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Efficacy of a mouthrinse based on hydroxyapatite to reduce initial bacterial colonisation *in situ*

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Highlights:

- Hydroxyapatite particles are proposed to prevent biofilm formation *in situ*.
- The effect is rather antiadhesive than antibacterial.
- Hydroxyapatite particles accumulate at the tooth surface *in situ*.
- The substantivity of this accumulation is limited.
- Hydroxyapatite particles accumulate around bacteria contained in saliva.

Abstract

Objective: The present *in situ* - investigation aimed to specify the impact of pure hydroxyapatite microclusters on initial bioadhesion and bacterial colonization at the tooth surface.

Design: Pellicle formation was carried out *in situ* on bovine enamel slabs (9 subjects). After 1 min of pellicle formation rinses with 8 ml of hydroxyapatite (HA) microclusters (5%) in bidistilled water or chlorhexidine 0.2% were performed. As negative control no rinse was adopted. *In - situ* biofilm formation was promoted by the intraoral slab exposure for 8 hours overnight. Afterwards initial bacterial adhesion was quantified by DAPI staining and bacterial viability was determined *in vivo/ in vitro* by live/ dead - staining (BacLight). SEM analysis evaluated the efficacy of the mouthrinse to accumulate hydroxyapatite microclusters at the specimens' surface and spit-out samples of the test solution were investigated by TEM.

Results: Compared to the control ($2.36 \times 10^6 \pm 2.01 \times 10^6$ bacteria/ cm²), significantly reduced amounts of adherent bacteria were detected on specimens rinsed with chlorhexidine 0.2% ($8.73 \times 10^4 \pm 1.37 \times 10^5$ bacteria/ cm²) and likewise after rinses with the hydroxyapatite test solution ($2.08 \times 10^5 \pm 2.85 \times 10^5$ bacteria/ cm², $p < 0.001$). No demonstrable effect of HA-particles on *Streptococcus mutans* viability could be shown. SEM analysis confirmed the

temporary adsorption of hydroxyapatite microclusters at the tooth surface. Adhesive interactions of HA-particles with oral bacteria were shown by TEM.

Conclusion: Hydroxyapatite microclusters reduced initial bacterial adhesion to enamel *in situ* considerably and could therefore sensibly supplement current approaches in dental prophylaxis.

Keywords: hydroxyapatite, pellicle, bacteria, bioadhesion

Introduction

Hydroxyapatite particles and clusters of different size may have the potential to develop into a valuable agent in preventive dentistry (Hannig et al., 2013a; Lelli et al., 2014). Although the authority of fluoride application as premier choice of standard oral prophylaxis measures is still undisputed, so far, neither bacterial adhesion nor acid-associated demineralization of dental hard tissue can be prevented completely. Alternative preventive treatment strategies aim to affect the physical, electrostatic and thermodynamic interactions that promote initial microbial adhesion and biomineralisation at the tooth surface (Besinis et al., 2015; Hannig and Hannig, 2010b; Kensche et al., 2016; Wessel et al., 2014). In this context, the acquired pellicle qualifies as the key structure to be modified by oral health care products (Hannig et al., 2013a; Hannig and Hannig, 2009; Weber et al., 2015). However, the wide variety of individual test preparations as well as the complexity of their potential active compounds has to be considered critically when evaluating the validity of studies testing oral health care preparations. This sometimes makes determination of effective antibacterial agents difficult, as most commercially available products contain a mixture of several active compounds (Hannig et al., 2013a). The pure components have rarely been tested *in situ*.

In recent years, growing attention has been drawn to nanoparticles, especially nanoparticulate metals and metal oxides (Allaker, 2010; Besinis et al., 2014; Hannig and Hannig, 2010b). Their possible benefits in biofilm management are suggested to be due to their shape, size-dependent active surface area, chemical reactivity and bacterial interactions (Allaker and Memarzadeh, 2014; Beyth et al., 2015; Hannig and Hannig, 2010b). In preventive dentistry, biomimetic micro- or nanocrystallite hydroxyapatite or other calcium-phosphate based systems such as CPP-ACP are promoted for their caries-preventive effect (Grychtol et al., 2014; Hannig et al., 2013a; Hannig and Hannig, 2010b; Najibfard et al., 2011). Unfortunately, most studies rather focus on their potential to repair demineralized surface lesions instead of investigating their ability to decrease bacterial adhesion or -growth (Lelli et al., 2014; Lu et al., 2007; Tschoppe and Kielbassa, 2011; Zhang et al., 2015).

With the aim to clarify the antibacterial potential of hydroxyapatite at the tooth surface, an *in situ* study performed by our group investigated the effect of different subfractions of a commercially available preparation (*Biorepair*) on initial bacterial colonization (Hannig et al., 2013a). The results indicated no noticeable antibacterial but a strong antiadhesive effect of zinc-carbonate hydroxyapatite (Hannig et al., 2013a). In line with our findings, Palmieri et al. observed an inhibitory effect of a comparable preparation on *Streptococcus mutans*- and *Mitis Group Streptococci* colonization of saliva-conditioned microtiter plates *in vitro* (Palmieri et al., 2013). The inhibition of biofilm formation by zinc oxide has repeatedly been shown, wherefore zinc-ions are added to some oral health care products (Grenho et al., 2015; Gu et al., 2013; Lynch, 2011). In comparison, the antibacterial or anti-adhesive potential of pure hydroxyapatite has so far not been sufficiently investigated and is rather based on hypothesis. In theory, hydroxyapatite particles could impair bacterial adhesion due to their pH-dependent surface charge influencing electrostatic interactions (Harding et al., 2005; Venegas et al., 2006). Furthermore, blocking of specific bacterial adhesins has been suggested based on *in vitro* investigations (Venegas et al., 2006).

In order to contribute to the assessment of pure hydroxyapatite microclusters as possible components of oral health care products, the present study investigated their potential to affect an *in situ* formed pellicle and to influence initial microbial adhesion and viability.

Therefore a pure hydroxyapatite microclusters containing mouthrinse was prepared and investigations were based on *in situ* experiments. The hypothesis of this study claims that the purely hydroxyapatite particles based mouthrinse would not reduce bacterial adhesion and -viability *in situ* within the first 8 hours of biofilm formation as effectively as the goldstandard chlorhexidine-digluconate.

