistance to oral chemotherapeutics than the so called planktonic or dispersed bacteria [Shani et al., 2000]. Moreover, a microbial community with metabolic cooperativity and cell-to-cell-signalling [Kolenbrander et al., 2006] failed to be established with the plate culture technology *in vitro*. Studies on the vitality pattern in biofilms are an example that the findings obtained *in vivo* do not match that from *in vitro* [van der Mei et al., 2008; van der Mei et al., 2006].

Obviously, considering the mentioned drawbacks of *in vitro* models, the *in situ* model has the advantage that it can precisely draw the picture of the situation in the oral cavity. It can also reflect the actual capacity of mouthrinses, and it is significantly relevant to the clinical efficacy. Thus, the *in situ* model has been applied in the present experiments.

5.1.2.2 The removable minisplint

The splint is an optimal tool in investigating intraoral biofilms. The application of splints results from the observation that the biofilm formed on the mounted enamel samples is identical to that on the natural tooth [Macpherson et al., 1991]. In this study, all the samples are mounted in splints by means of the silicone impression material (light body) in order of not disturbing the adhering biofilm when removing samples. As a result, mounting of the

samples at defined and reproducible sites is possible [Hannig, 1997, 1999]. Most importantly, this splint model is well accepted by subjects, as oral hygiene measurements could be performed without disturbing biofilm formation on the specimens.

5.1.2.3 Experimental conditions

In order to reduce inter- and intra-individual variations, the experimental conditions were strictly regulated and had been informed in detail.

- Each round began at 8:00 a.m. with an expectation of excluding the impact of circadian rhythm of salivary flow on biofilm formation.
- Toothbrushing without toothpaste has been required to be conducted 30 min before the experimental trail.
- In order to rule out the inferring factor of consumption of acidic beverages [Hannig and Balz, 1999], subjects were refrained from any beverages with an exception of water in the course of each round.
- In this study, the molar region in the upper jaw was selected, because the biofilm formed in this area has been investigated in numerous previous studies. Thus, this intraoral site allowed a comparison with former studies. Also, the exact and well defined position of the samples was able to preclude any topic influences / interferences on biofilm formation [Hannig, 1999].
- Each course of experiments lasted 24 h and 48 h, like many other studies on the longitudinal effect of influencing factors or antibacterial substances with regard to biofilm formation [Zaura-Arite et al., 2001].

The limitation of the present study is the small number of subjects. The huge amount of different experimental groups and the complexity of experimental and analytical methodology required the study to enroll a small number of volunteers which is also true in many other previous *in situ* studies [Auschill et al., 2001].

5.1.3 *Bac*Light™ viability assay

In the majority of previous studies, the quantification of bacterial colonization and antimicrobial activity of oral chemotherapeutics was performed using plate culture microbiological technology in combination with determination of the colony-forming units (CFU) [Embleton et al., 1998; Shani et al., 2000]. However, the reliability of these

technologies is questionable due to the following limitations. First, with the CFU method, only 50% of the oral bacterial strains are cultivaiable [Aas et al., 2005]. Second, ultrasonication is performed to detach the adherent bacterial cluster from the sample surface, resulting in the separation of bacterial aggregates and therefore, leading to potential overestimation [Hannig et al., 2007; Tawakoli et al., 2013]. These disadvantages might be overcome with the fluorescence staining techniques followed by fluorescence microscopic analysis. Some techniques such as fluorescence *in situ* hybridization (FISH) and 4', 6'-diamidino-2-phenylindole (DAPI) allow selected bacterial species or all the bacterial cells to be stained. Also, the fluorescence-based, two-colour viability assays (Live / Dead staining), which are based on the used fluorochromes' impact either on the membrane permeability or metabolic activity, could differentiate between the living and dead cells, so as to provide an insight into the quantification of bacterial colonization as well as bacterial viability pattern [Auschill et al., 2001; Netuschil et al., 1998]. Reasonably, it is possible to discriminate potential antibacterial and antiadherent effects of mouthrinses by means of how many adherent bacteria are existing, together with how vital they are during biofilm formation [Al-Ahmad et al., 2008; Weiger et al., 1998]. Although the cellular elements (*e.g.* epithelial cells) or contaminating materials are stained too, this has no adverse effects on the results.

