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Effect of Different Mouth Rinses on Salivary Bacteria *in vivo*

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1. Berichterstatter: Prof. Dr. M. Hannig

2. Berichterstatter:

To my Parents

To my beloved husband and my angels

Für meine Eltern

Für meinen geliebten Mann und meine Engel

Abbreviations

AmF	Amine fluoride
cSt	Centistokes
DAPI	4′, 6-diamidino-2-phenylindole
EO	Essential oil
EOMW	Essential oil mouth wash
G	gravitational force
H	Hour
HA	Hydroxyapatite
LDS	Live / Dead staining
max.	Maximum
Mg	Milligram
Min	Minute
min.	Minimum
Ml	Millilitre
nm	Nanometre
pH	Potential of hydrogen
PI	Propidium iodide
Ppm	Parts per million
REO	Rosemary essential oil
Rpm	revolutions per minute
S	Second
Ml	Microlitre

Content

1. Abstract

1.1 English

Effect of Different Mouth rinses on Salivary Bacteria *in vivo*

Objectives: Much research has been conducted to investigate the effect of mouth rinses on oral biofilms. However, only few studies examined the antibacterial effect of mouth rinses on the salivary flora. Therefore, the purpose of this *in vivo* study was to investigate the antibacterial impact of three varied mouth rinses and an oral spray on the salivary flora.

Materials and Methods: Five adult volunteers who presented good oral status performed a single mouthwash or applied an oral spray (Chlorhexamed Forte, BioRepair Zahn- Mundspülung, Elmex Kariesschutz, Theranovis oral spray). Each rinsing/spraying was performed in separate experimental trials with non-stimulated samples of saliva being collected from each volunteer under baseline conditions and at 30 s, 1 h, 6 h and 12 h after performing the tested mouth rinses/ oral spray. The samples were centrifuged and bacterial pellets isolated. Subsequently vital and non-vital bacteria were stained with SYTO 9 and propidium iodide (BacLight^{TM} viability assay) and examined microscopically. Additionally, the bacterial pellets were processed for transmission electron microscopic analysis.

Results: BacLight^{TM} viability assay confirmed with TEM analysis showed that the bacterial viability was reduced after using the tested mouth rinses/ oral spray compared to baseline. As expected, CHX showed high level of antibacterial activity up to six hours. Elmex Kariesschutz has a similar or better immediate antibacterial potential as chlorhexidine, whereas after one hour an obvious recovery in the bacterial vitality was detected. BioRepair revealed also good antibacterial effect up to one hour. However, Theranovis had the weakest antibacterial action on the salivary flora as only a slight decrease in the bacterial vitality was detected one hour after rinsing.

Conclusion: The results of the present study revealed initial antibacterial effects of all tested mouth rinses/ oral spray on the salivary bacteria. This study also allows classification of the tested mouth rinses/ oral spray according to their effectiveness on the salivary flora in terms of amount of leftover living bacteria. Under *in vivo* condition, the classification was as follows: CHX forte has good antibacterial effect up to 6 h, BioRepair and Elmex Kariesschutz are mouth rinses with moderate antibacterial effects and Theranovis oral spray revealed a non-considerable antimicrobial effect on the salivary flora.

1.2 German

Antibakterielle Wirkung von verschiedenen Mundspüllösungen auf die Speichelflora *in vivo*

Ziel der Untersuchung: Die Wirkung von Mundspüllösungen auf den Biofilm wurde bereits mehrfach untersucht. Allerdings wurde nur in wenigen Studien die antibakterielle Wirkung von Mundspüllösungen auf die Speichelflora analysiert. Das Ziel der vorliegenden i*n-vivo* Studie war es, die antibakterielle Wirkung von drei verschiedenen Mundspüllösungen und einem Mundspray auf die Speichelflora zu untersuchen.

Material und Methode: Fünf erwachsene Probanden mit gutem Mundhygienestatus führten jeweils Mundspülungen mit Chlorhexamed Forte (CHX), BioRepair Zahn- und Mundspülung oder Elmex Kariesschutz durch. Außerdem wurde von den Probanden ein Mundspray (Theranovis) angewendet. Die Speichelproben wurden wie folgt von jedem Probanden entnommen: Nicht-stimulierter Speichel als Ausgangswert sowie 30 s, 1 h, 6 h und 12 h jeweils nach der Anwendung. Die Proben wurden zentrifugiert und ein bakterielles Pellet isoliert. Anschließend wurden vitale und avitale Bakterien mit SYTO 9 und Propidiumiodid (*Bac*Light™ viabiltiy assay) angefärbt und mikroskopisch untersucht. Zusätzlich dazu wurden die Bakterienpellets mittels Transmissionselektronmikroskopie (TEM) analysiert

Ergebnis: Wie mittels *Bac*Light™-Färbung gezeigt und durch TEM Untersuchungen bestätigt wurde, ist die bakterielle Vitalität nach der Anwendung der verschiedenen Mundspülungen bzw. des Mundsprays im Vergleich zum Ausgangswert reduziert. CHX zeigte eine erhebliche antibakterielle Aktivität bis zu 6 Stunden, Elmex Kariesschutz und BioRepair Zahn- und Mundspülung zeigten eine gute Wirkung bis zu einer Stunde, wobei das Theranovis-Mundspray die schwächste antibakterielle Wirkung aufwies.

Zusammenfassung: Die vorliegende Studie zeigt den antibakteriellen Einfluss aller getesteten Mundspüllösungen sowie des Mundsprays auf die Speichelmikroflora. Sie ermöglicht auch eine Klassifizierung der getesteten Mundspüllösungen bzw. des Mundsprays aufgrund ihrer Wirksamkeit auf die Speichelflora unter dem Aspekt der Anzahl residualer vitaler Bakterien. Unter *in vivo*-Bediengungen ergab sich die Klassifizierung wie folgt: CHX hat eine starke antibakterielle Wirksamkeit bis zu sechs Stunden. BioRepair und Elmex Kariesschutz zeigten eine gute antibakterielle Aktivität bis zu einer Stunde. Daher sind Elmex Kariesschutz sowie BioRepair als Mundspüllösungen mit moderater antibakterieller Wirkung einzustufen. Theranovis-Mundspray hat die geringste Wirkung auf die Speichelmikroflora.

2. Introduction

Thoughout history mankind has practiced oral hygiene. Today, the use of toothbrushes along with toothpaste containing fluoride is globally accepted (van der Weijden & Hioe, 2005).

Over the past 50 years there is a general health understanding amongst the masses of how important oral hygiene and the prevention of teeth related problems (e.g. cleaning teeth with a toothbrush, mouth rinsing, the use of fluoride) led to the decline of prevalence of oral diseases (caries and periodontal diseases) during the past 50 years (Longbottom *et al.*, 2009; Wolff & Larson, 2009). However, there is still a need for efficacious prevention strategies as these oral diseases continue to be an issue for many people around the world (Burt, 1998; Sarner *et al.*, 2012).

The choice of oral care products which people can use at home is extensive. Mouth rinsing has grown in popularity as an important step after brushing for the prevention of oral problems (Jardim *et al.*, 2009; Sarner *et al.*, 2012) and is one of the most effective ways to reduce oral microorganisms (Yousefimanesh *et al.*, 2015).

There has been much research into how mouth rinses are effective against both plaque and inflammation (Gusberti *et al.*, 1988; Solis *et al.*, 2011). It was found that mouthwashes are very useful in reducing bacterial plaques (Yousefimanesh *et al.*, 2015).

The purpose of this study was to investigate *in vivo* the reducing of salivary bacteria after mouth rinsing. We chose for this investigation two classic mouthwashes (chlorhexidine solution, fluoridated mouthwash), a new commercially available mouth rinse containing zinc-carbonate hydroxyapatite nanoparticles, and essential oils in oral spray form.

2.1 Review of Literature

2.1.1 Oral flora

The oral cavity provides many soft and solid surfaces, each coated with a plethora of bacteria organized in biofilms. The human oral microbiome is the most studied human microflora (Chen *et al.*, 2010). Several of these microorganisms have been involved in both caries and

periodontitis, these are almost the most widespread bacterial diseases today (Aas *et al.*, 2005). Furthermore, specific species of oral microorganisms have been found to play a role in certain systemic diseases like bacterial endocarditis (Berbari *et al.*, 1997), pediatric osteomyelitis (Dodman *et al.*, 2000), aspiration pneumonia (Scannapieco, 1999), the low weight of premature babies (Offenbacher *et al.*, 1998; Buduneli *et al.*, 2005) or cardiovascular disease (Beck *et al.*, 1996; Wu *et al.*, 2000). The oral flora comprises one of the densest and most variable microbial populations in the body and its composition has been the focus of many investigations.

The salivary flora consists of 619 taxa of oral bacteria according to Dewhirst *et al.* (2010), Table 1 shows a breakdown of the phylogenetic constitution of these taxa. As can be seen 65.6% of the taxa have been cultured. This is quite excessive when compared to natural environments where less than 1% has would have been cultivated. 96% of the taxa is found in the six major phyla: *Spirochaetes, Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria.* The other 4% is found in the he residual phyla: *Synergistetes, Tenericutes Euryarchaeota, SR1, Chlamydia, Chloroflexi* and *TM7*.

Table 1 Phylogenetic distribution of 619 taxa in HOMD (Human Oral Microbiome Database) version 10

Phylum	No. (%) Taxa	Named species	Unnamed cultivated taxa	Unnamed uncultivated taxa
Bacteria				
Firmicutes	227 (36.7)	120 (52.9)	45 (19.8)	62 (27.3)
Bacteroidetes	107 (17.3)	39 (36.4)	27 (25.2)	41 (38.3)
Proteobacteria	106 (17.1)	70 (66.0)	9 (8.5	27 (25.5)
Actinobacteria	72 (11.6)	37 (51.4)	25 (34.7)	10 (13.9)
Spirochaetes	49 (7.9)	11 (22.4)	3 (6.1)	35 (71.4)
Fusobacteria	32 (5.2)	12 (37.5)	4 (12.5	16 (50.0
TM7	12 (1.9)	0 (0.0)	0 (0.0)	12 (100.0)
Synergistetes	10 (1.6)	2 (20.0)	0 (0.0)	8 (80.0)
Chlamydiae	1 (0.2)	1 (100.0)	0 (0.0)	0 (0.0
Chloroflexi	1 (0.2)	0 (0.0)	0 (0.0)	1 (100.0)
SR1	1 (0.2)	0 (0.0)	0 (0.0)	1 (100.0)
Archaea				
Euryarchaeota	1 (0.2)	1 (100.0)	0 (0.0)	0 (0.0)
Total	619 (100)	293 (47.3)	113 (18.3)	213 (34.4)

Since the oral cavity is a constantly changing ecosystem with multiple microflora. No clear no advantage would arise from removing all bacteria in order to control plaque-associated infections. A more advisable approach might be to eliminate only the most cariogenic and periodontopathic microorganisms from the oral microflora, while allowing the non-pathogenic bacteria to remain (Slee & O'Connor, 1983). Nowadays several antiseptic agents are available in the market, for the purpose of reducing oral bacteria and biofilms management.

2.1.2 Mouth rinses

Mouth rinses have been in use for centuries as breath fresheners, medicaments and antiseptics. Only in more recent times, however, mouth rinses have been given much credence as preventive agents against mouth and dental disease (Mandel, 1988). The most chemical plaque-control agents, which exist in the toothpaste, have been evaluated and later formulated in the mouth rinses. Mouth rinses formulas are basically simpler than toothpastes. They can be simple aqueous fluids, in order for them to be consistent and accepted in taste makes it important to add some components for flavoring, coloring and preserving such as sodium benzoate. Anionic detergents, such as phosphate, sulphate and chloride, are included in some products but they cannot be formulated with cationic antiseptics such as cetylpyridinium chloride or chlorhexidine. There are two main indications for using mouth rinses - promotion of oral mouth and dental health. This could be the elimination of plaque and gingivitis or hindering infection caused by oral flora in specific cases such as intraoral surgical procedures, or immuno suppression after transplantations or cancer therapies (Cannell, 1981; Ciancio, 1994).

In this study, four different mouth rinses available in the market (Chlorhexamed 0.2%, Elmex Karies schutz, BioRepair, Theranovis spray) were included and compared regarding their antiseptic effect on the salivary flora.

2.1.2.1 Substantivity of Mouth rinses

Weiland *et al*. (2008) defined Substantivity as the duration in which a formulation performs with a persistent action *in vivo*. The most effective plaque inhibitory agents in the antiseptic or antimicrobial mouth rinses are those showing persistence of effect in the mouth for hours. This persistence of action, termed substantivity (Kornman, 1986), appears dependent on several factors:

1. Adsorption and prolonged retention on oral surfaces.

2. Maintenance of antimicrobial activity once adsorbed primarily through a bacteriostatic action against the primary plaque forming bacteria.

3. Minimal or slow neutralization of antimicrobial activity within the oral environment or slow desorption from surfaces.

2.1.3 Chlorhexidine

Chlorhexidine is an antimicrobial (antiseptic) agent with low mammalian toxicity and a strong tendency to bind to mucous membranes (Denton, 2001). It is effective in preventing and controlling plaque formation, inhibiting and reducing the development of gingivitis (Lang, 2006). At physiological pH, chlorhexidine is a big cationic molecule, with the positive charge found over the nitrogen atoms on both sides of the hexamethylene bridge. Chlorhexidine can be adsorbed not only into pellicle and saliva, but also into other parts of oral cavity (tooth and mucosa membrane (Jones, 1997).

2.1.3.1 Antibacterial activity of Chlorhexidine

Chlorhexidine has a membrane-active antimicrobial effect attacking different types of bacteria both gram-positive and gram-negative, yeast and fungi including *candida*, selective dermatophytes, lipophilic pathogens and some viruses including *HBV* and *HIV*.

Unlike other antimicrobials, chlorhexidine has demonstrated some effectiveness against microorganisms in other forms and states as well. This includes *bacterial spores* and *protozoa* (McDonnell & Russell, 1999). Its effect differs according to concentration; the agent demonstrates a bacteriostatic effect at low concentration and a rapidly bactericidal effect at higher concentration. (Woodcock, 1988)

As the bacterial cell is negatively charged, the cationic chlorhexidine molecule is rapidly attracted to its Surface, thereby retaining phosphate-containing compounds. This process leads to a change in the integrity of the bacterial cell membrane causing the chlorhexidine to target the inner cell membrane. Chlorhexidine bonds with phospholipids, causing an increased permeability of the inside membrane and low-molecular-weight constituents like potassium irons to leak. (Denton, 2001). The higher the concentration of chlorhexidine, the greater the loss in permeable species from the cell and hence the greater the damage to the membrane.(Rolla & Melsen, 1975; Kuyyakanond & Quesnel, 1992)

2.1.3.2 Substantivity of Chlorhexidine

In the mouth chlorhexidine readily adsorbs into surfaces. Once adsorbed, and unlike some other antiseptics, chlorhexidine acts persistently bacteriostatic (high substantivity) for a duration of approximately 12 hours (Cousido *et al.*, 2010) (Tomas *et al.*, 2010). And in fact,

chlorhexidine's antimicrobial activity has been documented to last at least 48 hours on the skin (Hibbard, 2005). Additionally numerous authors have shown that CHX has a greater substantivity than other oral antiseptics (Moran *et al.*, 1992; Jenkins *et al.*, 1994; Elworthy *et al.*, 1996; Balbuena *et al.*, 1998) and has a persistent bacteriostatic action (high substantivity) lasting from 12 up to 24 hours (Bonesvol *et al.*, 1974).