2. Materials and Methods

Subjects and specimens

The *in-situ* experiments were realized with the participation of 9 volunteers, all healthy members of the laboratory staff or students aged 23 to 45. As prerequisite, all investigations corresponded to an ethics committee's review and approval (EK 147052013) and all volunteers had given informed written consent about participation in the study. The participants confirmed to be non-smokers. Initially, good oral health with no signs of gingivitis, caries or unphysiological salivary flow rate was ensured on the basis of an oral examination by an experienced dentist. In preparation of the *in situ* experiments, individual upper jaw splints were adjusted for all participants. In order to gain *in situ* pellicle samples, cylindrical enamel slabs measuring 5 mm in diameter and 1 mm in height were prepared as test specimens from bovine incisor teeth of 2-year old cattle (Hannig et al., 2013a; Kensche et al., 2013; Kensche et al., 2016). The surfaces were wet-ground and polished in a standardized grinding procedure with up to 4000 grid abrasive paper and the resulting smear layer was removed by steam jet and ultrasonication (US) with 3% NaOCl for 3 min.

Afterwards, all slabs were washed twice for 5 min in distilled water activated by US, followed by disinfection in 70% ethanol for 10 min (US), before finally being washed and stored in distilled water for 24 h.

Tested preparations and application protocol

In order to enable the exposure of 4 test specimens to the oral environment at a time, cavities were prepared in the buccal aspects of the splints in region of the premolars and the first molar of every quadrant. The samples were placed on each splint with polyvinyl siloxane impression material (Provil novo light regular set, Heraeus Kulzer, Germany) so that only the specimens' surfaces were exposed to the saliva. Before insertion of the splints, participants were instructed to brush their teeth without toothpaste and to rinse thoroughly with tap water. After the splints have been carried intraorally for 1 min to allow initial pellicle formation on the surfaces, rinses with 8 ml of the test preparations described below were carried out for one minute, followed by continuous intraoral exposure of the slabs for 8 hours overnight (23 pm – 7 am). In the morning at the lab, the slabs were removed from the splints and rinsed thoroughly with running tap water to remove any non-absorbed salivary remnants (Grychtol et al., 2014; Hannig et al., 2013a; Kensche et al., 2013). Further processing was performed *in vitro*.

The tested experimental rinsing solution was supplied by Dr. Kurt Wolff, Bielefeld, Germany. It contained only pure hydroxyapatite (HA) microparticles in distilled water (5 g HA dispersed in 100 ml bidistilled water). Referring to dynamic light scattering measurements performed by the company, the median particle size of the crystallites was 130 nm varying from 105 nm - 400 nm. A chlorhexidine-based mouthwash was used as reference (0.2% chlorhexidine-digluconate, meridol med CHX 0.2%, GABA, Lörrach, Germany) and samples carried intraorally without being subjected to any mouthrinse served as negative control. To avoid mutual interferences of the test solution and the control, experiments were carried out on different days.

Fluorescence Microscopy

Fluorescence microscopic detection of bacteria as well as determination of their viability was performed on the basis of 10 randomized microscopic ocular grid fields per sample (Jung et al., 2010; Kensche et al., 2013). The particular size of the ocular grid fields (0.0156 mm²) allowed calculating the number of cells per square centimeter. All epifluorescent analyses were performed at 1000fold magnification (Axioskop II, ZEISS, Oberkochen, Germany).

DAPI staining and glucan visualization

As a standard method of fluorescent microscopic investigation, DAPI (4', 6-diamidino-2-phenylindole) was used to visualize bacteria present in the investigated samples. The dye is taken up into bacteria where it binds to adenine/thymidine-nucleic acids of double-stranded DNA, forming fluorescent units. DAPI is applicable for the detection and quantification of adherent microorganisms, however, without differentiation of viability. Additionally, glucans, as major structure molecules of the extracellular matrix were fluorescence microscopically visualized by Alexa Fluor 574 conjugated Concanavalin A. The staining was conducted as described in earlier studies (Hannig et al., 2007; Jung et al., 2010; Kensche et al., 2013). In brief, the enamel slabs were washed in saline solution and then covered with 1 ml DAPI-Methanol working solution (1 µg/ ml) or, as simultaneous staining of glucans and bacteria was performed, 1,5 µl DAPI stock solution (1 mg/ ml Methanol) were added to 498,5 µl of PBS and 10 µl of ConA stock solution. After 15 min of incubation in a dark chamber at room temperature the solution was poured off and the slabs were rinsed with methanol and left to air-dry at room temperature. Finally the slabs were fixed to a slide and allocated to fluorescence microscopic investigation.

BacLight viability assay

The LIVE/ DEAD BacLight bacterial viability assay consists of two nucleic acid stains - the green fluorescent SYTO 9 stain (component A) and the red-fluorescent propidium iodide stain (component B) (Invitrogen, Molecular probes, Darmstadt, Germany).

In vitro experiment

All labeling was performed in a 96-well plate reader according to the manufacturer's instructions. After overnight cultivation, a suspension of *Streptococcus mutans* in saline solution was adopted and a part of it was inactivated by heating (95°C for 1 h). A dilution with saline solution (0.9% NaCl, sterile) was prepared from each test preparation (CHX, HA-particles) and mixed with an equal amount of the vital bacteria suspension. After incubating these for 10 min, the solutions were mixed with heat-inactivated bacteria (0 : 100; 5 : 95; 25 : 75; 45 : 55; 50 : 50). For fluorescent labeling, 0,5 µl of the BacLight stock solution were added to 250 µl of the suspensions and incubated for 10 min in a dark chamber before a volume of 100 µl from each sample was given into a microtiter plate and investigated by fluorescence microscopy. The excitation wavelength was 470 nm; emission was recorded at 530 nm for the vital and at 620 nm for the avital cells. All measurements were performed in duplicates to equalize inhomogeneity of the suspensions. In order to evaluate the mouthrinses' effect on bacterial viability, the ratio of vital and dead bacteria was calculated (emission vital/ emission dead bacteria) while saline solution served as reference.