The conventional LDS technology applies two fluorescent dyes, fluoresceine diacetate (FDA) and ethidium bromide (EB), to stain the living (green) and dead (orange) cells respectively, and it was first introduced by Netuschil [Netuschil, 1983]. With respect to the total amount of bacteria, this LDS has revealed a good correlation compared to results from DAPI staining [Hannig et al., 2007]. With the development of fluorescent dyes, a number of fluorescence microscopic assays with various combinations of fluorochromes, such as Syto 9 / PI (*Bac*Light), FDA / Sytox red, Calcein AM (acetoxymethyl) / Sytox red and CFDA (carboxyfluorescein diacetate) / Sytox red, establish alternatives to the toxic and instable staining procedure (EB). *Bac*Light™ viability assay has been used in former studies in consideration of its rapidity, high reliability and simplified preparation procedures [Hannig et al., 2013a; Hannig et al., 2013c]. In addition, this technology is preferred to assess the viability pattern, as the dead bacteria are not overestimated [Tawakoli et al., 2013]. Thus, *Bac*Light™ viability assay was adopted in the present study.

The counting technology was applied in former studies to evaluate the bacterial colonization [Hannig et al., 2013b; Hannig et al., 2007; Hannig et al., 2013c; Tawakoli et al., 2013]. However, single bacteria are difficult to be differentiated in the colonies and within the dentinal tubules, resulting in a possible underestimation of the number of bacteria. Thus, a

scoring system has been adopted in the present work for assessment of the amount of bacterial colonization. Nevertheless, the results recorded are corresponding to previous studies [Hannig et al., 2013a; Hannig et al., 2013b], and seem to be characteristic for bioadhesion in the oral cavity.

5.1.4 Electron microscopic investigations

The prevailing knowledge on the ultrastructural pattern and morphological appearance of biofilms is mainly based on conventional transmission and scanning microscopic investigations [Hannig and Joiner, 2006].

SEM is the most commonly used microscopic examination method for biofilms. It allows a direct detection of the morphological appearance at high magnification (*e.g.* 2.000 x or even 20.000 x). At the micro-scale, bacterial aggregates, the biofilm matrix as well as the bacterial interplay are strikingly illustrated. However, only the morphogenesis of biofilms on the dentinal surface rather than subsurface and deep parts of tubules is accessible. Thereby, an investigation on fractured samples is necessary.

TEM is the gold standard for the investigation on biofilm at the micro- and even nano-scale level. In addition to a thorough observation of ultrastructural features, it is feasible to accurately measure the biofilm thickness. Moreover, a detailed visualization of the bacterial morphology and in particular the interactions of bacteria with the pellicle layer as well as the microbial penetration into dentinal tubules are possible. Due to huge efforts for preparation, TEM is tedious, time consuming and inappropriate for a large number of samples. Another related issue is the tiny examined ultrathin-section (50-80 nm) obtained from a dentinal specimen (2 mm x 2 mm). However, it is meaningful to get an overall impression of biofilm at the sub-micron scale.

5.1.5 The methodological combination of fluorescence microscopy and electron microscopy

As described above, the vital fluorescence technology provides an insight into the bacterial colonization and vitality pattern. SEM (surface and fractured specimens), combined with TEM, is able to re-construct the ‘three-dimensional’ ultra-structure of the biofilms formed in the oral cavity, providing the morphological appearance at the sub-micron scale. Therefore, the combination of the vital fluorescence technique and the electron / fluorescence

microscopic technology allows investigation of the effects of mouthwashes on biofilm quantity and biofilm quality. This is of considerable relevance for assessing the efficacy of oral health care products in biofilm management.

5.2 Discussion of results

5.2.1 Descriptive results

The inter-subject variations in the pellicle structure and biofilm thickness evaluated by SEM / TEM might be related to a few individual factors, such as saliva flow rate, biopolymer supply and dietary habits.