2.1.3.3 Chlorhexidine products

Chlorhexidine is available in the form of solutions (0.12% and 0.2%), gels (1%), sprays (0.1% and 0.2%), dentifrices (0.4%), chewing gum, and varnishes (1%, 10%, 20%, and 35%) (Lindhe, 2007).

2.1.3.4 Clinical uses of Chlorhexidine

A number of clinical applications has been recommended for chlorhexidine reviewed by Addy and Moran 1997 (Addy & Moran, 1997). These applications include:

1. adjunct to mechanical oral hygiene
2. secondary prevention following oral surgical procedures
3. oral hygiene for the mentally and physically disabled
4. patients with intermaxillary fixation
5. medically compromised patients predisposed to oral infections
6. high-risk caries patients
7. patients suffering from minor recurrent aphthous ulceration
8. patients wearing removable and fixed orthodontic appliances
9. immediate preoperative chlorhexidine rinsing and irrigation.

2.1.3.5 Side effects of Chlorhexidine

Used as a mouthwash, reports show that chlorhexidine has many local side effects. (Flotra, 1971):

1. brown discoloration of the teeth, tongue and some restorative materials
2. taste perturbation affecting salty food and beverages, leaving them bland
3. rarely seen oral mucosal erosion, desquamation of the oral mucosa
4. extremely rare unilateral or bilateral parotid swelling
5. enhanced supragingival calculus formation.

Thus, mouth rinses containing CHX cannot be used for long-term oral care. The effect of chlorhexidine might be increased by restricting the consumption of specific foods and beverages during treatment with CHX, in particular just after applying the chlorhexidine formulation. It would, therefore, be reasonable to avoid the consumption of tea and coffee immediately after rinsing with chlorhexidine. Similarly, it would be recommendable to use the mouthwash as a final oral hygiene measure at night since no beverages will be consumed during sleep (Jones, 1997).

2.1.4 Fluoride

One of the most important milestones in the history of dentistry was the discovery of the anticardiogenic properties of fluorides (Peter, 2009). Fluorides are important for the improvement of oral health and are commonly found in oral hygiene products. They perform a dual function – enabling the remineralization and hindering the demineralization of the enamel. Furthermore, fluorides promote antibacterial activity especially when combined with amine or tin (Van Loveren, 2001).

It has been known for a while that amine fluorides (AmF) actually decreases caries and dental plaque. When oral hygienic products containing AmF as an AmF/SnF2 combination are used frequently, dental plaque is prevented from building up, thus dental disease (Madlena, 2013).

2.1.4.1 Amine fluorides in dentistry

Amine fluoride (AmF) is an organic type of fluoride. This type of fluoride is known for reducing the adhesiveness of dental plaque, due to the compatibility of the hydrophilic counterions to the enamel surface. It spreads quickly over all surfaces in the oral cavity, shows longer

clearance in the oral cavity and dental plaque, and has a pronounced activity on plaque (Madlena, 2013). Muhlemann and co-workers in 1957 and 1960 analysed the difference between organic and inorganic fluoride in relation to their impact on preventing enamel solubility. They discovered organic fluoride like amino fluoride were highly effective compared to inorganic fluorides (Muhlemann, 1957). Based on favorable characteristics detected in *in vitro* and *in vivo* studies, amine fluoride products were suggested as alternate or adjuncts for systemic fluoridation by Muhlemann (1967) and Schmid (1983) (Muhlemann, 1967; Schmid, 1983). Amine fluoride is available in the form of dentifrices, gels, mouth rinses, and for healthcare professionals in the form of prophylaxis pastes (Madlena, 2013).

2.1.4.2 Substantivity of Amine fluoride

In fact, previous studies, which investigated fluoride retention in oral cavity, provided evidence of amine fluoride lasting much longer than sodium fluoride (Hassell *et al.*, 1971; Muhlemann & Rudolf, 1975). AmF is strongly glycolytic (for 3-6 hours) and develops a highly bacteriostatic and bactericide effect (Brecx, 1997; Madlena, 2013). It was also found that amine fluoride has good substantivity and antibacterial efficacy up to 8h (Weiland *et al.*, 2008).

2.1.4.3 Clinical studies with amine fluoride containing products (Elmex Kariesschutz)

Clinical studies with amine fluorides have been performed using gel, toothpaste, combinations of toothpaste and gel/fluid, or combinations of toothpaste and mouth rinse (Madlena, 2013). In the early period clinical studies were published on AmF toothpaste, where the first was published by Marthaler (1968). Most of them showed considerable reduction in the recurrance of caries [in DMFT (Decayed, Missing, Filled Teeth)/ DMFS (Decayed, Missing, Filled Surfaces) values between 7.1 and 35% DMFT-S]. Similar studies were performed on amine fluoride gel showing caries reductions of 31-53% DMFS (Madlena, 2013).

2.1.4.4 Effects of amine fluorides

1. 1. Fluoride ions have the potential to impact the tooth enamel. Either through reacting with hydroxyapatite present in the dental enamel or creating a calcium fluoride depos-

it. Exactly how much fluoride absorbed is dependent on the PH of applied fluoride solutions. Absorption and long-time retention of fluoride ions can only take place under weak acidic conditions allowing a reaction between fluorides and dental enamel. The weak acidic nature of dissolved amine fluorides has a slightly acidic PH ranging from of 4.5 to 5.0. This acidic PH value together with the surface activity cause the dental enamel to absorb fluoride ions and a long-lasting depot to build. As demonstrated *in situ* by Klimek *et al.* (1998), a tooth paste containing amine fluoride (pH 5.5) led to an increased amount of fluoride on enamel (KOH-soluble fluoride or bound fluoride) compared to one containing a sodium fluoride (pH 7.0)

2. Remineralizing the enamel

Fluoride is the most commonly used remineralizing agent because it facilitates remineralization by promoting the growth of enamel crystallites which had been previously demineralized. AmF compounds showed a better improvement in enamel micro hardness when compared to NaF (Priyadarshini *et al.*, 2013).

3. Enhanced remineralization of initial caries lesions

The effect of inhibiting the formation of dental caries is known as the *cariostatic* action of fluorides. This is achieved by the building up of fluorhydroxyapatite which aid the remineralization of the initial carious lesions. Consequently, the speed at which demineralization occurs is reduced (Lata *et al.*, 2010)

As a calcium fluoride protective layer forms on the enamel, it provides an oral fluoride deposit. This enables fluoride ions to be released slowly but constantly over an extended period. However, increased quantities are released when an acid attack occurs. It is the low levels of fluoride which are always present in saliva, that expediate the natural remineralization process. This process results in the mineral deficit being recovered and the damage being reversed. Buchalla *et al* (2002) showed positive results *in situ.* To stimulate mineral growth in enamel lesions just one dose of amine fluoride (Elmex fluid®, 10000 ppm F resulted in remineralization of initial caries lesions.

4. Inhibiting acid production by plaque bacteria (antiglycolytic efficacy)

Bacteria convert sugar enzymatically into energy, thereby forming acids. These acids demineralize and damage the dental enamel. Amine fluoride reduces caries by inhibiting acid pro-

duction of plaque bacteria (Capozzi *et al.*, 1967). Bibby and van Kesteren (1940) were the first to demonstrate the inhibition of carbohydrate metabolism of pure cultures of oral streptococci and lactobacilli by fluoride.

Since then, numerous papers have been published reporting on the influence of fluoride on sugar metabolism in dental plaque, salivary sediment, and pure cultures of oral bacteria (Hamilton, 1977; Hamilton, 1988). The impact of fluoride on oral microorganisms and plaque has been extensively researched (Bowden, 1990; Hamilton, 1990; Tatevossian, 1990).

Proton-extruding ATPase as well as enzyme enolase can both be inhibited due to the mechanisms that enable fluoride to interfere with bacterial metabolism and dental plaque acidogenicity. Moreover, Fluoride ions potentially also have an effect on intracellular or enzymes associated with plaque like catalase, pyrophophatase, perixodase and acid phosphatase. (Hamilton, 1990). Bacterial cell functions such as macro-molecule synthesis and glycolysis are affected by fluoride in such a way that enolase, phosphoenolpyruvate phosphotransferase and H+-ATPase are inhibited (Hamilton, 1988).

Fluoride can inhibit bacterial enzymes, however, this inhibition is only effective at high fluoride concentrations (Hamilton, 1988). However, this Concentrations cannot be achieved in the plaque by using fluoride-containing toothpastes and mouth washes. For example, no different levels of mutans streptococci or lactobacilli were found regardless of whether subjects used fluoridated toothpaste or not (Petersson *et al.*, 1991). There were no differences in the distribution of children with *Streptococcus mutans* between water-fluoridated and non-fluoridated areas (Burt *et al.*, 1986).

5. Anti-adherent and plaque reducing efficacy of AmF

An *in vitro*-test was conducted on the surface of rat molars. The molars which were treated with AmF and AmCl showed resilience to the build-up of *Streptococcus mutans* OMZ 176, whereas when treated with NaF there was no impact at all.

These finding may explain why AmCl is present in so many topical applications of amine compounds as cationic and detergent agents. The reason is most likely the anti-plaque impact as opposed to the transformation of hydroxyapatite to fluorapatite in the molar surfaces (Balmelli *et al.*, 1974). In another study, *in vivo* results obtained by examining pretreated enamel surfaces with AmF, AmCl, and Na_2PO_3F proved that the amine group reducing surface energy, and not the fluoride incorporation into the enamel surface, is a major factor in

plaque prevention (Weiss *et al.*, 1977). Thus, organic fluorides, like amine fluorides (AmF) are better anticaries agents, have a significant antibacterial activity against cariogenic bacteria (Schmid *et al.*, 1984; Rosin-Grget & Lincir, 1995), and an antibacterial effect against oral and periodontal pathogens (Bullock *et al.*, 1989; Bansal *et al.*, 1990).

In summary, two dynamic elements make up the AmF molecule. First, that which provides anticaries properties – this is the floride ion. Second, a cationic surface-active agent – known as the organic amine.

Since amine groups are positively charged, amine excesses of amine fluorides have specific possess antibacterial properties. This enables them to decrease the acid production of bacteria by hindering metabolic activity. Tests *in vitro* were undertaken by Gehring (1983) into how *Streptococcus mutans* reacted to the antibacterial effects of amine fluoride and sodium fluoride. It was found that, unlike sodium fluoride, Amine fluoride was effective in breaking down bacterial acid production and in doing so destroyed salivary plaque bacteria. Thus the effect of AmF could be caused by preventing bacterial adhesion to teeth, directly killing bacteria, impeding the increase of the cariogenic streptococci (Bramstedt & Bandilla, 1966; Shern *et al.*, 1970) and maybe hindering enolase and phosphoglyceromutase (Harding *et al.*, 1974; Schneider & Mühlemann, 1974).

2.1.5 Apatite-containing Mouth rinses

Increasing the calcium concentration in oral fluids enhances lesion remineralization (Featherstone, 2009) . This was endorsed through a study comparing the remineralization of sodium fluoride (NaF) combined with nano-HA on primary carious lesions which clarified that the larger the amount of nano-HA in NaF mouth rinse, the higher the remineralization of enamel. This was a significant statement as it observes a synergetic role for nano-HA combined with a fluoridated mouth rinse (Xiangcai *et al*, 2005). A further study suggested that the micro-structural surfaces of the demineralized enamel showed signs of hardening with increasing treatment time and nano-HA concentration. This shows us that the effect in the NaF groups was higher than with distilled water and almost proves nano-HA to create a synergistic role in remineralization with almost all fluoride mouth rinses (Kim *et al*. 2007). For these re-

sons, research was redirected to develop preventive agents that can act in a quasi-manner to fluoride as an adjunct or independent of it.

2.1.5.1 Hydroxyapatite

The first commercial interest in hydroxyapatite was initiated by the Japanese company Sangi Co. Ltd. In 1970. They purchased the rights from NASA (U.S. National Aeronautics and Space Authority). Previous investigations into the health impact of astronauts had found that a loss of gravity had led to a decrease in the mineral density of teeth and bones. To remedy this NASA proposed using a material for repair - synthetic hydroxyapatite. In 1978, the Sangi Co. Ltd introduced a toothpaste containing for the first time nano-hydroxyapatite that could repair the tooth enamel (Apadent). In 2006, Europe saw the launch of a toothpaste which was an alternative to fluoride. The new toothpaste contained synthetic hydroxyapatite biomimetic, the active ingredient for remineralization and repair of tooth enamel (Pepla *et al.*, 2014).

2.1.5.2 Nano-hydroxyapatite

Nano-hydroxyapatite (n-HA) is regarded as a highly biocompatible and bioactive materials and has therefore been widely accepted in the fields of medicine and dentistry. (Hannig & Hannig, 2010). Nano-hydroxyapatite is able to dramatically remineralize initial enamel lesions in both restorative and preventive dentistry and has shown good results affecting tooth sensitivity (Pepla *et al.*, 2014). Nano-HA has been extensively applied in oral implantology, oral and maxillofacial surgery and periodontology due to its unique properties. Firstly, it can chemically bind itself to bone; secondly, via interacting with the osteoblasts it can stimulate bone growth and thirdly, it does not promote toxicity or inflammation. Compared to typical HA, nano-HA possesses certain distinct properties. These include greater surface energy, enhanced solubility, and optimal biocompatibility. Nano-HA is also a good substitute for bone and tooth as it has several advantages including high toughness, high strength, high density, long shelf life and optimal biocompatibility (Suchanek & Yoshimura, 1998). Another study evaluated the effect of nano-HA solution on erosive lesions and showed that enamel microhardness enhanced significantly after exposure to nano-HA solution (Haghgoo *et al*, 2011). Additionally, nano-HA is used as a filler, patching little holes and hollows on the tooth's surface. (Pepla *et al.*, 2014).

2.1.5.3 Antibacterial\ anti-adherent effect of nano- HA

Besides the remineralization effect of nano-HA, also in addition to its well-accepted effects, it has an anti-adherent effect. This anti-adherent property can be ascribed to the dimensions of the adopted biomimetic hydroxyapatite nano particles which mimic enamel crystallites (the smallest structure units of dental enamel). BioRepair's microclusters are composed of nano-sized crystallites (Hannig *et al*., 2013a).

Moreover, due to size effects, hydroxyapatite nanoparticles can enter into direct interaction with the bacterial membrane, thus facilitating the interactions with the bacteria. An *in vitro* study showed that both non-aggregated and accumulated hydroxyl-apatite nano-crystallite molecules (average size 100×10×5 nm) can cling onto the bacterial membrane, influencing the adhesins in order to reduce bacterial adherenc (Venegas *et al.*, 2006).

Another nano-sized (diameter of 2.12 nm) substance comprising of casein phosphopeptide stabilized amorphous calcium phosphate compounds has displayed a noticeable degree of attraction to the bacterial film in general and in particular to the bacterial surface. Probably, the bacterial receptors shut down; coaggregation and bonding to the pellicle are minimised.

Similiar processes may be happening with the tested nano-sized particles of the micro clusters known as hydroxyapatite crystallites (Rose, 2000a; b; c). The adhesion of small HA particles onto the larger bacteria was also seen by SEM in sonicated media (Venegas *et al.*, 2006). The antiadhesive effects can be ascribed to the liquid phase's components like xylitol. It can also be attributed to the hydroxyapatite micro clusters. However, it is components of the liquid phase rather than the hydroxyapatite micro clusters that seem to promote the antibacterial characteristics. In a previous *in situ* study (Hannig *et al.*, 2013a), reduction of the initial bacterial colonization of the enamel surface was observed after application of the customary preparation containing zinc-carbonate hydroxyapatite microclusters (Biorepair) and of the separate components. After 12 h, $1.3\times10^7\pm2.0\times10^7$ bacteria/cm² were found on control samples without rinsing,including DAPI staining; $2.4\times10^6\pm3.3\times10^6$ after using BioRepair. Also pure hydroxyapatite microclusters in saline solution ($2.1\times 10^6\pm3.0\times10^6$) and the liquid devoid of particles ($5.1\times10^5\pm3.3\times10^5$) reduced the amount of adherent bacteria. Moreover, antimicrobial effects on *Streptococcus mutans* were observed *in vitro* (Hannig *et al.*, 2013a).