In situ experiment

Based on previous studies, the assay was adopted for the visualization of vital/ avital cells on enamel slabs after exposure to the oral cavity. A stock solution was prepared from equal amounts of both components (Syto9 1.67 mM/propidium iodide, 1.67 mM, 300 µL DMSO) and B (Syto 9 dye, 1.67 mM/propidium iodide, 18.3 mM, 300 µL DMSO) and 2 µl were pipetted to 1 ml of saline solution. The slabs were incubated in this solution for 10 min at room temperature. Subsequently the solution was rinsed of with saline solution and fluorescence microscopic investigation could be performed using a fluorescein diacetate and an ethidium-bromide filter.

Statistics

All data were statistically processed by SPSS 22.0. The Kruskal-Wallis test as well as additional pair-wise comparison in terms of the Mann-Whitney U test were performed ($p < 0.001$).

Electron microscopic investigations

In order to visualize the potential adherence of the apatite microparticles/ -clusters from the rinsing solution onto the pellicle surfaces, additional enamel specimens were carried intraorally by 4 subjects. After 1 min of *in situ* pellicle formation, subjects rinsed with 10 ml of the hydroxyapatite – containing mouth rinsing solution for 60 s. Subsequently, the rinsing solution was spat out and the specimens' intraoral exposure was continued for different periods of time, 0, 30 or 120 min respectively. After oral exposure the pellicle covered enamel specimens were gently rinsed with water and air – dried. After carbon sputtering the enamel surfaces were analyzed by SEM (XL 30 ESEM FEG; FEI, Eindhoven, The Netherlands) at magnification up to 20.000 fold. Furthermore, the hydroxyapatite particles that had been used for preparing the apatite mouth-rinsing solution were directly applied to the SEM aluminum stub sample holder and analyzed by SEM.

Additionally, samples of the spit-out testsolution mixed with saliva obtained directly after the 60 s rinse were collected and prepared for analysis by transmission electron microscopy. Two milliliter of the spit-out samples were centrifuged at 4000 rpm for 10 min. The clear supernatant was discarded and 1 ml of fixing solution containing 2.5% glutaraldehyde was applied to the pellet for 1 h at 4°C. Afterwards the samples were washed with 0.1 M cacodylate buffer, centrifuged again and the supernatant was discarded and exchanged by 1 ml Osmium tetroxide (1%) for 1h for postfixation. Subsequently, the samples were washed with 0.1 M cacodylate buffer, as described above. After dehydration in an ascending series of alcohol, embedding took place in araldite CY212 (Agarscientific, Stansted, UK). Ultrathin sections were cut with a diamond knife in an ultramicrotome (Ultracut E, Bensheim, Germany), mounted on Pioloform-coated copper grids and contrasted with aqueous solutions

of uranyl acetate and lead citrate. TEM-analysis took place in a TEM TECNAI 12 Biotwin (FEI, Eindhoven, The Netherlands) at magnification of up to 100,000-fold.

Results

All fluorescence microscopic assays were performed to successfully label bacteria present in the initial oral biofilm after 8 hours of intraoral specimens' exposure. Besides the visualization of adherent bacteria by DAPI-labeling, further differentiation of antibacterial effects in terms of bacterial viability could be derived from the application of the BacLight live/ dead staining. Also, additional staining of glucan formation was performed to display the accumulation of extracellular matrix components.

Microbial adhesion

Regarding the enamel slabs of the control-group, without having experienced any rinses, an obvious adhesion of bacteria could be detected after 8 hours of intraoral exposure with a bacterial monolayer covering the majority of the investigated slab-surface ($2.36 \times 10^6 \pm 2.01 \times 10^6$ bacteria/cm²). Glucan visualization substantiates the appearance of promoted biofilm formation as there was a dense accumulation of glucans around the adherent bacteria. Some samples showed an almost closed layer of labeled extracellular matrix glucans. In contrast, as expected, 0.2% chlorhexidine reduced bacterial colonization significantly and only a few isolated bacterial accumulations or even single bacteria were found throughout the microscopically investigated sample fields ($8.73 \times 10^4 \pm 1.37 \times 10^5$ bacteria/cm²). In line with inhibited bacterial adhesion, glucan formation or -accumulation was also reduced and only a few filamentous molecules were detectable in immediate proximity to adherent bacteria. Interestingly, the test solution composed purely of 5% hydroxyapatite in bidistilled water reduced bacterial adhesion almost as effectively as the positive control ($2.08 \times 10^5 \pm 2.85 \times 10^5$ bacteria/cm²). According to the Mann-Whitney U test no significant difference was determined for the amount of adherent bacteria after rinses with chlorhexidine 0.2% and the

hydroxyapatite test solution ($p = 0.323$). Again, only occasional accumulations of few bacteria were randomly distributed at the surface of the investigated samples. Likewise, glucan detection was reduced noticeably.

Bacterial viability

In-vitro experiments

Briefly, no noticeable antibacterial effect could be determined for the application of the hydroxyapatite-based test solution to a suspension of *Streptococcus mutans in vitro*. The BacLight viability assay was performed to reveal if addition of the tested preparation had an impact on the ratio of viable to dead bacteria due to affecting bacterial viability in solution. In contrast to the positive control 0.2% chlorhexidine whose antibacterial effect was significant in comparison to the referring saline solution, bacterial viability appeared to be rather unaffected by the presence of hydroxyapatite particles in solution.

In-situ experiments

In comparison to the negative control, 0.2% chlorhexidine as well as the hydroxyapatite particles in bidistilled water reduced the amount of detectable adherent bacteria significantly. Eight hours after the adoption of the rinses and intraoral slab exposure significantly less viable ($p < 0.001$) as well as dead bacteria ($p < 0.001$) were visible adhering to the specimens' surface and the initial biofilm than for the control, respectively. Although the antibacterial effect of chlorhexidine hasn't quite been achieved by the test solution, the difference was not significant (viable: $p = 0.134$, dead: $p = 0.214$). Interestingly, as suggested by the *in vitro* results, the proportion of dead to vital cells increased after the adoption of chlorhexidine (13.56). However, it was not affected by the test solution (5.59), if compared to the negative control (8.82), or was even slightly shifted towards the proportion of vital cells, respectively.