The dentinal biofilm was characteristic by the preferential pellicle formation and bacterial colonization in tubules. This phenomenon can be explained by the well accepted concept that surface irregularities are the starting points of the initial pellicle formation and bacterial adherence [Hannig and Hannig, 2009; McConnell et al., 2010]. Moreover, irregularities above the critical surface roughness of 0.2 μm are supposed to be beneficial for the microbial retention [Bollen et al., 1997]. Presumably, tubules served as a shelter for microorganisms from shear forces and, therefore, provided a suitable environment for bacterial growth. Thus, the presence of dentinal tubules facilitates the incipient pellicle formation and bacterial colonization. Tubules free of bacteria were not observed in any test group, which post a challenge to the antimicrobial agent. However, caution should be taken when interpreting the present results, since the bovine dentin reveals a smaller density of tubules in larger dimension than human dentin [Jung et al., 2010].

TEM analysis indicated that the 24- / 48-h biofilm was characterized by a homogenous layer with a granular appearance, in agreement with previous investigations on enamel pellicle [Hannig, 1999]. Different from the evident two-layered enamel pellicle (the basal layer and the outer layer), the basal pellicle is unrecognizable on the dentinal surface. Such a vague initially adsorbed pellicle layer might be ascribed to the comparatively rough dentin surface generated from the polishing procedures.

The combination of SEM and TEM investigations on the undisturbed biofilm from control groups offered an insight into the ultrastructural morphogenesis of dentinal plaque. As stated by Hannig and Joiner [Hannig and Joiner, 2006], a 24-h plaque was identified as a microbial community embedded in a network comprising of granular, globular and fibril-like structures, indicating a heterogeneous complexity in the mature biofilm. It is noticeable that series of SEM images demonstrated tiny particles (50-100 nm in diameter) adsorbed on bacterial cells. It might be a presumable evidence for the specific bacterial attachment in the mature stage [Hannig and Joiner, 2006]. In general, the progressive biofilm development could be observed in aspects of an increase in bacterial and pellicle volume, the emergence of large aggregates,

including density and the diversity of microbiota. Larger pellicle agglomerates and a dense arrangement were evident in the 24-h biofilm, even noticeable after 48 h, providing evidence that the protein aggregates are crucial to the later pellicle maturation [Hannig and Joiner, 2006]. Furthermore, the universal presence of bacterial cell division and fibril-like structures between bacteria in 48-h bioiflm implies that bacterial proliferation and microbial co-aggregation give rise to the further microbial development, forming a monolayer confluent or multilayered colony.

As evaluated by TEM, the pellicle thickness for the observation period of 24 h varied between 100 nm to 1.300 nm, in agreement with previous studies conducted on enamel [Hannig, 1999]. After another intraoral exposure time of 24 h (= 48-h biofilm), the biofilm thickness remained at the same level. However, investigations carried out by Brecx *et al.* [Brecx et al., 1985; Brecx et al., 1981; Brecx et al., 1983] revealed that the plaque on plastic films reached a thickness ranging from 7 to 14 µm after an intra-oral exposure time of 24 h. These results were confirmed by other studies using CLSM techeniques [Auschill et al., 2001; Netuschil et al., 1998], which also established a thickness of up to 35 µm after 48-h formation on enamel [Netuschil et al., 1998]. It is reasonable to ascribe the observed inconsistency to the different experimental conditions, for instance, supporting materials and analytical methods applied.

In spite of the similar pellicle morphogenesis detected in CHX-treated samples compared to control samples, a low incidence of bacteria or even lack of microorganisms were observed after CHX rinsing, supporting the well established antimicrobial properties of chlorhexidine [Jones, 1997]. However, individual bacteria were present in tubules. A possible explanation might be that tubules protect the colonizing bacteria against chlorhexidine. Based on series of micrographs, it might be an indication that the target of chlorhexidine is the microorganisms, rather than pellicle proteins or the process of pellicle formation. On the other hand, such a dramatic reduction in bacterial surface coverage might suggest the possibility that chlorhexidine modifies the pellicle surface, and thereby, impeding bacterial adherence. However, the exact mechanism could not be established precisely by electron microscopy evaluation exclusively in this study. In contrast to the undisturbed biofilm, the pellicle structure treated by CHX seemed to be more electron-dense, which might be associated with an increment in the calcification levels or denatured pellicle proteins due to the chlorhexidine treatment [Hannig and Joiner, 2006].