2.1.5.4 Zinc

Many studies demonstrate that Zinc salts supress the formation of *Streptococcus* bacteria (Gallagher & Cutress, 1977; Bates & Navia, 1979; Oppermann *et al.*, 1980). It was also demonstrated that the zinc ions hinder both the adsorption of bacteria to the tooth surface and the growth of existing ones (Harrap *et al.*, 1984; Saxton, 1986). But it is worth to mention that further research was carried out by Sodeberg *et al.* (1990). Findings concluded that zinc ions had the greatest influence on gram-positive bacteria, whereas there was little or no impact on gram-negative aerobic bacteria, even when the zinc concentration was high (1024 µl/ml)(Soderberg *et al.*, 1990).

There may be several mechanisms for the antibacterial action of the zinc ion. One explanation put forward is that zinc attaches itself to the membranes of microorganisms, compariable to mammalian cells (Sugarman, 1983). When zinc is applied at a high concentration it hinders the cellular function. Potent levels of zinc - between 10^5 M and 10^3 M, have the effect of reducing sugar transport, amino acid uptake, and electron transfer in bacteria. This is in part attributed to the attachment of zinc to sulfhydryl or histidine deposits and the consequential demise of the enzyme function (Kasahara & Anraku, 1972; Singh & Bragg, 1974).

More recently a comparative study of six metal oxide nanoparticles found that ZnO nanoparticles were clearly more effective in restraining the growth of various pathogenic bacteria (Jones *et al.*, 2008). The fact that high concentrations of zinc remain in saliva even after 24 hours of rinsing verify such claims about Zn in the oral cavity (Burguera-Pascu *et al.*, 2007).

2.1.5.5 Substantivity of BioRepair

A previous study looking at BioRepair and its subfractions, found there were noticeable effect after 6 to 12 hours on the number of adherent bacteria observed with DAPI staining and *Bac*-Light. These findings are similar to those witnessed after using chlorhexidine as a mouth rinse (Hannig *et al.*, 2013b).

2.1.6 Essential oils

Essential oils are the volatile oily composites which are aromatic in nature. They are derived from plant sources including flowers, roots and seeds. Many processes have been developed for the collection of such oils - expression, fermentation, enfleurage or solvent extraction. Most commercial production of EOs utilizes steam distillation (Burt, 2004). Throughout the ages, people have used essential oils and other extracts of plants as a part of everyday life and as cures for everyday ailments (Jones, 1996). Now research is being conducted into their impact on infectious diseases. It was the World Health Organisation (WHO) that acknowledged how many people around the world rely on such traditional cures as the main medicine for primary care (Prabuseenivasan *et al.*, 2006). Other uses of plant based oils and extracts are food preservation, natural therapies as well as in pharmaceuticals (Martindale, 1996; LisBalchin & Deans, 1997). It has been found that plants can have antibacterial, antiviral, insecticidal and antioxidant properties. Since they contain large amounts of biologically active compounds, certain oils have been beneficial in cancer treatments. One of the major consumers of oils is the fragrance industry. (Prabuseenivasan *et al.*, 2006; da Silva *et al.*, 2008).

A good example to cite is a member of the mint family – a plant called rosemary. This is widely grown evergreen fairly fragrant shrub (Moss *et al.*, 2003) and often used in food preservation. Studies done on rosemary essential oil (REO) have verified its naturally strong antioxidant qualities which are beneficial in deterring both fading of color and solube oxidation (Aruoma *et al.*, 1992; Balentine *et al.*, 2006; Hussain *et al.*, 2010). In a further *in vitro* study REO was found to deter borne pathogens (Smith-Palmer *et al.*, 1998; Hammer *et al.*, 1999). In a recent study, antibacterial properties of REO were further examined. The study concentrated on the effects of these properties on various types of bacteria. There are 3 types of properties – Minimal bactericidal concentration (MBC), Minimum inhibitory concentrations (MICs), and Time–kill dynamic processes. The rrosemary essential oil and 1,8-Cineole and –Pinene were tested on the following bacteria and fungi: Three gram-positive bacteria (*Staphylococcus epidermidis, Staphylococcus aureus and Bacillus subtilis*); three gram-negative bacteria (*Proteus vulgaris, Pseudomonas aeruginosa and Escherichia coli*) and two fungi (*Candida albicans and Aspergillus niger*). For all the tested microbes from the above categories, the REO displayed the most antibacterial and antifungal activity compared to 1,8-Cineole and Pinene (Jiang *et al.*, 2011). In particular, REO hindered the development of three different types of pathogenic bacteria (*Escherichia coli, Streptococcus indiana, and Listeria*

innocua), however it only succeeded in eliminating *Escherichia coli*. Celiktas *et al.* (2007), also verified these findings in relation to *Escherichia coli* (Knobloch, 1989; Yesil-Celiktas *et al.*, 2007).

Another common oil, widely cultivated in Europe, USA and Japan is the essential oil of peppermint (*Mentha piperita-Lamiaceae/Labiatae*). It is used on a commercial scale mostly by the personal care, food and pharmaceutical industries. Essential oil of peppermint is obtained by harvesting the leaves and then extracting oil from by steam distillation. The resulting oil is constituted of the following: Between 38 to 48% was menthol – falling under the category of monoterpinic alcohol; between 20 and 30% was menthones – falling under the category of ketones; lastly, the category of monoterpens and oxides was represented. While the aroma of peppermint essential oil (*Mentha piperita*) may be synonymous in some minds with the candy, it is a lot more than flavoring. It is a good antiseptic, antibacterial and antiviral, helpful for neuralgia and treating general feelings of illhealth as well as common complaints like headaches and migraines.

For centuries for it has been used for digestive problems given its anti-spasmodic properties, as well as sinus and respiration complaints (Thosar *et al.*, 2013). Frequently it is used in toothpaste and chewing gum for oral health as peppermint oil gives a refreshing feeling in the mouth, has a pleasant smell and tastes good. Another benefit is its ability to stimulate salivation thus preventing foulness of breath – halitosis caused by dryness of mouth. (Dawes & Macpherson, 1992). It has cooling effect in case of varicose veins and hemorrhoids (Ratan, 2006).

In an *in vitro* study, peppermint oil showed no antibacterial activity against *streptococcus mutans* (Chaudhari *et al.*, 2012); whereas Shapiro et al. (1994) demonstrated that the antimicrobial properties of oils from peppermint were one of the most potent against facultatively anaerobic oral bacteria (Shapiro *et al.*, 1994). The essential oil of peppermint showed the same antibacterial effect when tested on eighteen different types of bacteria (including gram-positive cocci, rods, gram-negative rods) across the spectrum. Eleven fungi were tested using the disc diffusion technique and also displayed the same results (Pattnaik *et al.*, 1996).

The antimicrobial activity of essential oils has been the subject of both *in vivo* and *in vitro* tests, only certain oils have a shown this as a potential quality. When demonstrated it has been a primary action of attacking the cell membrane, breaking it down until it leaks and eventually

dies. A secondary action could be preventing membrane synthesis and preventing cellular respiration (da Silva *et al.*, 2008). Since essential oils have a high volatility and lipophilicity they are able to easily penetrate into the cell membrane and break it down biologically (Inouye, 2003). It has been shown that essential oils are active against pathogens which cause oral infections. In comparison to other antimicrobial agents they are a low cost and effective method to control such bacteria. However, further research *in vivo* is required into the safety and reliability of such products (Chaudhari *et al.*, 2012). Commonly available essential oils mouthwashes (EOMW), were seen to be a long term viable option for those not wanting to use chlorhexidine mouthwash for gingival inflammation (Van Leeuwen *et al.*, 2011). But according to systematic reviews, an analysis of long-term studies lasting four weeks or over provided evidence of chlorhexidine mouthwash providing substantially better plaque control than EOMW (Van Leeuwen *et al.*, 2011).

2.1.6.1 Substantivity of Essential oils

Few studies measured the substantivity of the EOMW (Quintas *et al.*, 2015), they only assessed the immediate antimicrobial effect (Shapiro *et al.*, 1994; Chaudhari *et al.*, 2012). Only one *in situ* study recorded the substantivity of EOMW, and showed them to be present for at least 7 h after its application (Quintas *et al.*, 2015).

2.2 Aim of this work

Much research has been conducted to investigate the effect of mouth rinses on oral biofilms (Shani *et al.*, 2000; Hannig *et al.*, 2013b) , whereas only few studies investigate the antibacterial effect of mouth rinses on the salivary flora (Addy *et al.*, 1991; Cousido *et al.*, 2010). Therefore, the aim of this study was to:

1. Investigate the *in vivo* antibacterial effects of three mouth rinses (Chlorhexamed forte, BioRepair Zahn- und Mundspülung, Elmex Karies schutz) and one spray (Theranovis spray) on the salivary flora up to 12 h after rinsing/ spraying.
2. Compare the antibacterial efficacy of the four tested products on the salivary flora.

3. Material and Methods

3.1 Subjects

5 adults between the ages of 20 and 40 participated in the study. They belonged to the staff of the Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University. The volunteers were selected on the basis of their overall oral health using the following criteria: At least 24 permanent teeth without the presence of oral diseases such as gingivitis or periodontitis (Community Periodontal Index score=0) (WHO, 1997) and no caries. Exclusion from the study was on the grounds of: being a smoker, having dental prostheses or orthodontic devices, taking undergoing antibiotics or regularly using oral antiseptics over the last 3 months. Furthermore, it was necessary to detect if any systemic disease as this could result in a change in the production or composition of saliva. Prior to the study, all participants were subjected to a professional tooth cleaning. On the night before the experiment, they were forbidden to undertake any kind of oral hygiene. On the experimental day, no food or drink was allowed over the duration of the experiment including an hour before when the saliva was collected. The project was approved by the Ethics Committee of the Medical Association of Saarland (238/03-2012).

3.2 Salivary Samples

Non-stimulated samples of saliva were collected from each volunteer under base line conditions as well as at 30 s, 1 h, 6 h and 12 h after performing the following mouth rinses/ oral spray in separate experimental trials:

1) a single, 30-s mouth rinse with 10 mL of Elmex Kariesschutz (Table 2).

2) a single, 30-s mouth rinse with 10 mL of BioRepair Zahn- und Mundspülung (Table 2).

3) a single, 60-s mouth rinse with 10 mL of Chlorhexamed Forte (Table 2).

4) four times spraying with Theranovis oral spray (Table 2).

The non-stimulated saliva samples were collected using the spitting method.

The subjects participated in 4 complete mouth rinse tests each. A three days gap was scheduled in between each test.

Table 2 Principal composition of mouthrinses and spray tested in this study

Dental product, manufacturer	Code	Main ingredients	Percentage
Chlorhexamed Forte Alkoholfrei GlaxoSmithKline GmbH&Co.KG, Bühl, Germany	CHX	Chlorhexidinbis(D-gluconat) Peppermint aroma Macrogolglycerolhydroxystearat Glycerol Sorbitol solution purified water	0.2 Not known Not known Not known 70 Not known
BioRepair Dr. Kurt Wolff, Bielefeld, Germany	BioRepair	Water Sorbitol Alcohol denat. Glycerin Xylitol Cellulose gum Zinc PCA Zinc hydroxyapatite Aroma Sodium lauryl sulphate Silica Ricinus communis seed oil Ammonium-acryloyldimethyltaurate / VP-copolymer Sodium saccharin	>50 <25 4 <5 <5 <5 <1 <1 <1 <1 <1 <1 <1 <1 <1

		Sodium benzoate Benzyl-alcohol Phenoxyethanol Limonene	<1 <0.1 <0.1 <0.1
Elmex® Kariesschutz, GABA, Lörrach, Germany	Elmex	Water PEG-40 Hydrogenated castor Oil Olafluor (amine fluoride) Sodiumfluoride Aroma Potassium Acesulfame Polyaminopropyl Biguanide Hydrochloric Acid	Fluoride content:250 ppm
Theranovis Oral Spray Theranovis KG, Bingen, Germany	Theranovis	Aqua Alcohol Peppermint oil Rosemary oil Neem oil Thyme oil Grape seed extract Grapefruit seed extract	72,25 25 1,2 1,2 0,1 0,05 0,1 0,1

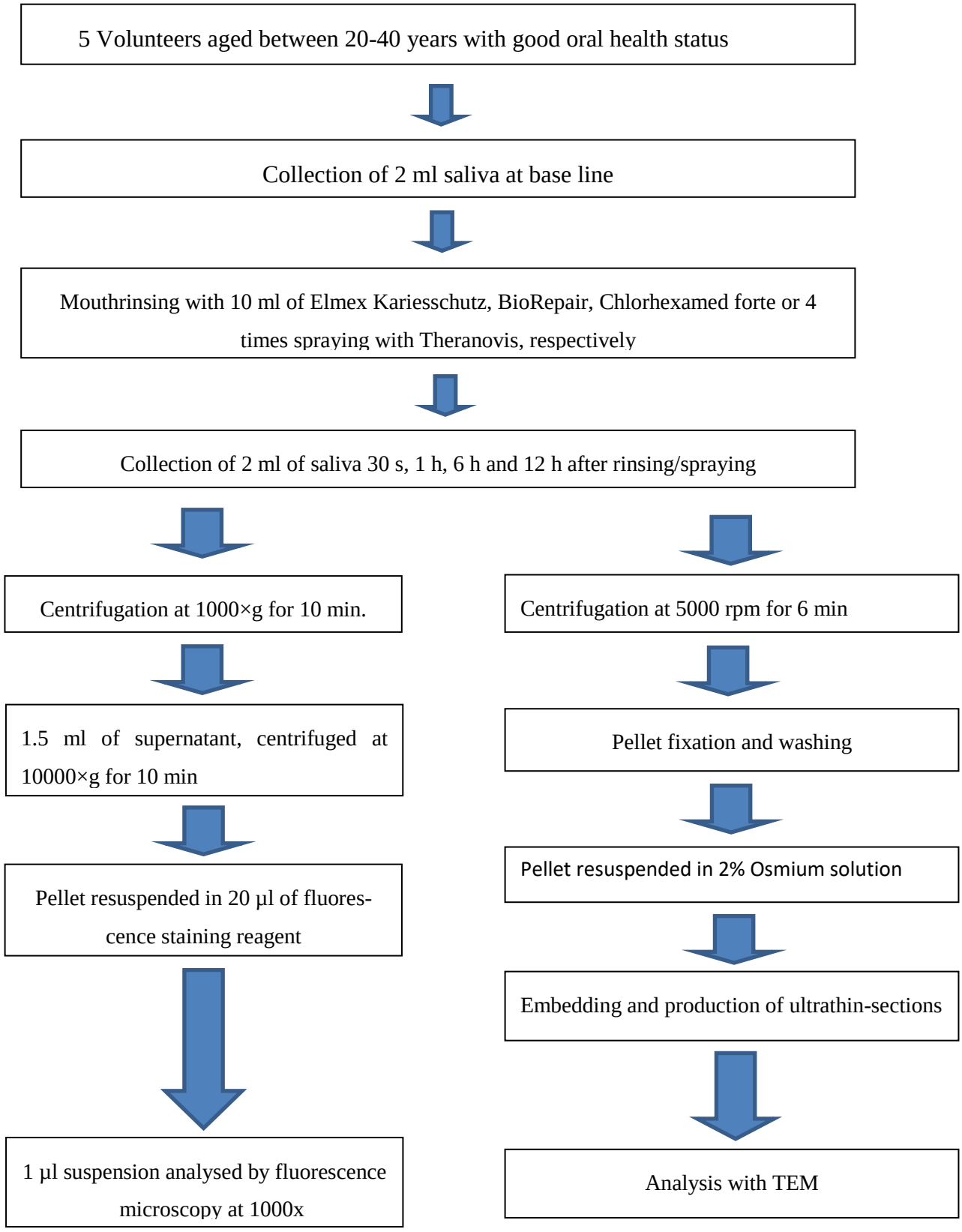


Fig. 1: Flowchart of the *in vivo* experiments and subsequent analyses.