Electron microscopy

Scanning electron microscopy at up to 20,000-fold magnification allowed the visualization of hydroxyapatite particles and – clusters in the low micrometer range and below.

Representative micrographs of the tested underlying hydroxyapatite powder as well as the pellicle covered specimens after intraoral exposure and application of the 5% hydroxyapatite test solution are depicted in fig. 5. Different times of continued pellicle formation were performed to investigate the course of potential accumulation processes at the pellicle surface. The analysis of the pure hydroxyapatite powder showed fragment-like formed hydroxyapatite aggregates of a few micrometers in size which were composed of smaller particles. Due to not being dissolved in a solution, larger clump - like aggregates with a diameter of 10 to 15 μm were detectable as well (fig. 5 a, b). Furthermore it could be shown by SEM that rinsing the mouth with the 5% hydroxyapatite test solution promotes the accumulation of hydroxyapatite microclusters and – particles at the pellicle surface, which could still be detected after 30 min of continued intraoral slab exposure (fig. 5 c - f). The spherically shaped particles were randomly distributed on the specimen's surface and compared to the undissolved hydroxyapatite powder, the individual hydroxyapatite clusters appeared to be smaller and less agglomerated. Similar to the provided data from the dynamic light scattering measurements, several particles of approximately 150 nm in size could be detected. However, after prolonged intraoral slab exposure, only isolated remains of the hydroxyapatite aggregates could be detected (fig. 5 g, h) and the durability of the adsorption process appeared to be limited.

The additional analysis of spit-out samples of the test solution mixed with saliva obtained directly after performing the mouthrinse confirmed that a considerable amount of dissolved hydroxyapatite microclusters was distributed in the oral cavity (fig. 6). It must be assumed that the oral fluids had been enriched with remnants of the test solution. In accordance with the SEM findings, the diameter of definable crystalline microclusters in solution varied from 100 nm to 500 nm, including dense particle accumulations as well as seemingly isolated particles. Furthermore, bacteria from saliva were also detectable in the spit-out samples. Bacterial fimbriae surrounding the cell wall could be visualized. As shown in figure 6, the

hydroxyapatite microclusters appeared to accumulate evenly around the present bacteria in a wrapping-like manner, apparently interacting with the fimbriae of the bacterial cell wall (fig. 6 b).

Discussion

It is a unique feature of this study that a mouthrinse purely prepared of hydroxyapatite microparticles was investigated for its effect on initial bacterial colonization of the tooth in situ. Given the morphological similarities between enamel crystallites and synthesized hydroxyapatite particles, an accumulation of the material at the pellicle surface is perfectly conceivable (Bradna et al., 2015; Lelli et al., 2014; Tschoppe and Kielbassa, 2011). However, according to the SEM analysis results of an in vitro investigation by Bradna et al., the formation and sustainability of these material deposits so far had to be questioned (Bradna et al., 2015). Furthermore, little is known about the specific influence of hydroxyapatite microclusters on pellicle- or bacterial biofilm formation (Aykut-Yetkiner et al., 2014; Hannig and Hannig, 2010a; Kensche et al., 2016; Poggio et al., 2014). Compounding this is the fact that customary health care preparations often contain a multitude of components which might influence oral bioadhesion processes (Babu and Garcia-Godoy, 2014; Hannig et al., 2013a). Therefore, a specific correlation of bacterial growth, - adhesion or - viability with the presence of hydroxyapatite particles has not been confirmed yet. It is precisely for this reason that only hydroxyapatite particles dissolved in bidistilled water were used as the test solution in this study. In order to gain a more differentiated idea of the materials' morphology as well as its biological characteristics, in vitro- as well as in situ investigations were performed. The electron microscopic investigations showed a fine distribution of the hydroxyapatite powder in solution, yielding hydroxyapatite microclusters in the low micrometer range varying from 100 nm to approximately 500 nm (fig. 5, 6). Van der Waals- and electrostatic forces derived from the dipole properties of hydroxyapatite might be responsible for the persisting cohesion of the particles in form of clustering (Bystrov et al.,

2009). The sparse literature on this subject discusses the individual particle size of nano- and microstructured hydroxyapatite microclusters to be relevant for their interaction with surrounding biopolymers whereby smaller particles or clusters exhibit a greater active surface area (Abdulkareem et al., 2015; Westas et al., 2014). This possibly influences the stability of their incorporation in the initial biofilm at the tooth surface as well as their interactions with bacteria (Kensche et al., 2016). Physicochemical interactions such as Van der Waals forces, electrostatic interactions and hydrogen bonding between pellicle components and bacteria contained in saliva are considered the decisive driving force of initial bacterial biofilm formation at the tooth surface (Busscher and van der Mei, 1997; Costerton et al., 1995). This implies that effective prophylactic agents can either work by a direct antibacterial or an (anti-)adhesive effect. The latter may involve hampering the adsorption of pellicle components acting as receptors or interacting with bacteria to keep them from irreversibly attaching to the tooth surface (Hannig et al., 2013b; Hannig et al., 2009; Weber et al., 2015).

Numerous studies have confirmed the advantages of chlorhexidine to substantially reduce bacterial vitality in the oral cavity, thus it served as the positive control in this study (Hannig et al., 2013b; Ribeiro et al., 2007; Vitkov et al., 2005). The cationic agent binds to the anionic wall of bacteria cells causing dysfunctions of the cell's osmoregularity, metabolism and structural integrity (Quintas et al., 2015). Consistent with the literature the epifluorescence microscopic investigations performed in this study confirmed a significant reduction of bacterial biofilm formation at the tooth surface even 8 hours after the 0.2% chlorhexidine mouthrinse in situ (Quintas et al., 2015). The BacLight in vitro investigation suggests that the reduced amount of adhering bacteria is predominantly derived from its strong bactericidal effect. Alternatively, noteworthy indications for the efficacy of a hydroxyapatite microparticles based mouthrinse to reduce bacterial colonization of enamel arose from a previous study which closely investigated the impact of a customary mouthrinse and its solid and liquid components on initial biofilm formation in situ (Hannig et al., 2013a). It was shown, that the mouthrinse as well as the separate components significantly reduced the amount of adherent

bacteria in general as well as the amount of viable bacteria detectable by epifluorescence microscopy on enamel specimens exposed in the oral cavity for 6 or 12 hours (Hannig et al., 2013a). Nevertheless, uncertainty remained about whether and to what extent the observed effects had to be attributed to the incorporated zinc-carbonate within the apatite lattice, whose antibacterial effect has already been emphasized (Palmieri et al., 2013). Therefore, the present study focused on the investigation of pure hydroxyapatite microclusters.