Elmex Kariesschutz represented a comparatively thicker, more homogenous surface layer of deposits in SEM micrographs, in good consistence with the densely, globular structured layer

illustrated by TEM images. Elmex Kariesschutz produced a pronounced pellicle layer masking the original surface characteristics, in accordance with its anti-erosive properties of amine fluoride in previous studies [Naumova et al., 2012; Wegehaupt et al., 2011]. This can be the result of CaF_2-like precipitations. Under high fluoride concentration (>100 ppm F), calcium fluoride is theoretically formed [ten Cate, 1997]. Indeed, randomly distributed globules with diameters between 200 nm and 300 nm were detected on the surface. However, it is not determined in the present work whether such globules are actually CaF_2 particles. On the other hand, a reduction of bacterial adherence to a moderate degree corresponds to the mild antimicrobial capacity of amine fluoride [Hannig et al., 2013b]. As shown in TEM micrographs, individual bacteria entrapped within the pellicle showed an obstacle cell membrane and vague cell structures, implying also inhibitory effect of Elmex Kariesschutz on microorganisms.

Several specific structures were observed only after application of the BioRepair mouth rinsing. First, globular structures in varying diameters between 50-100 nm were dispersed on the surface and in the tubules. This observation should not be misinterpreted as the parotid proteins, because of the different sizes [Hannig and Joiner, 2006]. In addition, the presence of clusters of mineral structures throughout the pellicle drew attention. Presumably, these structures might be apatite nanoparticles contained in the mouthrinse because of their similar appearance and dimension when compared to pure nano-apatite clusters (see Fig. 25). In fractured specimens, tubular occlusion to different extents after rinsing with BioRepair, was demonstrated. This occlusive property is of great clinical relevance, in accordance with the anti-erosive and anti-caries capacity of BioRepair rinsing derived from previous *in vitro* studies [Poggio et al., 2010; Tschoppe et al., 2011]. Moreover, a significant decline in bacterial surface coverage was observed. However, bacterial remnants were presented as single bacteria or micro-colonies, in agreement with previous promising results from *in vitro* and *in vivo* studies on the short-term antimicrobial properties of the BioRepair mouth rinsing solution [Hannig et al., 2013a]. In some areas, a continuous pellicle layer surrounding a mono-layer of microorganisms could be noticed. The general bacterial adherence onto the pellicle might suggest an inhibitory effect of BioRepair on the spatial microbial co-adherence. Furthermore, morphological alterations in adherent bacteria were observed in TEM / SEM evaluations. Loss of cellular integrity and cell wall features as well as the possible microbial denaturation provided evidence for the inhibitory effect of BioRepair on microorganisms. However, the exact antimicrobial mode of action of BioRepair has not been clarified, yet.

5.2.2 Quantitative results

5.2.2.1 CHX

As expected, CHX significantly reduced the bacteria incidence on the dentinal surfaces in accordance with previous published investigations [Brecx et al., 1990; Tomás et al., 2008]. As gold standard for chemical plaque control [Jones, 1997], chlorhexidine established pronounced anti-plaque properties based on its powerful antibacterial behavior and substantivity. Once applied in the oral cavity, the inhibition of initial bacterial attachment is achieved by the immediate antibacterial activity [Jenkins et al., 1988]. Chlorhexidine acts on the bacterial cell wall and disrupts the membrane integrity [Jones, 1997]. With the formation of vesicles and precipitation in the cytoplasm, the bacterial cell is killed [Brecx and Theilade, 1984; Davies, 1973]. As time passes, chlorhexidine is present at a low concentration as the result of the dilution by saliva and exhibits bacteriostatic effects [Brecx, 1997; Tomás et al., 2008]. Therefore, the further bacterial adherence is hindered up to 7 hours [Newcombe et al., 1995; Tomás et al., 2009], which is consistent with the present study.

5.2.2.2 Elmex Kariesschutz

Elmex Kariesschutz revealed a moderate behavior against oral microorganisms in the present study. In contrast to the controls, no obvious antibacterial property was observed, which was indicated by the similar amounts of dead bacteria cells in Elmex Kariesschutz group and the control groups. Such observation differs form the previous studies on the promising antimicrobial effect of fluoride and amine.