3.3 *Bac*Light™ viability assay

LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Art. No. L7012, Invitrogen, Molecular probes, Eugene, Oregon, USA) uses the membrane quality of cells as a means by which to determine and count the number of dead and alive cells in salivary samples. Bacteria with an intact membrane were stained green, whereas the red stained cells with a compromised membrane were considered to be dead.

Two dyes were used, SYTO 9 green-fluorescent nucleic acid stain and propidium iodide ($C_{27}H_{34}I_2N_4$, PI) the red-fluorescent nucleic acid stain. They differ in their characteristics and how they penetrate healthy microorganisms, no matter if used individually or in tandem.

When used alone, the SYTO 9 stains all bacteria, whereas PI penetrates only bacteria with damaged cell walls. When both dyes are used together, PI diminishes the SYTO 9 dye fluorescence. The ideal solution is to use the right proportions of both together.

Thus, the total count of bacteria and the correlation between the live (green) and damaged (red) bacteria can clearly distingushed (Boulos *et al.*, 1999).

3.3.1 Preparation of staining solution

The dual fluorescence staining (LIVE/DEAD® *Bac*Light™) was prepared following the manufacturer's recommendations. The exact amount of each substance was mixed together with 1µl SYTO 9 and 1µl PI in 1 ml 0.9% saline solution (B. Braun Melsungen AG, Melsungen, Germany). It was stored for 15 min at 20ºC before use.

3.3.2 Centrifugation and staining of salivary samples for analysis in fluorescence microscopy

The salivary samples were centrifuged at 1,000×g for 10 min to remove the epithelial cells. Subsequently, 1.5 ml of the supernatant was centrifuged at 10,000×g. The resulting supernatant was discarded and the pellet obtained was resuspended in 20 µL fluo rescence solution (*Bac*Light Live/Dead staining, Art. No. L7012, Invitrogen, Molecular probes, Eugene, Oregon, USA). After homogenizing the bacterial suspension by shaking, it was stored in the dark

at room temperature for 15 min. The detailed experiment is summarized in the flowchart of Fig. 1.

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

Observations of 1 µl of each sample were performed using a fluorescence microscope (Leica DMRB, Leica Microscope & System GmbH, Wetzlar, Germany). The counting of live and dead bacteria was performed at 1.000-fold-magnification on nine randomized microscopic fields per sample. The bacterial viability was assessed and scored according to the criteria described in Fig. 2.

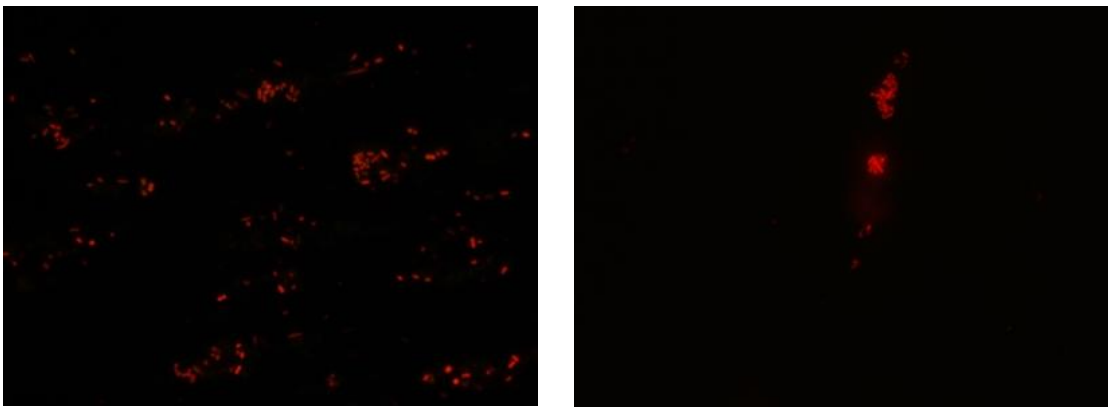
Fig. 2 Criteria for scoring the vitality of Bacteria

Fig. 2 a: **Score 1:** living bacteria 0 (only dead bacteria)

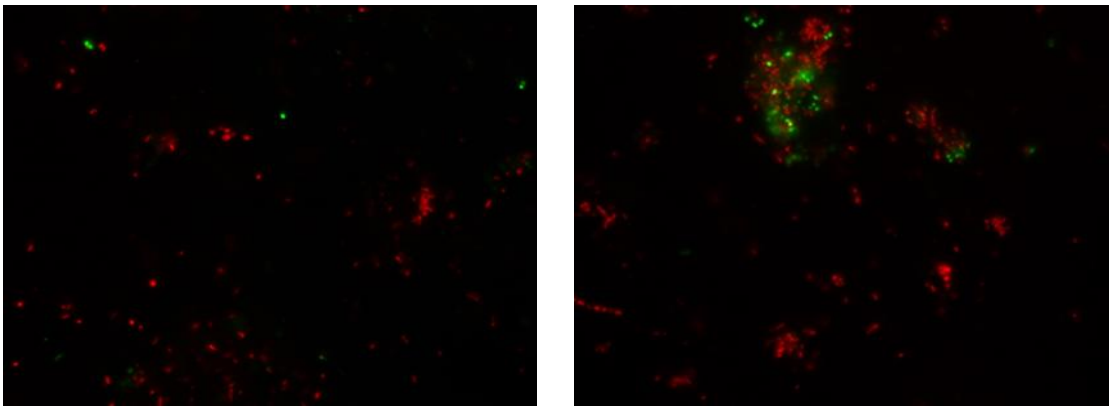
Score 2: living Bacteria less than 25%

Score 3: living bacteria from 25% and less than 50%

Example: Score 1:



Example: score 2



Example: score 3

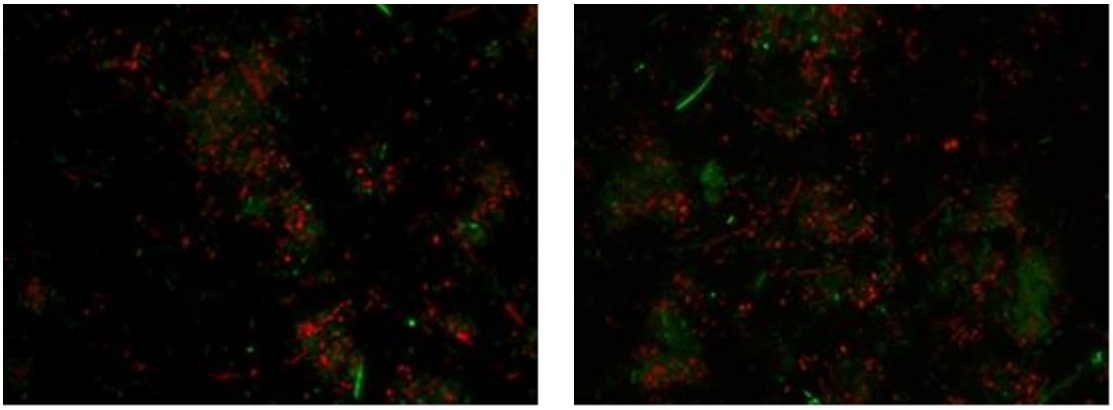
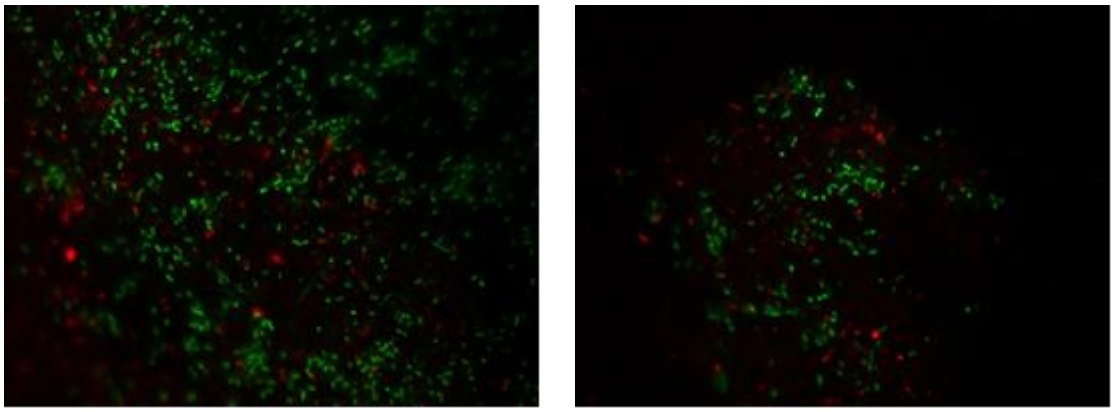


Fig. 2 b: **Score 4:** living bacteria from 50% live and less than 75 %.

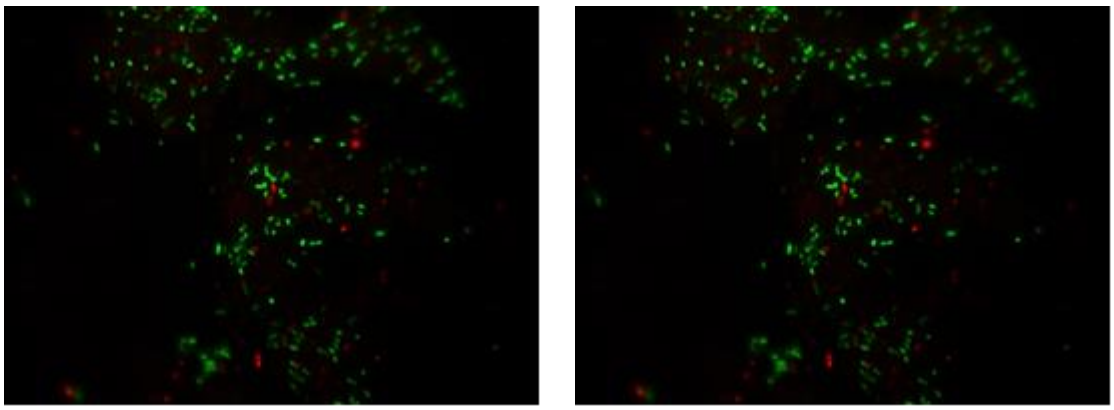
Score 5: living bacteria from 75%

Score 6: 100 % alive (livíng bacteria).

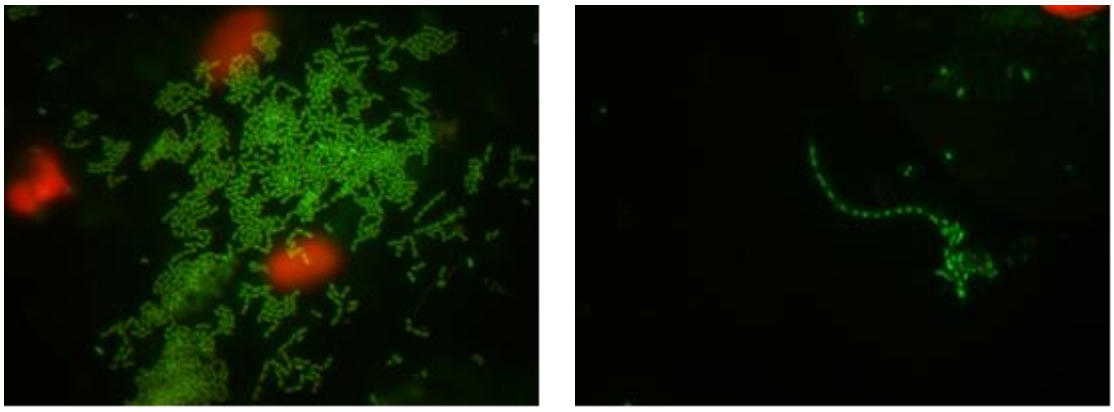
Example: score 4



Example: Score 5



Example: Score 6



3.4 Transmission Electron Microscopic (TEM)

3.4.1 Fixation of saliva samples for analysis with TEM

Fixation

The saliva samples were centrifuged at a consistent speed of 5,000 rpm for a duration of six minutes. The supernatant was discarded whilst the pellets were resuspended in glutardialdehyde solution for one and a half hour at room temperature. The solution contained 1% glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany), 1% formaldehyde (Science Services, München, Germany) in cacodylate buffer (pH 7.4, PAA Laboratories GmbH, Pasching, Austria).

Washing

The samples were centrifuged at 5,000 rpm for 6 min and the supernatant was discarded. The pellets were resuspended in cacodylate buffer and leaved for 10 min. Then, the samples were centrifuged at 5,000 rpm for 6 min. supernatant was discarded. Pellets werer resuspended again in cacodylate buffer. The samples were centrifuged at 5,000 rpm for 6 min, the supernatant was discarded. The washing was repeated once again. Pellets were resuspended last time in cacodylate buffer and stored at 4 °C.

3.4.2 Post fixation and Embedding

The samples were centrifuged at 5000 rpm for 10 min, the supernatant was discarded. For visualization of organic structures in the pellets, they were contrasted with 2% osmium tetroxide (O2SO4). After incubation with the osmium tetroxide for 1 h in shaking and dark, the samples were centrifuged at 5000 rpm for 10 min, the supernatant was discarded. Pellets were resuspended with a small volume of 0.1 M cacodylate buffer (Just that the pellet was covered with buffer to ensure a high concentration of the sample). Subsequently, 50 µl of the sample were resuspended in 50µl Low Melting Agarose and left to cure (about 15 minutes).

Samples were dehydrated five times in increasing concentrations of ethanol (50%, 70%, 90%, 100% water-free ethanol, 100% water-free acetone) (SERVA Electrophoresis GmbH, Heidelberg, Germany) (Hannig and Balz, 1999).
Incubation of the samples in a mixture of araldite M (Agar Scientific Ltd., Stansted, England) and acetone (1:1) overnight. Then, the sampled were placed in the silicone form and filled up with araldite M and hardened for 48 h at 65°C.

3.4.3 Production of ultrathin-sections

Ultrathin-sections of about 65 nm of the samples were produced. An ultramicrotome (Ultracut Leica EM UC7) was responsible for the cutting, using a diamond knife (Microstar 45°, Plano GmbH, Wetzlar, Germany). These cuttings were fixed onto a Pioloform-F coated cooper grid (slot-dazzle copper 1x2 mm (Plano GmbH, Wetzlar, Germany) and contrasted with uranylacetate (10 min) and lead citrate (5 min).

3.4.4 TEM analysis

An investigation was undertaken of the salivary bacteria which had been subjected to the various mouth rinses using a magnification strength ranging from 890- to 98.000-fold by a TECNAI 12 (FEI, Eindhoven, Netherlands). In the BioRepair group, the nanoparticles were also subjected to a TEM analysis (Fig. 11).