Both the epifluorescence microscopic examination as well as the electron microscopic investigations revealed demonstrable effects of the hydroxyapatite particles on initial bioadhesion processes. DAPI- as well as BacLight – *in situ* tests confirmed a clear reduction of initial bacterial biofilm formation after 8 hours of intraoral biofilm formation which was comparable to chlorhexidine. In terms of decreasing bacterial colonization alteration of both bacterial adhesion as well as bacterial metabolism is conceivable. Crucial evidence for a rather anti - adhesive than antibacterial effect of the hydroxyapatite particles was provided by the additional BacLight *in vitro* assay determining the vitality of *Streptococcus mutans* under the influence of the investigated hydroxyapatite test solution. However, Venegas et al., who examined the interaction of hydroxyapatite particles of low micrometer range with different bacterial cells in human saliva *in vitro*, suggest an electrostatically derived affinity of *Streptococcus mutans* and other bacterial strains to bind and coaggregate at the particles' surface visualized by SEM – analysis (Venegas et al., 2006). The findings of our transmission electron microscopic analysis of the spit-out mouthrinse - or saliva samples, respectively, further indicate considerable binding forces between bacterial cell wall components and hydroxyapatite particles (fig. 6). The structural composition of gram-positive bacteria adhesins is a subject of ongoing research (Vengadesan and Narayana, 2011). However, an earlier *in vitro* experiment of Ueshima et al. has shown that the adhesion and proliferation of gram-positive bacteria tends to increase on negatively charged hydroxyapatite surfaces (Ueshima et al., 2002). Alternatively, the bacterial biofilm reducing effect of hydroxyapatite particles could also be due to a modification of the *in situ* formed pellicle ultrastructure or - component adsorption, respectively, as previously seen for zinc-carbonate

hydroxyapatite particles (Kensche et al., 2016). Epifluorescence microscopic measures alone do not clarify if a reduction of initial bacterial colonization is due to a layer formation of hydroxyapatite particles at the pellicle's surface. Therefore, SEM analysis was performed as an appropriate method to visualize the morphology of the applied hydroxyapatite particles *in vitro* as well as to clarify their potential accumulation at the pellicle surface *in situ* at different times after application of the mouthrinse. For the first time, to the best knowledge of the authors, a mouthrinse-induced accumulation of hydroxyapatite microclusters at the tooth surface *in situ* was now confirmed. Microclusters measuring 50 nm to 400 nm could clearly be detected at the surface of the investigated enamel slabs over a period of up to 2 hours after the mouthrinse. Moreover, our SEM – analyses also revealed the dynamics of this accumulation process as after 2 hours only isolated microclusters were detected. For this reason, SEM – analysis of longer exposure times was omitted. The material deposits are not sustainably bound to the enamel surface and the detachment or desorption of the hydroxyapatite – aggregates must be concluded. However, an only brief accumulation of the microparticles at the enamel surface does not necessarily imply a failure of protective or desirable effects. Taken together, the results of the epifluorescent and electron microscopic investigation suggest that the inhibition of bacterial biofilm formation by hydroxyapatite microclusters are caused by different observed mechanisms of action. On the one hand, the proven accumulation of hydroxyapatite microclusters at the pellicle surface can shield pellicle-receptors from bacterial attachment. Bacteria adhering to the hydroxyapatite microclusters can also be eliminated by desorption of the bacteria - hydroxyapatite complexes from the pellicle surface, respectively. On the other hand, cell wall adhesins of bacteria contained in saliva can be blocked by the accumulation of hydroxyapatite particles and bacteria could even be agglutinated by the interaction with the hydroxyapatite microclusters. Consequently, those bacteria would not be available for the adhesion to the pellicle surface. These observations should be considered as important aspects contributing to the reduced amount of detectable adhering bacteria after the hydroxyapatite microcluster mouthrinse and 8 hours of intraoral biofilm formation.

Conclusion

From a clinical point of view the mouthrinse purely based on hydroxyapatite microclusters appears to be a promising biomimetic option to effectively control bacterial biofilm formation as a supplement of daily oral hygiene procedures. For the first time, an accumulation of hydroxyapatite aggregates at the tooth surface as well as to salivary bacteria *in situ* could clearly be confirmed. The material's antiadhesive effect significantly reduced the amount of bacteria adhering at the pellicle's surface and no significant inferiority was seen in comparison to chlorhexidine 0.2%. However, a long-term adsorption of the active aggregates could not be confirmed and closer investigation of the physicochemical interactions between the applied hydroxyapatite particles and the surrounding biopolymers as well as their interference in initial bioadhesion processes are necessary for a better understanding of the observed effects.

Acknowledgment and conflict of interest:

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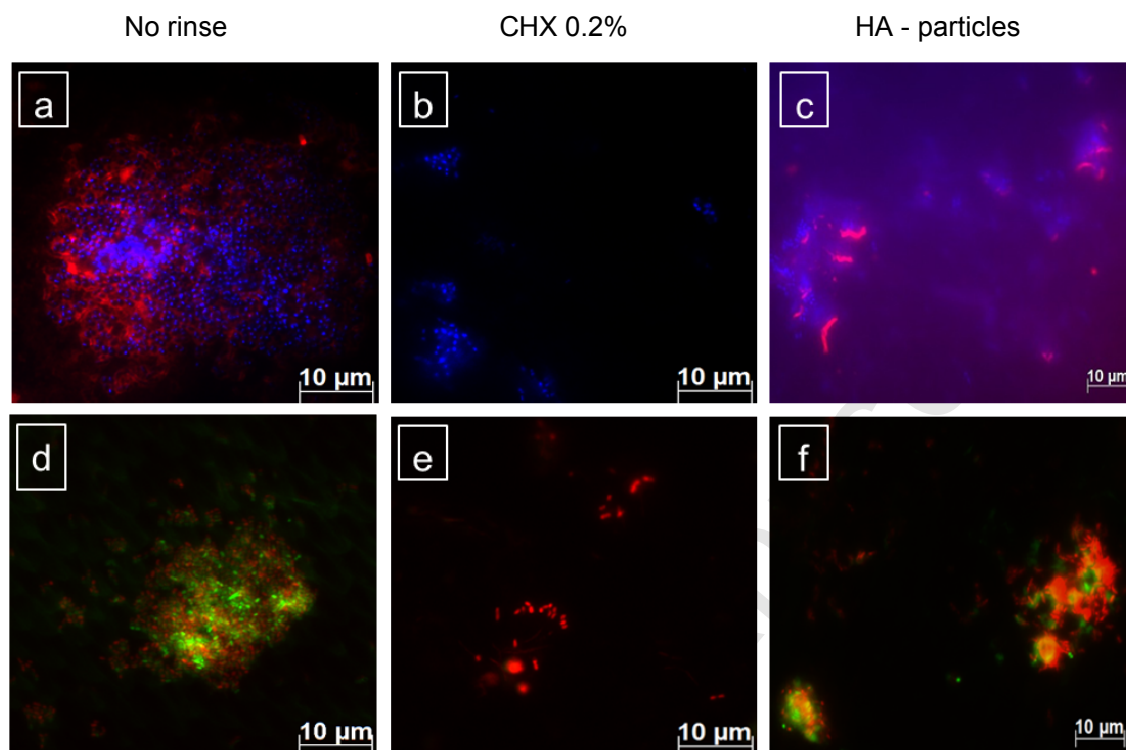


Fig. 1: Representative images of the fluorescence microscopic investigation: specific fluorescent labelling including DAPI (blue) and Concanavalin A (red) allows the visualization of bacteria and glucans adhering to the specimens' surface (a – c) as well as, considering BacLight LIVE/ DEAD – staining, the differentiation of the adherent bacteria according to their vitality, green – vital and red – avital (d – f). After 8 hours of intraoral exposure, the unrinsed enamel slabs of the negative control appeared densely colonized by bacteria – glucan agglomerates (a). BacLight – staining confirmed that the high amount of adherent bacteria in general (a) implied a high proportion of vital bacteria (d). Quite contrary to the slabs being rinsed with chlorhexidine, where initial bacterial colonization as well as glucan formation were significantly prevented (b) and almost no vital bacteria were detected (e). Furthermore, 8 hours after the rinses with the 5% hydroxyapatite test solution, only sparse bacterial accumulations were detected with little sign of extracellular matrix formation compared to the negative control (c). BacLight - staining obviously confirms the impact of the hydroxyapatite test solution on inhibiting bacterial colonization, although a verifiable shift from vital to dead bacteria could not be confirmed (f).

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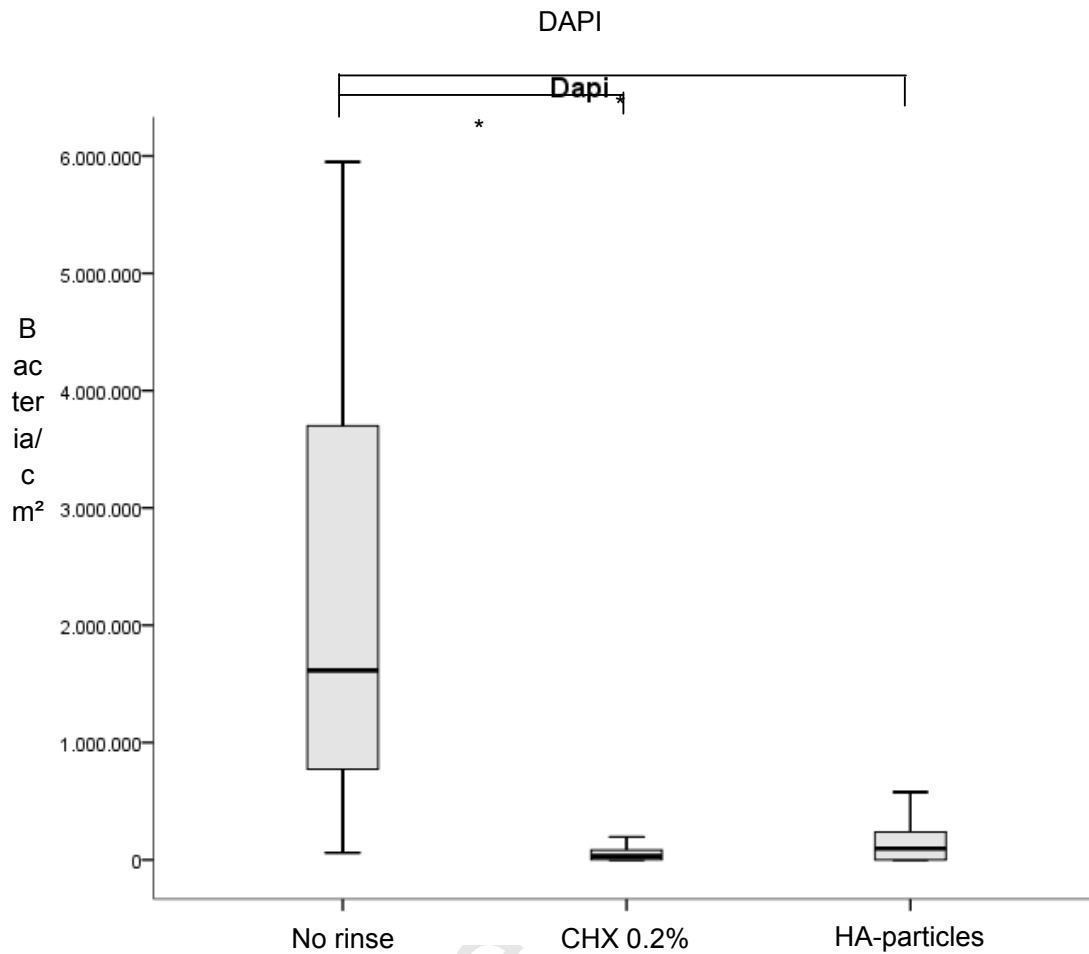