As demonstrated by a lot of studies on both biofilm and planktonic cells [Koo, 2008], an impaired effect of fluoride on the biological activity of acid-tolerant oral bacteria, including *S.mutans*, is achieved through the acid stress in cells and decreased acid production [Hamilton, 1990]. The mechanisms involved are supposed to be the inhibition of glycolytic enzymes (enolase) and proton-translocating ATPase as well as the cytoplasmic acidification in a bulk of *in vitro* studies [Van Loveren, 2001], which is mainly supported by experimental studies. Recently, a study on the metabolic effects of fluoride (225 and 900 ppm F^-) on the supragingival plaque provided the *in vivo* evidence [Takahashi and Washio, 2011]. The results showed that fluoride negatively influences the bacterial enzymes, not only in the Embden-Meyerhof-Parnas pathway (the EMP pathway), but also in the pentose phosphate pathway so as to repress bacterial growth and acid production. However, it is also supposed

that the major effect of amine fluoride is the antibacterial property derived from amine [Shani et al., 1996; van der Mei et al., 2008]. An antiglycolytic effect of amine fluoride is well documented [Ciardi et al., 1978; Embleton et al., 1998; Exterkate et al., 2010; Shani et al., 2000; van der Mei et al., 2008]. The results from Shani *et al.* [Shani et al., 2000] demonstrate that amine fluoride has a similar antibacterial potential as chlorhexidine, which is inconsistent with the present results. The discrepancy might be related to the *S.sobrinus in vitro* biofilm model in that study, resulting in different environments for biofilm development.

On the other hand, a reduced number of viable cells after 24-h intraoral exposure, even noticeably after the 48-h experimental period, were observed in the Elmex Kariesschutz group in comparison with the control group. It can not be fully explained by the unsurpassed antibacterial efficacy of AmF and may indicate the anti-adhesive potential of this substance. Fluoride is suggested by *in vitro* studies to inhibit bacterial adsorption to hydroxylapatite and subsequent microbial growth [Bradshaw et al., 2002; Rölla and Melsen, 1975]. However, the effective concentration is not easily to reach *in vivo* and further *in vivo / in situ* researches are needed [Van Loveren, 2001]. Moreover, a study using a biofilm model reveals no difference in the initial adherence of *S.mutans* to hydroxyapatite or fluoropatite, but the subsequent bacterial growth was retarded in the biofilm-coated fluorapatite at pH 5.5 [Li and Bowden, 1994]. Furthermore, the preincubation of AmF results in decreased adherence of *S.sobrinus* on HA [Shani et al., 2000]. Additionally, the adhesion of *S.mutans* to the pellicle is reduced after the treatment with AmF [van der Mei et al., 2008]. Through electrostatic interactions, the adsorption of AmF is shown to have considerable effects on hydrophobicity and surface charge of the pellicles [de Jong et al., 1984a, b; de Jong et al., 1984c; Shani et al., 1996; van der Mei et al., 2008]. Van der Mei and collegues also suggested that AmF might also influence the initial bacterial adhesion [van der Mei et al., 2008]. An adsorption of fluoride occurring at the nano-scale potentially adds different surface characteristics to dentin modulating the tenacity of initial unspecific bacterial adherence to the pellicle layer [Hannig et al., 2013b]. An anti-adhesive effect of amine fluoride might be anticipated *in vivo*.

AmF might have a similar mechanism compared to chlorhexidine in influencing biofilm formation [Hannig et al., 2013b]. A higher adsorption and lower desorption of AmF to hydroxyapatite than that of chlorhexidine has been demonstrated [Sefton et al., 1996; Shani et al., 2000], which indicates a more pronounced retention of AmF to tooth surface. Moreover, amine fluoride is assumed to have a high affinity to the proteinaceous pellicle layer, which serves as a reservoir for fluorides and facilitates desorption of the fluoride at the tooth surface

[Arends and Schuthof, 1981]. It is also postulated that the retained fluoride in the oral cavity (tooth surface, pellicle and oral mucosa) might affect the process of bacterial growth and bioadhesion while the plaque serves as a slow-release reservoir for fluoride [Otten et al., 2010]. Thus, a persistent efficacy is expected, which confirms the distinct reduction observed in viable cells after 48-h exposure time under the Elmex Kariesschutz rinsing regime.