4. Results

No side effects were experienced by any of the subjects. The semi-quantification of salivary bacteria and bacterial vitality using *Bac*Light™ viability assay as well as transmission electron microscopy (TEM) allows the investigation of the bacterial vitality without or with application of mouth rinses, also at the different times after rinsing.

4.1 *Bac*Light™ viability assay

4.1.1 Visualization of bacteria in the salivary samples

*Bac*Light™ viability assay enable the live and not alive bacteria in the salivary flora to be visualized at the same time and to be distinguished exactly (Fig. 3). The majority of bacteria found were cocci, rods, and seldom epithelial cells were also observed (Fig. 4 and Fig. 5). Additionally, *Bac*Light™ staining revealed visualization of the arrangement of microorganisms, such as individual bacteria, mono-layered chain, and accumulated colonies, etc. (Fig. 6). An overview of bacteria detected using *Bac*Light™ viability assay is shown in Fig. 7.

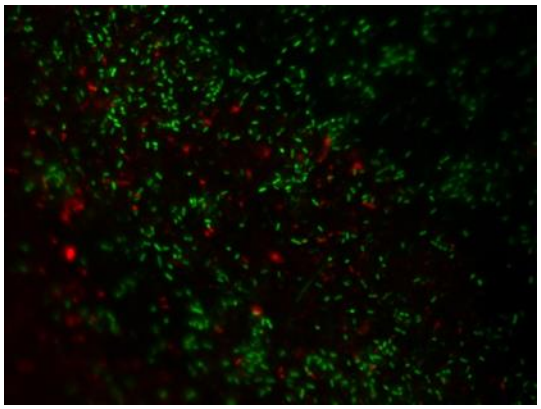
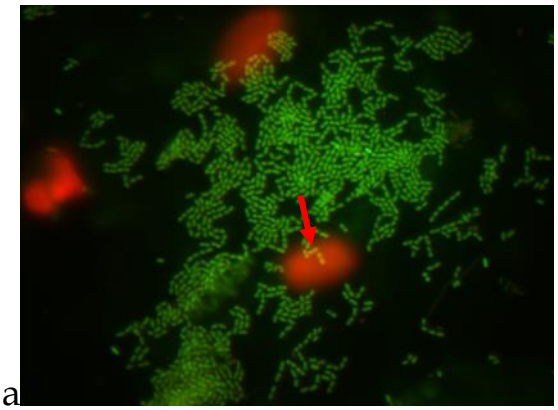


Fig. 3: Salivary samples after staining with the *Bac*Light™ viability assay: Visualization of green (living) and red (dead) cells. BioRepair group, 12 h after rinsing. Original magnification: 1.000-fold.

a 

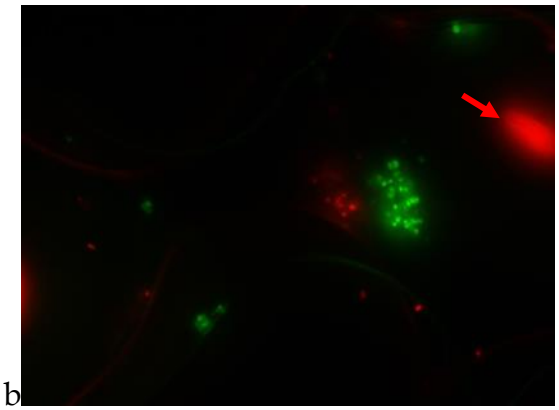
b 

Fig. 4: Salivary samples after staining with the *Bac*Light™ viability assay: Epithelial cells were detected in the salivary samples. Epithelial cells were excluded from quantification of bacteria. The nucleus of the epithelial cell is highlighted using a red arrow. (a) the CHX group, 30 s after rinsing; (b) BioRepair group, 6 h after rinsing. Original magnification: 1.000-fold.

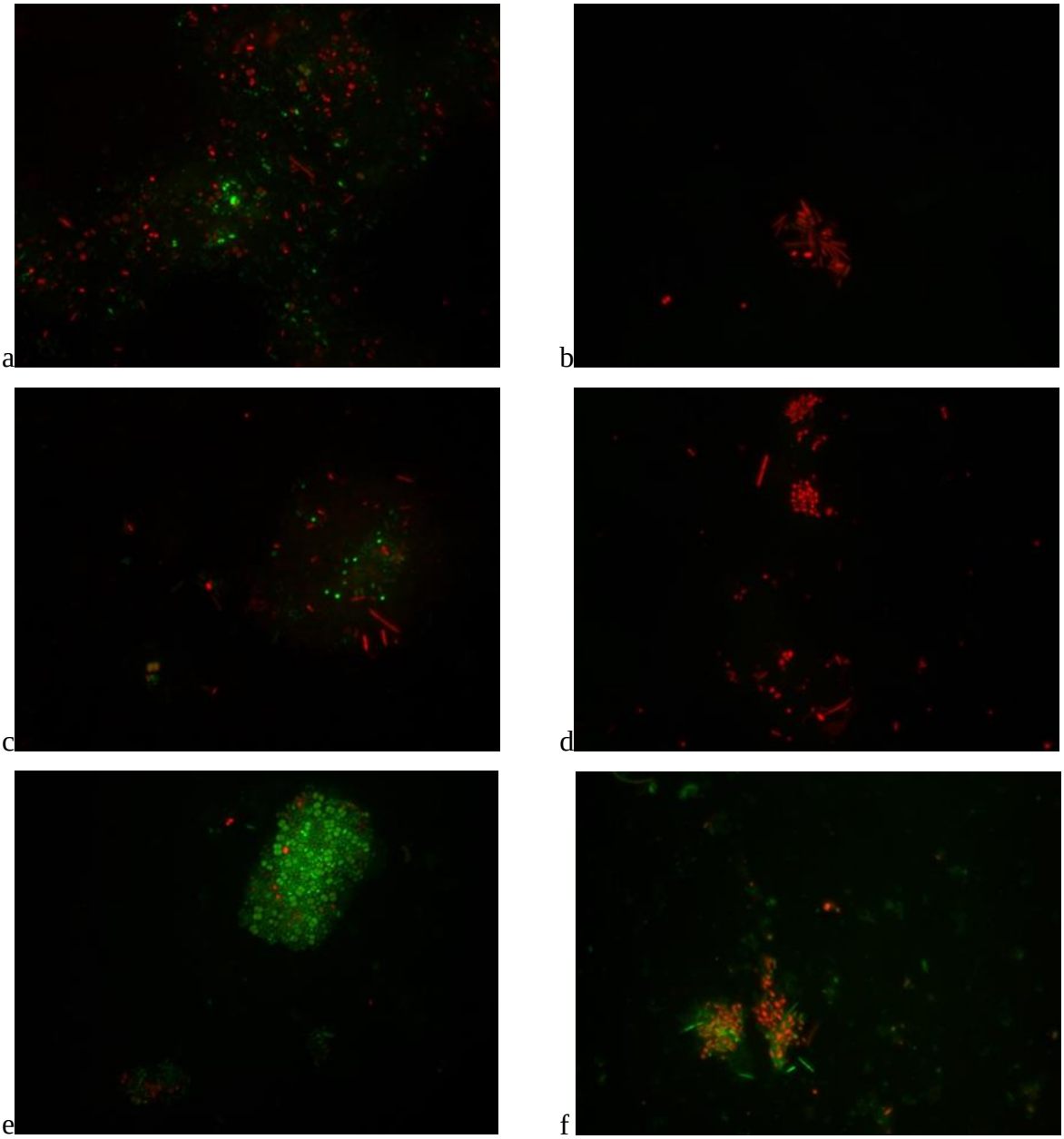


Fig. 5: Salivary samples after staining with the *Bac*Light™ viability assay: cocci as well as rods were detected in the salivary samples under *in vivo* condition without rinsing (a, c and e); 1 h after rinsing with CHX (b) and 6 h after rinsing with CHX (d and f) green: living; red: dead. Original magnification: 1.000-fold.

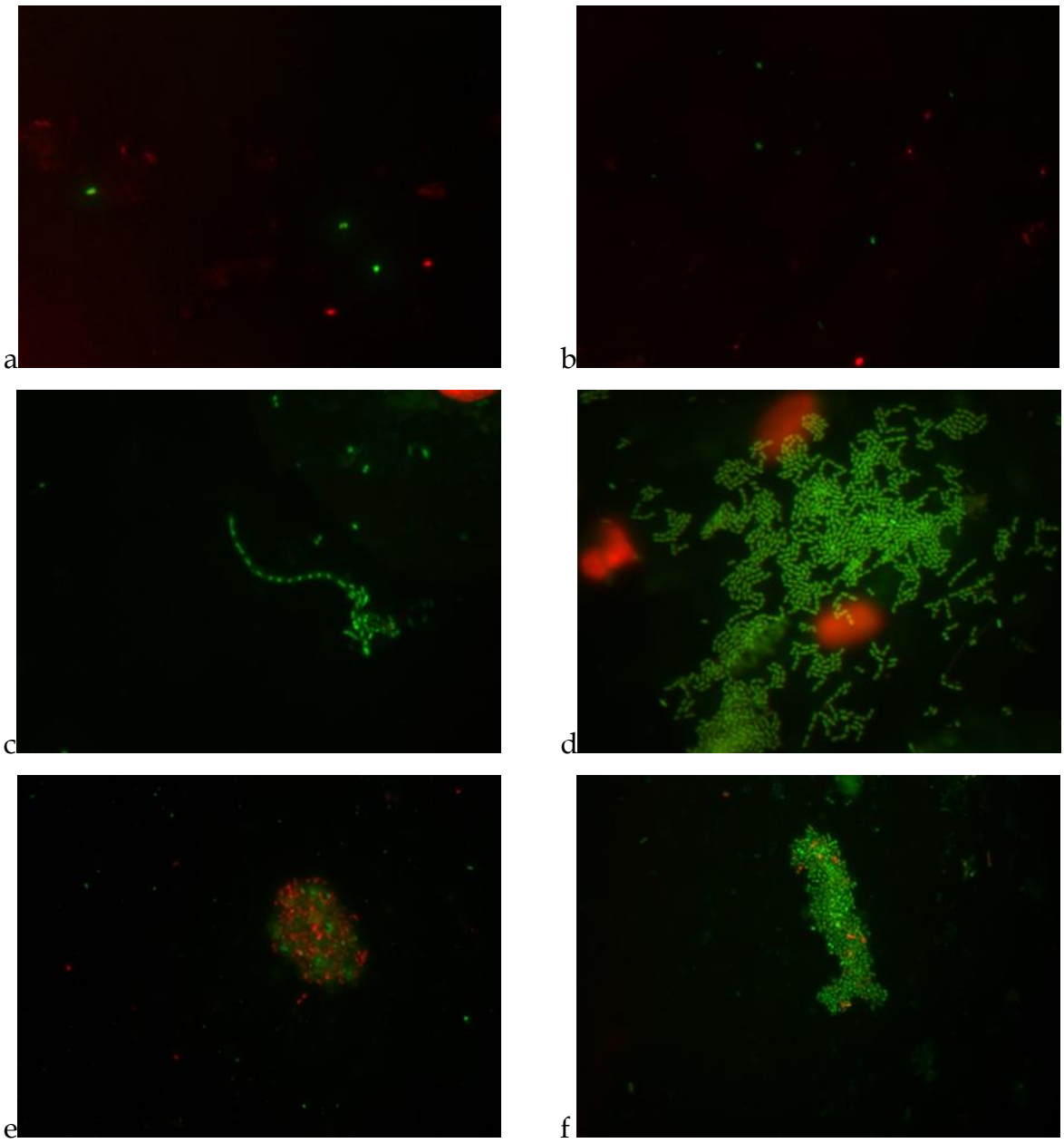


Fig. 6: Salivary samples after staining with the *Bac*Light™ viability assay: Visualization of differing compositions of bacteria in the salivary flora. Both single bacteria as well as colonies were observed. (a) 1 h after rinsing with Elmex Kariesschutz; (d) 30s after rinsing with CHX; (e) 30 safter spraying with Theranovis; (c and f) without rinsing and (b) 6 h after rinsing with BioRepair. green: living; Red: dead. Original magnificationon: 1.000-fold.

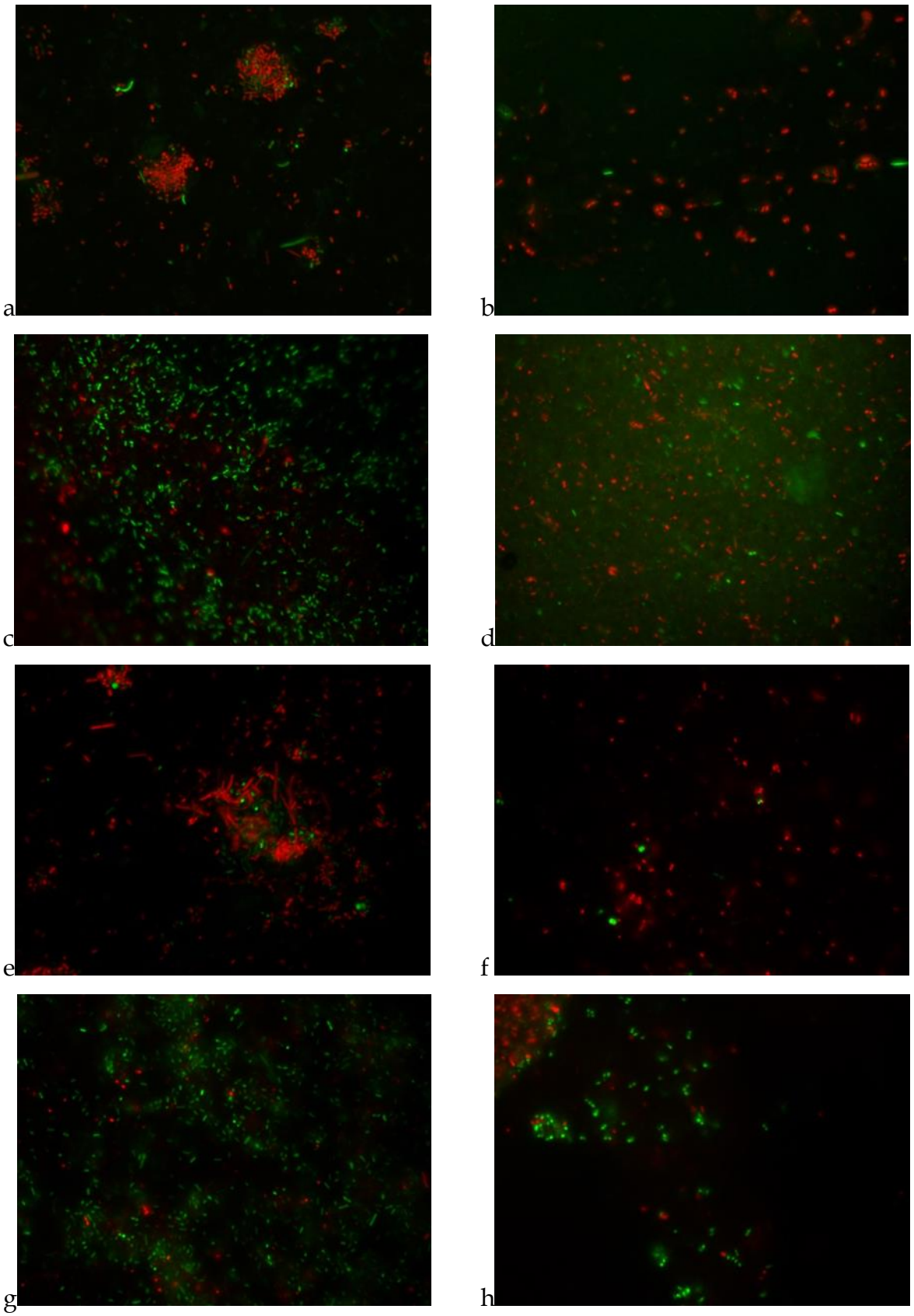


Fig. 7: Salivary samples after staining with the *Bac*Light™ viability assay: a display of salivary flora both without rinsing and prior to rinsing with the two mouth rinses in each experimental period. (a) 1 h after rinsing with CHX; (b) 6 h after rinsing with CHX; (c) 12 h after rinsing with BioRepair; (d) 1 h

after rinsing with BioRepair; (e) 30 s after rinsing with Elmex Kariesschutz; (f) 1 h after rinsing with Elmex Kariesschutz; (g) 6 h after spraying with Theranovis and (h) 30 s after spraying with Theranovis. green: living; Red: dead. Original magnification: 1.000-fold

4.1.2 Semi-quantification of bacteria viability

At the baseline as well as at 30 s, 1 h, 6 h and 12 h after each rinsing the number of microorganisms identified by *Bac*Light™ viability assay was based on the count outlined in Fig. 2. Medians and range of scores of the vitality of bacteria across all groups are summarized in Table 3. The results indicate that the tested mouthwashes had different effects on bacterial vitality in the salivary samples.