Fig. 2: Influence of 5% hydroxyapatite test solution and 0.2% chlorhexidine on initial bacterial adhesion to enamel *in situ*, visualized by DAPI – staining. After 1 min of pellicle formation participants of the experimental groups rinsed with 8 ml either of the hydroxyapatite test solution or chlorhexidine for 1 min and the intraoral exposure of the specimens was continued for 8 hours overnight. Samples exposed intraorally without rinsing served as control. All labeling was carried out in duplicate for $n = 8$ subjects. As confirmed by the Kruskal-Wallis test, both, the gold standard chlorhexidine but also the hydroxyapatite microcluster solution reduced initial bacterial adherence to the enamel slabs significantly compared to the control ($p < 0.001$). Additional pairwise comparison by Mann–Whitney U test yielded no significant difference between chlorhexidine 0.2% and the hydroxyapatite test solution regarding their effect on initial bacterial adhesion ($p < 0.001$).

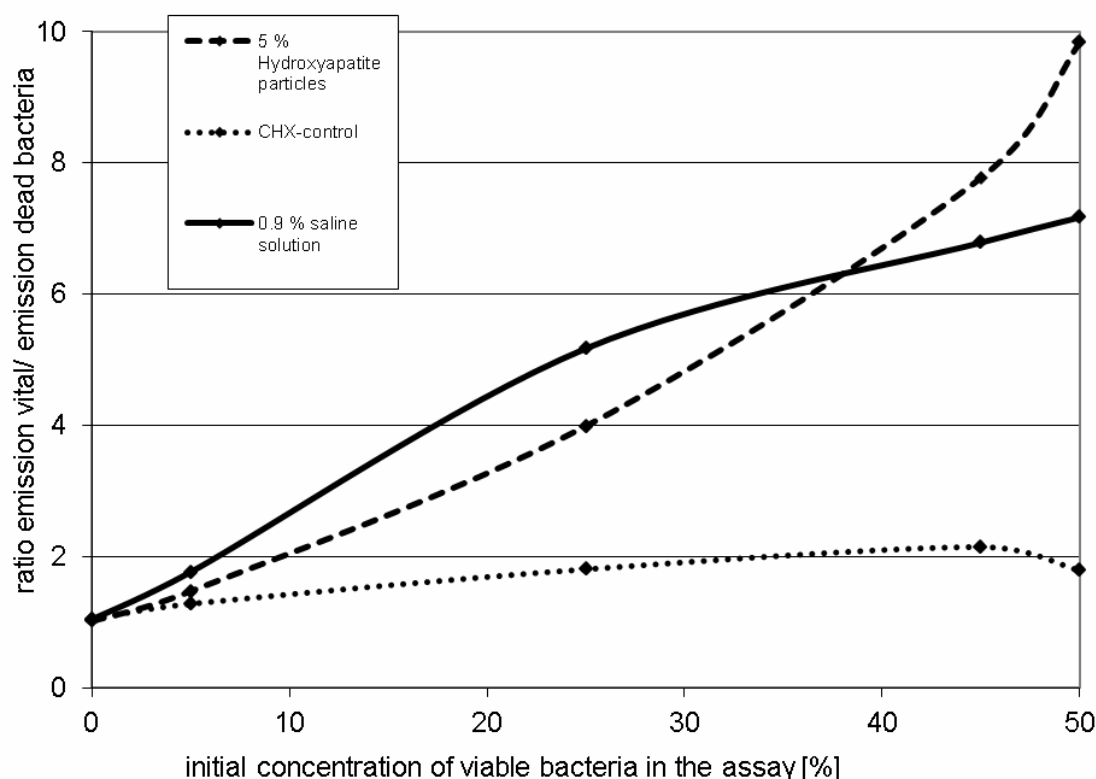


Fig. 3: Effect of 5% hydroxyapatite microcluster solution and of 0.2% chlorhexidine (positive control) in comparison to 0.9% saline solution (negative control) on the viability of *S. mutans* *in vitro*. In order to specify the antibacterial effect of the tested preparations they were incubated with a suspension of *S. mutans* *in vitro*. Afterwards, the samples were added to a suspension with a defined proportion of heat-inactivated bacteria and the final ratio of vital to dead bacteria was determined on the basis of BacLight LIVE/ DEAD - staining (emission vital: 530 nm/ emission dead: 620 nm). All measurements were carried out in duplicate. While 0.2% chlorhexidine reduced the amount of viable bacteria significantly, the hydroxyapatite microclusters appeared to have no noticeable bactericidal effect on *S. mutans*.