5.2.2.3 BioRepair

As shown by both, SEM and *Bac*Light™ semi-quantitative analyses, in the present study, BioRepair mouth rinsing exhibited less antibacterial properties, but similar inhibitory effects on bacterial colonization compared to the gold standard, chlorhexidine. These results correspond to a previous *in situ* study conducted on bovine enamel for experimental periods of 6 h and 12 h [Hannig et al., 2013a].

Up to date, the exact mode of action of the BioRepair mouth rinsing solution on microorganisms is not well established. The combined effect of several components of the liquid phase such as sugar alcohols (sorbitol and xylitol), detergent (sodium lauryl sulphate), or food preservatives (sodium benzoate) and the clustered nano-particles presumably account for the promising inhibitory effect on biofilm quantity (the total bacterial adherence) and biofilm quality (the microbial vitality) [Hannig et al., 2013a].

The zinc-carbonate hydroxyapatite has been considered to be the main active substance in the BioRepair product. It shows a promising performance in remineralization processes and treatment of dentin hypersensitivity [Orsini et al., 2010; Tschoppe et al., 2011]. The general mode of action of these biomimetic hydroxyapatite nano-particles (scattered or clustered) is based on the high bioactivity derived from the size (100 x 10 x 5 nm) which is similar to the natural hydroxyapatite. Thereby, similar biochemical properties of the natural hydroxyapatite and the synthic biomimetic zinc-carbonate nanoapatite could be established. Thus, such biomimetic nano-sized HA is theoretically able to repair the micro-defects resulting from acid attack or initial caries [Hannig and Hannig, 2010b; Roveri et al., 2008; Sarikaya et al., 2003]. Based on its size effect, the nano-sized HA can interact directly and intensively with the bacterial cell membrane, proved in an *in vitro* study [Venegas et al., 2006]. Therefore, a competing effect / retention on the adhesins of the bacterial cell membrane by the nanoparticles is expected. As such, adhesion sites on the adhered organisms might be blocked on the one hand and the adhered cells could also be sequestered on the other hand, which

leads to a slow down of microbial co-aggregation and bacterial co-adhesion to the initially formed biofilm. Such supposed mechanisms are similar to that of an another nano-material, which is based on CPP-ACP, and has already been well accepted [Rose, 2000a, b, c]. In addition, the presence of Zn PCA (pyrrolidone carboxylic acid complexed with zinc) in the BioRepair product is assumed to be beneficial with regard to biofilm control. The fibrillar networks derived from PCA [Kezic et al., 2009] can interact with the pellicle structures and, thereby, promote the immobilization and accumulation of the nanoparticle. Furthermore, zinc by itself might exhibit antimicrobial effects against oral microorganisms, including *S. mutans* [Burguera-Pascu et al., 2007; Fang et al., 2006].

Sugar alcohols such as sorbitol and xylitol in the liquid phase of the BioRepair rinsing solution, which amount to about 30% in the product, can be also related to the antimicrobial mechanisms [Bowen, 1994]. Xylitol is present naturally in vegetables and small fruits like berries [Granström et al., 2007] and is used as a non-sugar sweetener with the result of impressive caries reduction [Mäkinen, 2011]. It is claimed to reduce the plaque accumulation and decrease the growth and acid production of *S.*mutans [Söderling et al., 2011]. The antimicrobial mode of action is still not clear. *In vitro* studies provided two principle mechanisms. The first postulated mechanism involves the inhibitory effect of accumulated intracellular xylitol complexes on the glycolytic enzymes, resulting in an impaired bacterial growth and reduced acid production [Trahan et al., 1985]. Another possible explanation is generation of the energy-consuming 'futile xylitol-5-phosphate cycle'. Xylitol taken up by *S.mutans* is phosphorylated to a xylitol complex and then subsequently dephosphorylated and expelled as xylitol in the end [Trahan, 1995]. In this way, the growth of *S. mutans* is retarded. In addition, a recent *in vitro* study suggested that xylitol could decrease polysaccharide-mediated adhesion of *S. mutans* contributing to plaque accumulation with a mechanism not dependent on growth inhibition [Söderling and Hietala-Lenkkeri, 2010]. However, the two major hypotheses mentioned above have not been completely verified under *in vivo* conditions [Takahashi and Washio, 2011], and emphasis is placed on that xylitol is non-fermentable by acid-resistant bacteria in the oral cavity [Takahashi and Washio, 2011]. In spite of these disagreements on the antimicrobial mode of action, xylitol indeed possesses antimicrobial potential under *in vivo* conditions: reduction in the size and growth of *S. mutans* colonies with morphological changes [Lee et al., 2009] and decline in *S. mutans* counts in plaque after short-term xylitol consumption [Söderling et al., 2011]. Additionally, xylitol consumption over a protracted period is shown to result in the elevation of salivary peroxidase