All four tested mouthwashes and the spray reduced the vitality of bacteria in the saliva samples compared to the baseline samples before rinsing. The results revealed reduced bacterial vitality after washing with CHX, BioRepair and Elmex Kariesschutz in each experimental trial. However, less extent of inhibition of bacterial vitality was observed on samples following spraying with Theranovis. The baseline samples had median scores of 4 (50-75% living bacteria). The median scores at 30s after rinsing with CHX and BioRepair had the same value of 3 (25- 50% living bacteria) and the value of 2 (living Bacteria less than 25%) after rinsing with Elmex Kariesschutz. Nevertheless, the scores of Elmex Kariesschuz samples were between 1 and 3 whereas, the scores of samples after rinsing with CHX or with BioRepair ranged between 1 and 4. In the Theranovis group, the median scores remained for all experiment time at the same level of 4. A slight decrease in scores range was detected at one hour after rinsing. One hour following rinsing with CHX showed an increasing in the antibacterial effect, the median score changed from 3 into 2, as compared with BioRepair, which remained at the same median score of 3. However, one hour after rinsing with Elmex Kariesschutz an obvious recovery in the bacterial vitality was detected and the median score changed from 3 into 2. After six hours, it was observed that the efficacy decreased after rinsing with Elmex Kariesschutz and BioRepair, as the median score increased to 4, whereas CHX median score was 3. After 12 hours, the median scores after rinsing with CHX, BioRepair, Elmex and Theranovis were at the same value of 4 (Table 3).

Table 3 Viability of the salivary flora following rinsing with different mouth rinses evaluated by *Bac*-Light™ viability assay. Medians of scores of bacterial vitality in the salivary samples according to criteria defined in Fig. 2. Median (Min. - Max.).

Mouth rinses / Time after rinsing	Chlorhexamed Forte	BioRepair Zahn- und Mund-spülung	Elmex Kariesschutz	Theranovis oral spray
Unrinsed (control)	4(3-5)	4(3-5)	4(3-5)	4(3-5)
30 seconds	3(1-4)	3(1-4)	2(1-3)	4(3-5)
1 hour	2(1-4)	3(2-4)	3(2-4)	4(2-6)
6 hours	3(1-4)	4(2-5)	4(2-5)	4(3-5)
12 hours	4(2-5)	4(2-5)	4(2-5)	4(4-6)

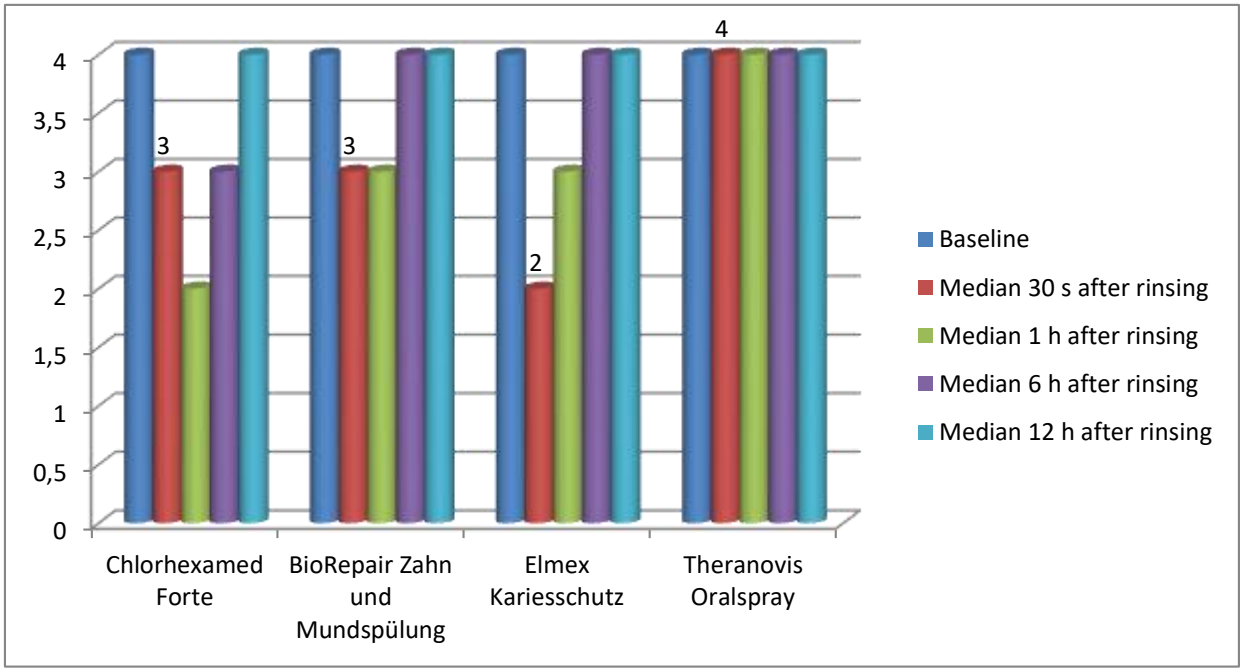


Fig.8: Median scores of bacterial vitality for all volunteers at the baseline and 30 s, 1 h, 6 h, 12 h following mouth rinses with the different rinsing solutions or after spraying respectively.

4.2 TEM

The results of TEM investigations on salivary samples were consistent with the findings of the *Bac*Light™ viability assay in term of the bacterial vitality in the different experimental times. A series of TEM images indicated the changes in the bacterial vitality after rinsing with different mouthrinses (Fig. 12, 13) as compared to control group (Fig. 9).

Untreated bacterial cells were observed to understand the variations between the control sample and the bacterial cells after treatment. Fig. 9 shows the morphological features of the control cells: : normal cell membrane, the outside cytoplasmic membrane, the internal periplasmic space, cytoplasmic components and few electron-dense areas. The observed bacteria in the salivary samples were either in single form or as multitude of bacteria adjacent to the epithelial cell membrane forming a multilayer. Cell division is frequently evident.

In Fig. 10, TEM images demonstrated the effect of the mouth rinses on the bacterial cells. Morphological changes are verified by the change of shape, promoting interactions and disruption of the cell wall, which leads to release of intracellular material.

A considerable microbial alteration was detected in CHX, Elmex Karieschutz and BioRepair groups at 30 s and 1 h after rinsing (Fig. 12, 13). However, the maximum antibacterial effect in the CHX group was present in the samples 1 h after rinsing. Whereas, in the Elmex Karieschutz group the highest effect was detected in samples 30 s after rinsing. A pellet with only few intact (living) bacteria was observed in the BioRepair treated sample, for 30 s as well as 1 h after rinsing (Fig. 12). In addition, clustered hydroxyapatite particles consisting of aggregated crystallites were present throughout the samples treated with BioRepair (Fig. 11). The images of Theranovis oral spray group 30 s and 1 h after rinsing did not show a considerable effect compared to the control group (Fig. 13).

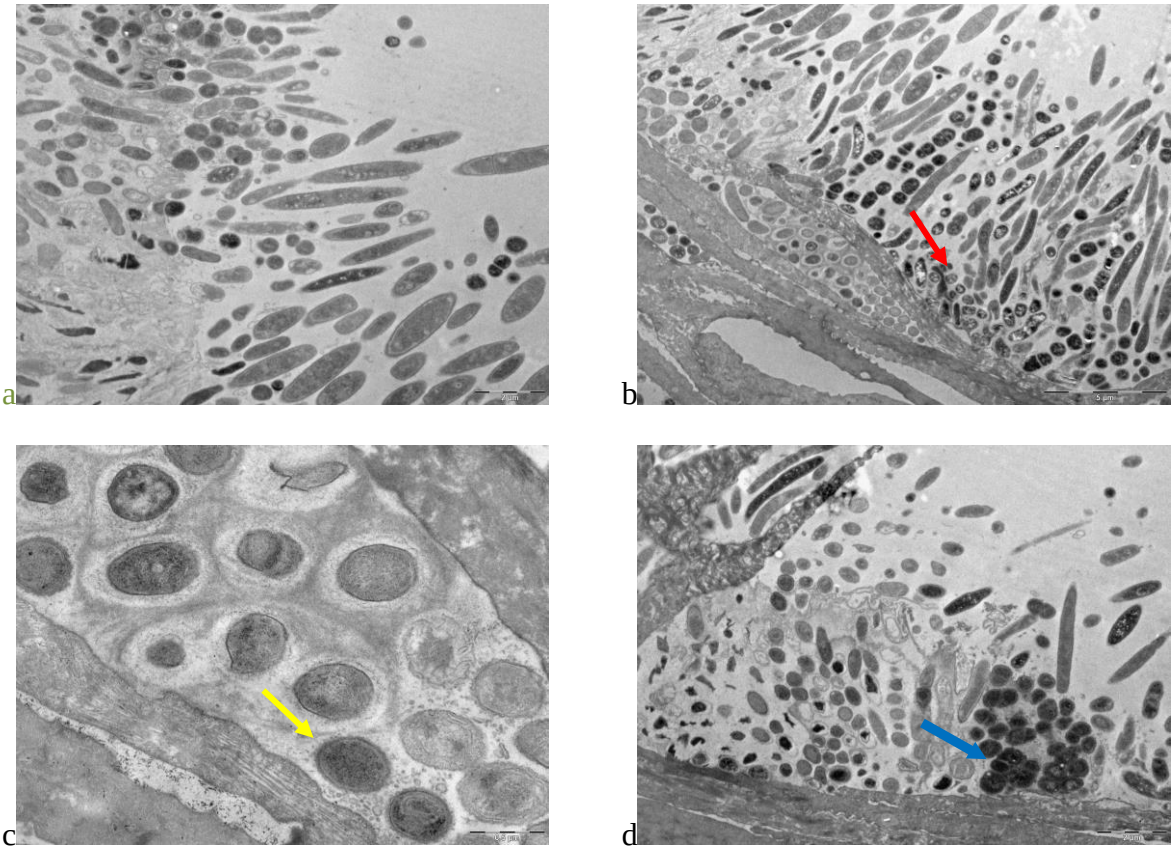


Fig 9: TEM micrographs: bacteria in salivary samples without rinsing (control group). The morphological features of cells (yellow arrow) and cell division (blue arrow) are evident. Red arrow showes a multitude of bacteria adjacent to the epithelial cell membrane. Original magnification: (a) 6800-fold; (b) 4800-fold; (c) 9300-fold; (d) 13000-fold.

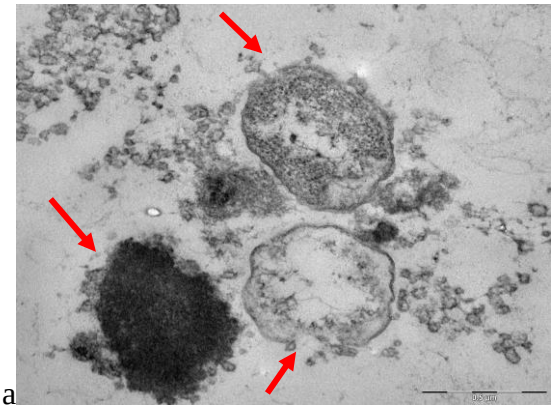
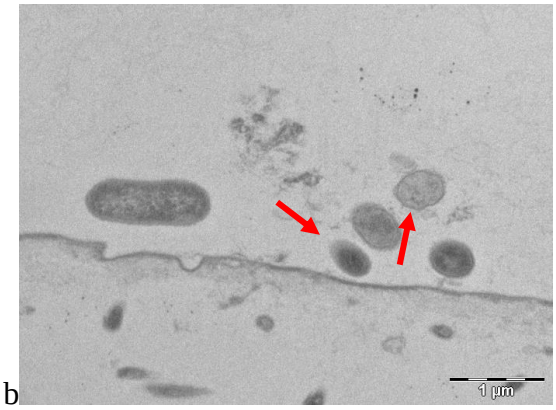
a  b 

Fig 10: TEM micrographs: changes in the morphological features of cells after rinsing. Disruption on most of all the bacterial cell walls (red arrows). (a) Salivary sample 30 s after rinsing with BioRepair, (b) salivary sample 1 h after rinsing with Elmex Kariesschutz. Original magnification: (a) 49000-fold; (b) 18500-fold.

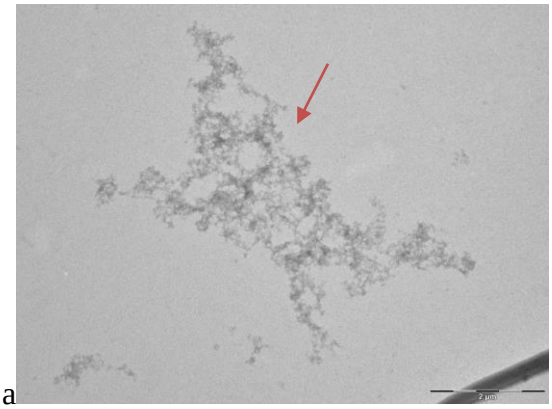
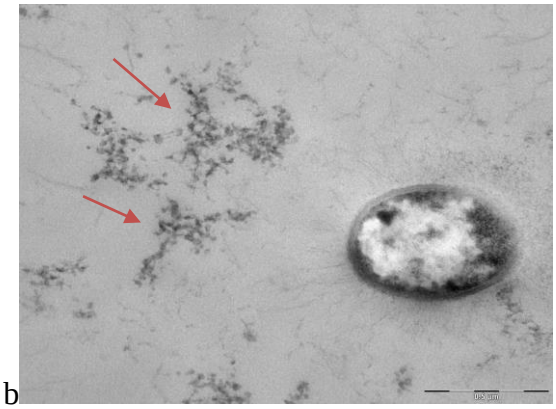
a  b 

Fig. 11: TEM micrographs: clustered hydroxyapatite particles consisting of aggregated crystallites (red arrows) are present throughout samples treated with BioRepair. (a) Salivary sample 1 h after rinsing with BioRepair; (b) salivary sample 30 s after rinsing with BioRepair. Original magnification: (a) 11000-fold; (b) 49000-fold.

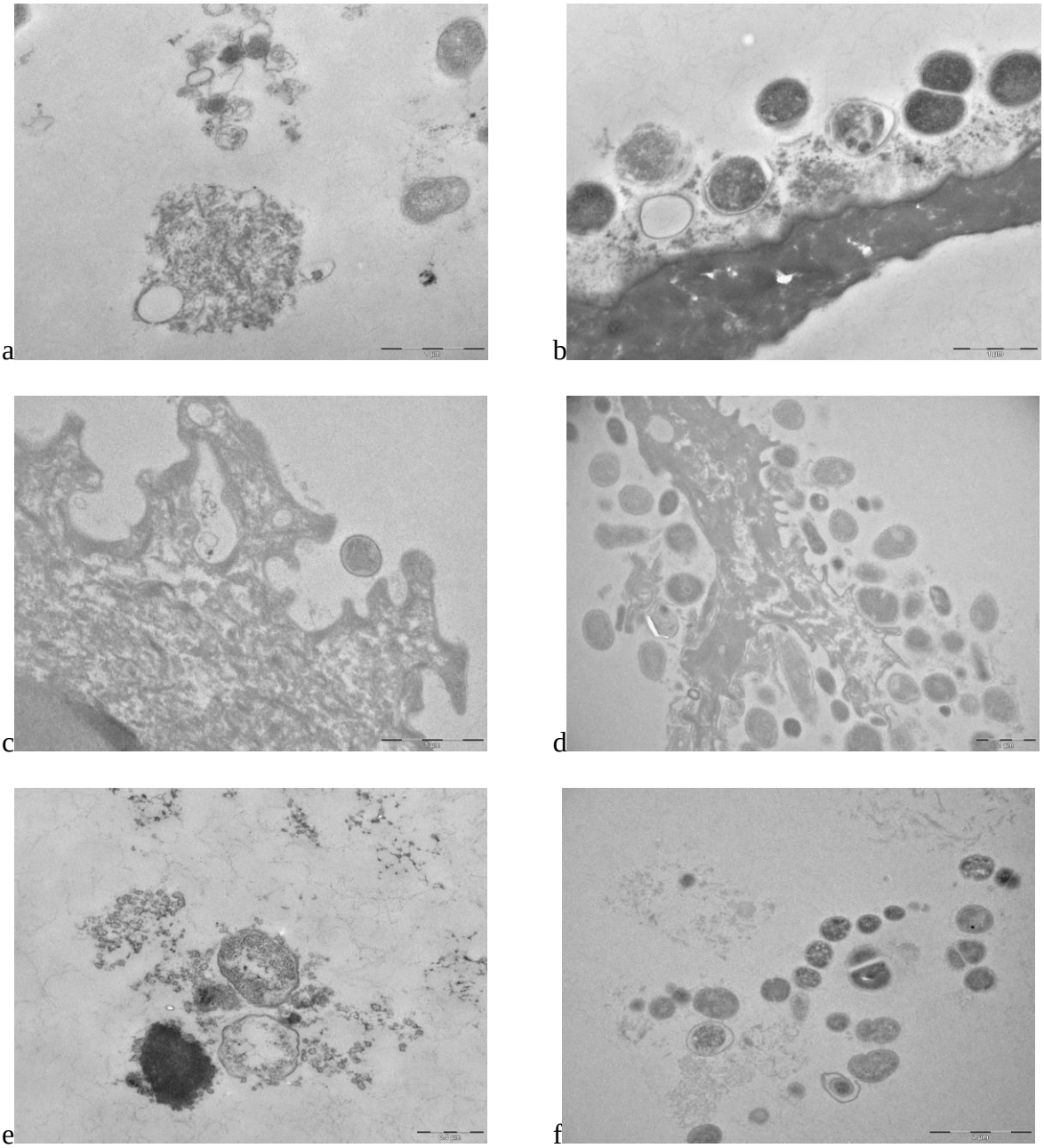


Fig. 12: TEM micrographs: images indicate the changes in the bacterial vitality after rinsing with CHX or BioRepair in each experimental period. (a, b) Salivary sample 30 s after rinsing with CHX; (c, d) Salivary sample 1 h after rinsing with CHX; (e) Salivary sample 30 s after rinsing with BioRepair; (f) Salivary sample 1 h after rinsing with BioRepair. Original magnification: (a) 18500-fold; (b, c) 23000-fold; (d) 13000-fold; (e) 30000-fold; (f) 11000-fold.

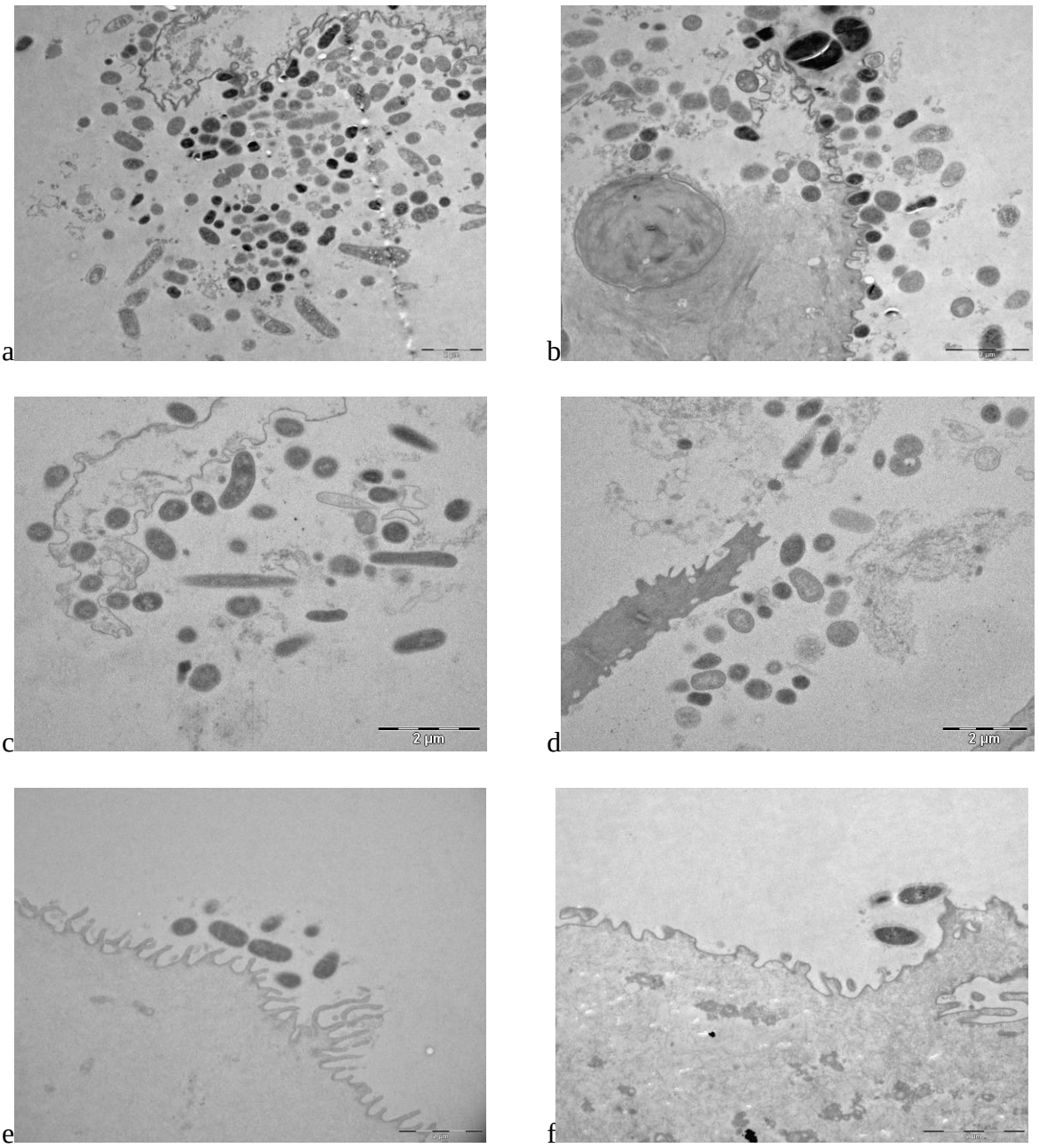


Fig. 13: TEM micrographs: images indicate the changes in the bacterial vitality after rinsing with different mouth rinses in each experimental period. (a,b) Salivary sample 30 s after rinsing with Elmex Kariesschutz; (c,d) Salivary sample 1 h after rinsing with Elmex Kariesschutz; (e) Salivary sample 1 h after spraying with Theranovis; (f) Salivary sample 30 s after spraying with Theranovis. Original magnification: (a) 6800-fold; (b) 9300-fold; (c,f) 11000-fold; (d,e) 9300-fold.

5. Discussion

5.1 Discussion of Materials and Methods

5.1.1 Limitations

To limit inter- and intra- individual variations during the experiment, the environment was strictly regulated:

- Each experimental trial started at 8:30 a.m. in order to minimize the impact of circadian rhythm on the production of saliva and its bacterial vitality.
- Subjects were not allowed to practice any oral hygiene 12 hours before the experimental trial to avoid any possible effect on the results of bacterial vitality. Only Toothbrushing without toothpaste was allowed.
- To rule out any effects of consumption of acidic beverages or food on the bacterial vitality, subjects refrained from any beverages or food for 1 hour before and during the course of the experiment (Hannig and Balz, 1999).

The limitation of this study is the small number of subjects. The long experimental time up to 12 hours and the application of several mouth rinses required the involvement of a small group of volunteers, as is normally the case in other *in vivo* studies (Hannig *et al.*, 2013 a).

5.1.2 Centrifugation

Centrifugation speed and time criteria to harvesting microorganisms from saliva depend on three factors: the salivary viscosity, the weight of the cells, and the bacterial damage through centrifugation. Saliva is intrinsically inhomogeneous, as it simultaneously consists of a liquid gasous phase and a gel phase (Schwartz, 1987). The complexity of this system decreases the accuracy in the evaluation of its characteristics and in particular of the viscosity (Foglio-Bonda *et al.*, 2014). Average viscosity of unstimulated whole saliva is 1.4 cSt (fresh) to 1.12 cSt at 25 min after collection (Foglio-Bonda *et al.*, 2014), whereas water has a viscosity of 1.0020 cSt at 20°C (Korosi & Fabuss, 1968). Due to Stoke's law, particle movement in liquids is in inverse linear proportion to viscosity. Thus, centrifugation time or speed in saliva should be about 1.1-1.4 times higher than the one in water (depending on time elapsed after collection of saliva). However, there is a lack in understanding how aging affects the viscosity value

of saliva (Foglio-Bonda *et al.*, 2014). Rantonen and Meurman (1998) found the stimulated salivary viscosity remains almost constant during the first 30 minutes after collection. Other authors affirmed that viscosity of stimulated saliva decreases within few hours (Schipper *et al.*, 2007), otherwise unstimulated saliva seems to be less stable (first 5-8 minutes) (Roberts, 1977). In the present study, the aging does not play an important role as we start centrifugation directly after saliva collection.

Differential centrifugation is the processing of particles which are of differing sizes and densities at various sedimentation rates. This technique has been applied in other studies where it was necessary to sort cells based on size using incremental centrifugation speeds (Catsimpoolas, 1976). By centrifuging at a rate of 10,000×g, Fliss *et al*. (1991) were able to cumulate *lactococcus species* in broth culture. Whereas, in another study bacteria were removed from saliva at 12000×g for 15 min (Oho *et al.*, 2000).

In order to harvesting microorganisms from liquid cultures, Centrifugation of the saliva is a prerequisit. The damage that may be caused by this procedure of centrifugal compaction is generally overlooked, as there is no simple way to assessment this (Peterson *et al.*, 2012). Collection of bacteria using high centrifugation speeds is preferred by many experimental protocols. There is an assumption that such a procedure would not harm bacterial cells or destroy them (Deupree & Schoenfisch, 2008) (Romani *et al.*, 2008). However, centrifugal damage can have an impact on the outcome of such research work. It can change both internal and external properties of bacterial cells as well as the DNA (Peterson *et al.*, 2012), for example centrifugation speed of 15000×g damages the cell surface (Peterson *et al.*, 2012). There are a lot of centrifugation speeds and times for bacteria on researchgate forums and in protocols for kits of DNA preparation. They were usually in the range of 2000-6000×g for 1-10 min. Thus, g-value for separating bacteria from saliva should be ~10000×g, high enough to oppose salivary viscosity and low enough to cause minimal damage to cells.

5.1.3 *Bac*Light™ viability assay

Many of the documented research finding available to date are based on studies of antimicrobial effect of rinsing solution on salivary bacteria has been performed using plate culture microbiological techniques (Jenkins *et al.*, 1994; Elworthy *et al.*, 1996; Auschill *et al.*, 2002). However, the reliability of this method is questionable due to the following limitations: First, the approach is seen as a retrospective way of determining bacterial viability (Berney *et al.*,

2007); Second, in terms of defining quantity – it is not the number of single bacteria that is counted rather number of CFU ml^{-1} (Weiger *et al.*, 1998); third, external and environmental factors which may impact reproducibility. These could include how the culture is transported, temperature at which it storage, environment and length of time in incubation (Boulos *et al.*, 1999; Lehtinen *et al.*, 2004); Fourth, with respect to *in vitro* cultivation, it is challenging to meet the physiological and metabolic requirements of a polymicrobial population (as found in saliva) (Nadkarni *et al.*, 2002; Biggerstaff *et al.*, 2006). Taking these factors into account, bacterial viability may be underestimated (Boulos *et al.*, 1999), or even overestimated for certain types of cells (Tomas *et al.*, 2009). These disadvantages might be overcome by applying fluorescence staining techniques and then a fluorescence microscopic analysis. As an alternative technique for cell quantification, some researchers have recommended trying epifluorescence microscopy with specific fluorochromes, such as the LIVE/DEAD® *Bac*Light™ solution. (Boulos *et al.*, 1999; Lahtinen *et al.*, 2006). Advantages of using this method include:

- it is both a quick and simple method, allowing real-time bacterial viability quantification (Boulos *et al.*, 1999).
- Use of fluorescence two-color viability assays to stain cells – one shows living and the other non-living. Fluorochromes are able to react to membrane permeability or metabolic activity to determine differences in cell vitality allowing quantification of bacteria as well as bacterial viability pattern (Netuschil *et al.*, 1998; Auschill *et al.*, 2001)
- the increased chance to find microorganism which cannot be cultivated using plate culture techniques (Joux & Lebaron, 2000; Berney *et al.*, 2007).

Other authors found that the immediate antibacterial effect could be similarly interpreted with both microbiological techniques (Tomas *et al.*, 2009). For example, using epifluorescence microscopy solution, Weiger *et al.* (1988) detected that under normal conditions, about 85% of salivary flora is active. These findings collate to those of Caballero *et al.* using the SYTO 9/PI solution (Garcia-Caballero *et al.*, 2009) and with our results obtained in the present study (vitality was around 75 % of living bacteria or score 4 according to criteria in Fig. 2).

There are many reasons as to why The *Bac*Light™ viability assay was preferred in previous research work - these include the high speed, reliable and easy preparation (Hannig *et al.*,

2013b). Furthermore, since the non-live bacteria are not overestimated, it enables the viability pattern to be mapped (Tawakoli *et al.*, 2013).

To summarise, epifluorescence microscopy with the SYTO 9/PI dual staining is an effective technique that enable evaluating the antibacterial effect of mouth rinses on the salivary bacteria in real time (Tomas *et al.*, 2009). Thus, *Bac*Light™ viability assay was adopted in the present study. The scoring system has been adopted in the present study for evaluation of the bacterial vitality through the percentage of the living bacteria to the dead and the results recorded are corresponding to previous studies.