[Mäkinen, 1976], which might be related to the depression of *S. mutans* growth. A recent *in vivo* investigation also demonstrated a significant reduction in the *S.mutans* colonization after a four-week-use of a 20% xylitol mouthrinse [ElSalhy et al., 2012]. A comparable effect of xylitol in the form of mouthrinse to that exhibited by other xylitol products is reasonably anticipated [ElSalhy et al., 2012].

Due to its hexitol nature, sorbitol, the other sugar alcohol contained in the BioRepair solution, is fermentable by several oral microorganisms. The process is yet slower than that of glucose. In addition, the end products are mainly carbon dioxide and fermate (such as hexane). Understandably, sorbitol is able to reduce caries incidence in comparison with glucose. When compared with xylitol, sorbitol possesses a weaker cariostatic efficacy, exhibiting no obvious impaired effect on the dental plaque development and even slightly stimulating the growth of some *S.mutans* [Giertsen et al., 2011]. Nevertheless, the combination of xylitol and sorbitol reduces the plaque mass and distinctly the counts of cariogenic bacteria [Mäkinen, 2011].

Sodium lauryl sulphate (SLS) in BioRepair amounts to about 1 wt%. Based on its well-known surface active properties, SLS had been considered to be the first generation of antimicrobial mouthrinses with excellent inhibitory effects on plaque mass [Mandel, 1994]. In the early 1990s, 1% SLS in mouthwashes was demonstrated to exert similar plaque inhibitory effects compared to a 0.2% triclosan containing mouth rinse [Jenkins et al., 1991]. Thus, it is reasonable that SLS will be involved in the antimicrobial mechanisms of the BioRepair product.

A food preservative contained in the BioRepair product is sodium benzoate, which is demonstrated to suppress the *S. mutans* biofilm formation under *in vitro* conditions [Al-Ahmad et al., 2008]. In an animal experiment, sodium benzoate combined with fluoride revealed a better performance on caries reduction than fluoride alone [Davis et al., 2001]. Moreover, acid killing of *S. sanguis* and *Actinomyces naeslundii* in biofilms has been shown to be enhanced by sodium benzoate [Marquis et al., 2003; Phan et al., 2000]. After application, the undissociated sodium benzoate has a high lipid solubility that allows its accumulation on the cell membranes or on various structures and surfaces of the microorganisms. In an acidic environment such as dental plaque, sodium benzoate is able to combine with a proton and cross the cellular membrane. In the cytoplasm, benzoate acid works as a weak acid through a similar mechanism as fluoride. Thus, the acid stress of plaque streptococci is expected to be enhanced [Marquis et al., 2003]. However, sodium benzoate rinse does not influence the glycolytic activity of dental plaque [Danielsen et al., 1996].

Collectively, the BioRepair mouth rinsing solution might reveal both antibacterial and antiadherent properties, which is in good consistence with the results of the present study. It is assumed that xylitol and zinc-carbonate hydroxyapatite apatites are the main active substances in the BioRepair product.

5.3 Conclusion

In conclusion, the present study confirms the promising antibacterial performance of chlorhexidine. Besides the minor antimicrobial effect, the moderate biofilm inhibitory capacity of the fluoridated mouthrinse is supposed to be associated with its antiadherent properties. In addition, the potent efficacy of the tested mouthrinse containing biomimetic HA nanoparticles to reduce bacterial biofilm formation is presumably ascribed to the antiadherent and antibacterial effects. The present study contributes to a better understanding of the antimicrobial behaviors of HA clusters *in vivo*. The tested 'new' mouthrinse with HA nanoparticles seems to be a reasonable amendment for biofilm management. However, long-term clinical studies based on a larger number of subjects are necessary to be conducted in future.

6 Reference

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8 Curriculum vitae

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