5.1.4 Transmission electron microscopic investigations

Supporting of the *Bac*Light™ viability assay findings, investigations on the morphological appearance of bacteria in the salivary samples were carried out using Transmission Electron microscopy. This is the most highly regarded method for the microbial analyzis at both micro and nano-scale level (Hazelton and Gelderblom, 2003). A further advantage of TEM is that it is possible to visualize the bacterial morphology and in particular the interactions of bacteria with the other samples ingredients. However, TEM requires great efforts for preparation and is time consuming. Therefore, it is not suitable for a large number of samples.

5.2 Discussion of Results

In the present study, we identified mouth rinse solutions with antiseptic impact that lead to an immediate reduction of vitality of salivary bacteria as well as to a reduction after 1 h, 6 h, and 12 h.

5.2.1 CHX 0,2%

The 0.2% CHX mouth rinse had the best antibacterial effect on the salivary microflora up to 6 h after its use. This is in accordance with the data from previous studies, in which an immediate antibacterial effect of a single application of CHX has been observed to remain stable for at least up to 7 after the mouth rinse (Addy *et al.*, 1991; Moran *et al.*, 1992; Briner *et al.*, 1994). However, by other authors in accordance with the results reported in some *in vitro*

studies (Toljanic *et al.*, 1992), a significant recovery in bacterial vitality was detected in 1 h\ and 4 h-salivary samples after CHX mouth rinses in comparison to the vitality at 30 s after the mouth rinse. In our study, a progressive antibacterial effect until 1 h and subsequent progressive recovery in bacterial vitality was observed, which was also detected by other authors (Jenkins *et al.*, 1994; Yates *et al.*, 1997). These variations in results may be attributed to differences in methodologies. Consequently, this emphazies the necessity to understand how the recovery of salivary flora is affected by CHX substantivity (Cousido *et al.*, 2010).

5.2.2 Elmex Kariesschutz

In good agreement with previous studies on the antimicrobial effect of sodium fluoride and amine fluoride (Hamilton, 1990; Brambilla *et al.*, 1999), Elmex Kariesschutz mouth rinse revealed a considerable effect against salivary microorganisms for the first hour in the present study. As demonstrated by many studies, fluoride affects in inhibiting demineralization and promoting remineralization of the teeth surfaces (ten Cate, 1999; Fejerskov, 2004). Furthermore, It inhibits the production of bacterial acid *in vitro* (Hamilton, 1990; Marquis, 1990; Jenkins, 1999) as well as plaque acid production *in vivo* (Tatevossian, 1990; Vogel *et al.*, 2002) by inducing acidic stress in cells and decreased acid production altering the biological activity of acid-tolerant oral bacteria, such as *streptococcus mutans* (Hamilton, 1990). In the majority of *in vitro* studies, there is an assumption that the two processes at work. Firstly, cytoplasmic acidification and secondly, the hindering of glycolytic enzymes (enolase) and proton-translocating ATPase (Van Loveren, 2001). In a previous study it has been indicated that fluoride has a detrimental impact on bacterial enzymes and consequently alters two pathways - the Embden-Meyerhof-Parnas pathway (the EMP pathway) and the pentose phosphate pathway, thus repressing bacterial growth and acid production. Nevertheless, the amine group is considered to be the main factor responsible for the antimicrobial properties of amine fluoride as demonstrated in previous *in situ* studies (Shani *et al.*, 1996; van der Mei *et al.*, 2006). An antiglycolytic effect of amine fluoride is well documented (Embleton *et al.*, 1998; Shani *et al.*, 2000; van der Mei *et al.*, 2008).

Our results demonstrate that amine fluoride has a similar immediate antibacterial potential as chlorhexidine (at 30 s after the mouth rinse). This finding also confirms the results obtained by Shani *et al.* (2000) . On the other hand, amine fluoride was identified by other authors as a

mouth rinse with limited antiseptic impact that lead to an immediate reduction of the vitality of the oral bacteria up to 1 h after its application (Pitten & Kramer, 1999). This is reasonable because of the fast recovery of the bacterial vitality after the first hour which was detected in the present study. Amin fluoride mouth rinsing may be useful as an adjunct to the daily performed oral hygiene (Pitten & Kramer, 1999). However, it is necessary to conduct studies investigating the effectiveness as well as substantivity of Elmex Kariesschutz after prolonged rinsing times, for example rinsing for 1 min instead of 30 s.

5.2.3 BioRepair

As shown by *Bac*Light™ semi-quantitative analyses, in the present study, BioRepair possessed less antibacterial properties than Elmex at 30 s after application, but similar effects on salivary bacteria like Elmex Kariesschutz at 1, 6 and 12 h after rinsing. Therefore, BioRepair can also be considered as mouth rinse with moderate antibacterial effects on the salivary microflora, whereas results of an *in vitro* study showed that BioRepair had pronounced effect on the viability of the bacteria, even when the solution was weak. When observed on *in situ* biofilm formation, The effects of the preparation were almost the same as those recorded for chlorhexidine (Hannig *et al.*, 2013b). In fact, most previous experiments to investigate the antibacterial efficacy of BioRepair were performed *in vitro*, which are difficult to compare with the present *in vivo* investigation.

So far, it has not been set, how BioRepair mouth rinsing affects the oral microorganisms. There is a synergy which occurs –the different ingredients when mixed together is seen to be more effective in preventing bacterial vitality. Each type of ingredient has its role either as sugar alcohol – the sorbitol and xylitol; as a food preservative – the sodium benzoate and as a detergent – the sodium lauryl sulphate (Hannig *et al.*, 2013a).

The zinc-carbonate hydroxyapatite is regarded as main active ingredient in the BioRepair product. The scattered or aggregated biomimetic hydroxyapatite nano-particles display increased bioactivity. This is due to its dimensions (100 x 10 x 5 nm) which are similar to the natural hydroxyapatite (Hannig & Hannig, 2010). Being of this minute size allows the nano-sized HA to effectively interact with the bacterial cell membrane – competing effect on the adhesins, as was shown to be the case in an *in vitro* study (Venegas *et al.*, 2006b)..

Additionally, BioRepair contains Zn PCA (pyrrolidone carboxylic acid complexed with zinc). PCA is a physiological component of human skin responsible for moisturizing (Kezic *et al.*, 2009). It may lead to a buildup of fibrillar networks as a result of pellicle and the reproduction of the tiny hydroxyapatite crystallites which are derived from hydroxyapatite micro clusters. Even as a single ingredient, zinc has proven antimicrobial properties against oral microorganisms, including *streptococcus mutans* (Fang *et al.*, 2006; Burguera-Pascu *et al.*, 2007). It was found, that zinc salts are equally active against *streptococcus mutans* at different concentrations. The unpleasant taste of zinc ions in mouth rinses is the only disadvantageous effect concerning its use, which were improved by the addition of suitable additives (Burguera-Pascu *et al.*, 2007).

BioRepair mouth rinse is made up of sugar alcohols including sorbitol and xylitol which have been shown to be related to antimicrobial effects (Bowen, 1994). Xylitol - a five-carbon polyol sweetener is a good example of this being of benefit to oral hygiene (Soderling *et al.*, 2011). Its use as a non-sugar based sweetener is widely accepted with the result of impressive caries reduction It is found naturally in vegetables and small fruits especially berries (Granstrom *et al.*, 2007). As with all polyol sweeteners, Xylitol increases the flow of saliva, thus enabling the mineralization process (Granstrom *et al.*, 2007; Soderling *et al.*, 2011). It has been demonstrated in numerous studies that xylitol decreases plaque accumulation (Soderling, 2009), it also decreases the numbers of mutans streptococci in both the plaque and saliva of xylitol consumers (Soderling & Hietala-Lenkkeri, 2010). Also, a xylitol-associated reduction in the mother-child transmission of *streptococcus mutans* has been observed. Mothers who were treated with xylitol, had reduced amounts of *streptococcus mutans,* compared to those given chlorhexidine or fluoride varnish (Isokangas *et al.*, 2000; Soderling *et al.*, 2000; Soderling *et al.*, 2001). Moreover, Thorild *et al.* (2004) and Soderling & Pihlanto-Leppala (1989) observed a substantial decrease in mother-child transmission of salivary *streptococcus mutans* by a maternal consumption of xylitol chewing gum containing chlorhexidine/xylitol. Furthermore, xylitol provides adhesion inhibition, possible with the consumption of potent xylitol products, as seen in saliva tests (Lif Holgerson *et al.*, 2006). Nevertheless, within a pilot study of Soderling *et al.* (2011) xylitol consumption reduced *streptococcus mutans* seen in plaque but did not impact the microbial properties of either plaque or saliva. However, the mechanism behind this antimicrobial action of xylitol is not well understood (Soderling & Hietala-Lenkkeri, 2010). There are two major hypotheses provided by *in vitro* studies. The first assumes, that xylitol hinders growth partly because of a glycolysis-inhibiting buildup of

xylitol-5-P on a intracellular level (Soderling & Pihlanto-Leppala, 1989). The second put forward the idea of how excessive energy is being used - 'futile xylitol-5-phosphate cycle'. Xylitol is consumed by *streptococcus mutans* and is then phosphorylated to a xylitol complex next dephosphorylated and finally released as xylitol (Trahan, 1995). Both hypotheses have not been completely verified under *in vivo* conditions, rather it was revealed that xylitol does not actually hinder the production of plaque acid, it is a sugar alcohol that non-fermentative (Takahashi & Washio, 2011). Despite this difference of suggestions, under *in vivo* conditions xylitol is shown as indeed possessing antimicrobial capability given the decline in both size and buildup of *streptococcus mutans* colonies with morphological alterations (Lee *et al.*, 2009). Furthermore, when xylitol is consumed over a longer period, salivary peroxidase are increased potentially causing a reduction in *streptococcus mutans* growth (Makinen, 1976). Moreover, a mouth rinse would not be seen as harmful for children since only a tiny amount of xylitol would be swallowed.

Significant reduction in the scores of *streptococcus mutans* was found after four-week-use of 20% xylitol mouth rinse in a recent *in vivo* study. The antimicrobial effect of xylitol mouth rinse on *streptococcus mutans* is comparable to other xylitol products, which is reasonably expected (ElSalhy *et al.*, 2012).

The other sugar alcohol contained in the BioRepair solution is sorbitol ($C_6H_{14}O_6$), a polyalcohol compound obtained from catalytic hydrogenation of starch hydrolysates (Rhodes & Kator, 1999). It is fermentable by several oral microorganisms because of its hexitol nature. When compared with xylitol, sorbitol has a weaker cariostatic effectiveness. Consequently, it does not hinder the plaque development, rather it may even encourage growth of some *streptococcus mutans* (Giertsen *et al.*, 2011). However, the amount of plaque and the levels of cariogenic bacteria are both decreased by the combining of xylitol and sorbitol (Makinen, 2011).

Sodium lauryl sulphate (SLS) constitutes 1 wt% in BioRepair mouth rinse. It is reasonable that SLS has a role in the antimicrobial mechanisms of the BioRepair as it was demonstrated to confer plaque inhibitory properties to toothpaste formulation (Jenkins *et al.*, 1991). It was seen as one of the initial generation of antimicrobial mouth rinses, able to reduce plaque considerably (Mandel, 1994). Such results revealed SLS in mouthwash formulations to produce reductions in salivary bacterial counts which remained significant up to 7 h. Mouth rinses containing 1% SLS were found to have similar plaque inhibitory effects compared to a 0.2%

triclosan containing mouthwash. Thus, SLS exerts considerable antimicrobial activity in the mouth (Jenkins *et al.*, 1991).

Sodium benzoate as a food preservative is also contained in the BioRepair solution, which might be related to the antibacterial effect of BioRepair. As food preservatives, sodium benzoates are used to kill bacteria or at least to inhibit its growth (Eklund, 1980; Salmond *et al.*, 1984). Some authors have argued that in theory the fall in the prevalence of caries could be explained by the huge rise in the consumption of food (Davis *et al.*, 2001). Based on the results of a previous *in situ* study, benzoate significantly inhibited biofilm thickness and vitality (Arweiler *et al.*, 2008). Also in an *in vitro* study, sodium benzoate was demonstrated to suppress *streptococcus mutans* biofilm formation (Al-Ahmad *et al.*, 2008) and prevent the increase of oral bacteria that cause dental caries. In an animal experiment, a combination of fluoride and sodium benzoate was more successful at inhibiting caries than fluoride alone (Davis *et al.*, 2001). Once administered, sodium benzoate builds up on and within the microorganisms as a result of excessive lipid soluability. Even if the conditions are more acidic like plaque deposits, sodium benzoate can combine with a proton and cross the cellular membrane. Just like fluoride, it also acts as a weak acid, in the cytoplasm. The result is an increase in the acidic stress of plaque streptococci. (Marquis *et al.*, 2003). Nevertheless, sodium benzoate neither affects removal of plaque nor seems to influence the glycolytic potential of the plaque (Danielsen *et al.*, 1996).

To conclude, the BioRepair mouth rinsing solution reveals antibacterial effects, thus validating the findings of the present study.

5.2.4 Theranovis

In the present study Theranovis oral spray did not reveal important antibacterial effect on the salivary flora. Slight antimicrobial effects were detected 1 h and 6 h after spraying. However, it was much less than after rinsing with CHX or even with Elmex Kariesschutz and BioRepair. Other authors had observed that, rinsing with solutions based on plant extracts significantly reduces the number of bacteria and the bacterial viability in biofilms (Quintas *et al.*,

2015), but these effects are related to reduced bacterial adhesion and not to a reduction of bacterial vitality in the salivary flora.

The use of essential oils in complementary medicine is widely practiced for numerous ailments including bacterial and fungal infections, e.g., gingivitis and vaginal candidiasis.

It has long been the case that till such claims can be scientifically validated they remain outside of conventional medicine practices (Hammer *et al.*, 1998).

Many studies (Chaudhari *et al.*, 2012; Celiktas *et al.*, 2007) conducted to investigate the antimicrobial effect of essential oils and most of them proved this effect, but results varied in relation to the degree of sufficiency of these oils.

For example, a recent *in vitro* study (Thosar *et al.*, 2013) to examine what could be used against oral pathogens a plant based oil was shown to be an effective intracanal antiseptic mouth rinse. The oil was peppermint. This does not agree exactly with the results of the present *in vivo* study. We can conclude the following: The present *in vivo* study tested the antibacterial activity of the essential oils rosemary and peppermint, which are the main active components of Theranovis spray. They revealed even slight effectiveness and this is at least a proof of their antibacterial efficacy. Hence, both *in vitro* and *in vivo* studies containing these oils or essential oils of other kinds of plants, as well as with different ways of application, concentration and different times of rinsing are recommended to identify the best antimicrobial efficacy of essential oils of plants.

5.3 Conclusion

The present study demonstrates the antibacterial performance of all tested mouth rinses/ oral spray on the salivary bacteria. The results of this study allow also classification of the tested products due to their efficacy in decrease

ng the level of bacterial vitality of the salivary flora under *in vivo* condition as follows:

- CHX had potent antibacterial effectiveness up to six hours.
- BioRepair (Zinc Hydroxyapatit) had moderate antimicrobial activity with an effect up to one hour.

- Elmex Kariesschutz has the best immediate antibacterial effect. However, it is also considered to be a mouth rinse with moderate antimicrobial activity with an effect up to an hour thereafter an obvious recovery in the bacterial vitality was detected.

- Theranovis oral spray showed no considerable antibacterial effect on the salivary flora.

However, there is a need for further *in vivo* studies based on a larger number of subjects, to characterize the antibacterial effects of the mouth rinses on the salivary flora in more detail.

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7. Publication/ Acknowledgements

7.1 Publication

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