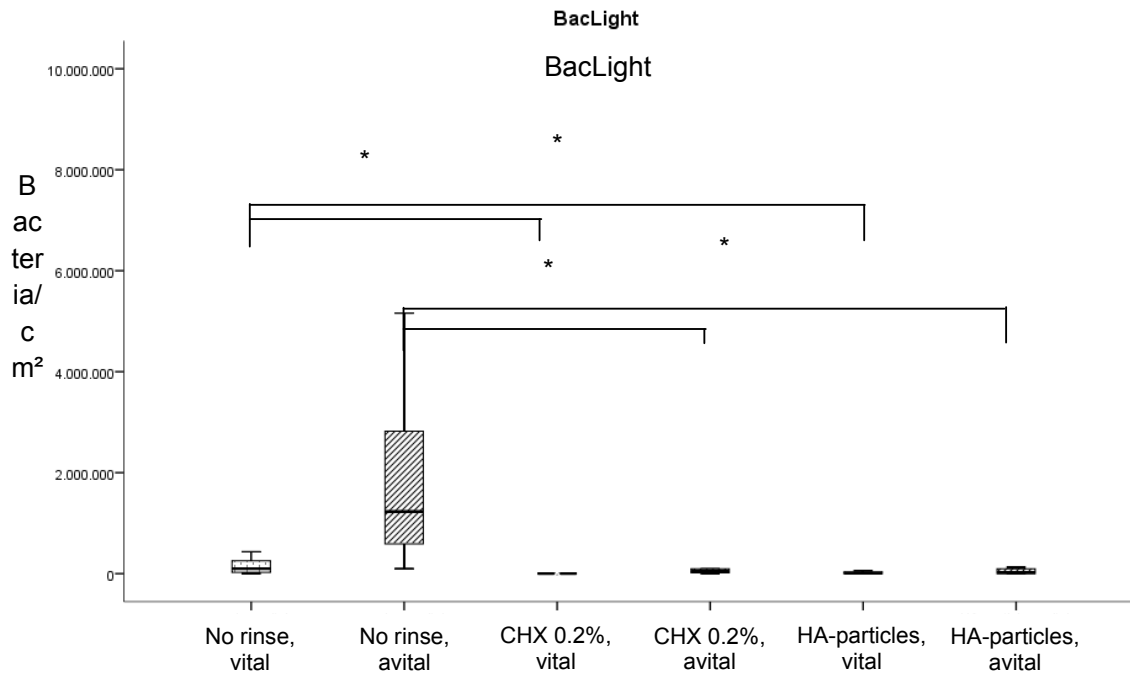


Fig. 4: BacLight LIVE/ DEAD – staining provided a differentiated consideration of the rinses' impact on the viability of the bacteria adhering to the enamel specimens after 8 hours of biofilm formation *in situ*. *In - situ* pellicle formation was allowed for 1 min before rinses with 8 ml either of the hydroxyapatite test solution or chlorhexidine were conducted for 1 min. The intraoral specimens' exposure continued for 8 hours overnight. Samples exposed intraorally without rinsing served as control. All labeling was carried out in duplicate for $n = 8$ subjects. According to the results of the Kruskal-Wallis test both rinses had a significant impact on the reduction of vital as well as dead bacteria detectable on the specimens' surface compared to the control ($p < 0.001$). Furthermore, no significant differences were revealed when comparing the rinses by the Mann-Whitney U test ($p < 0.001$).

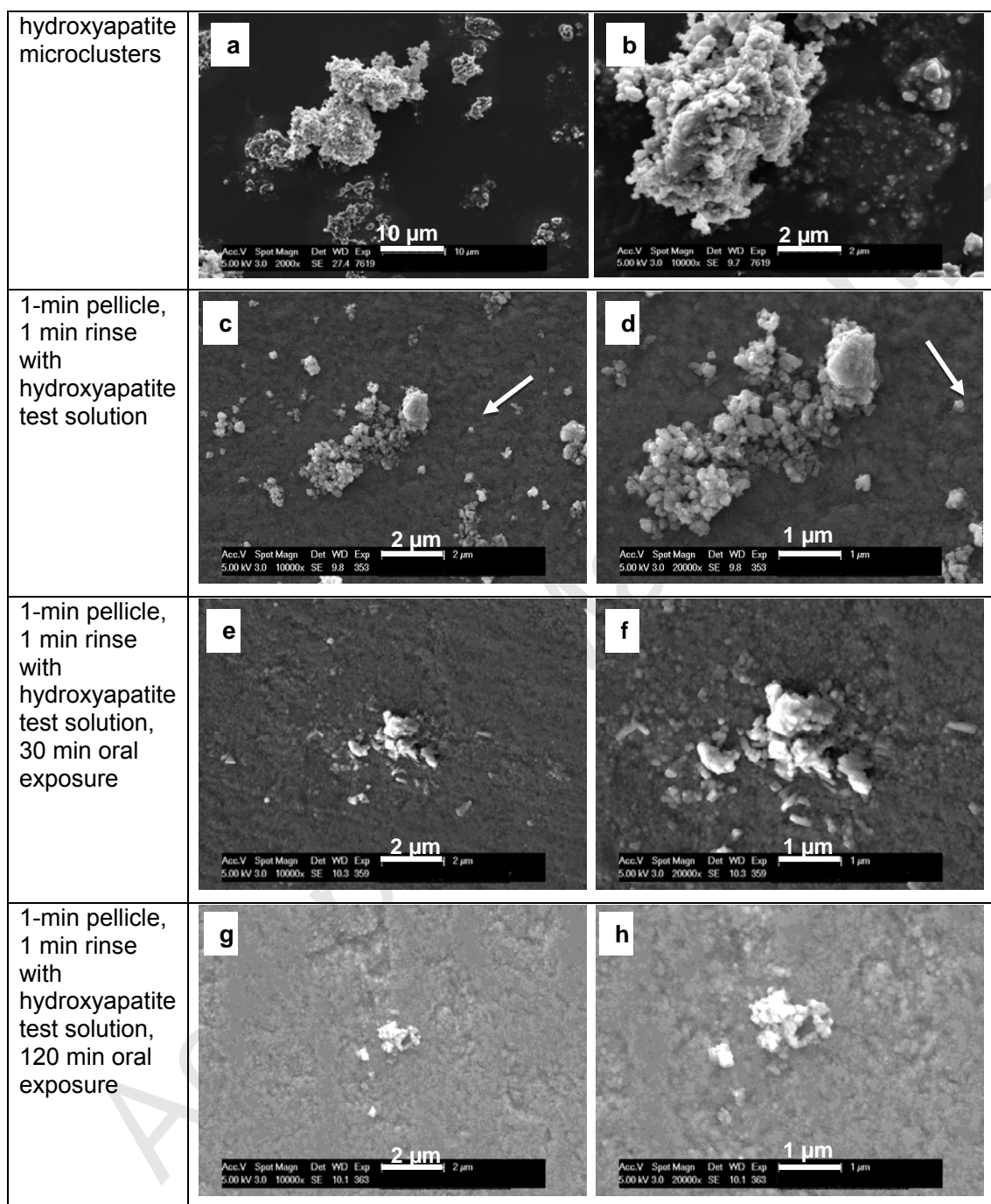


Fig. 5: Representative images of scanning electron microscopic analysis revealing the course of the hydroxyapatite microcluster accumulation and desorption at the tooth surface *in situ*. Close investigation of the hydroxyapatite powder at 2000 fold and 10000 fold magnification visualized the aggregation of hydroxyapatite particles in microclusters in small micrometer range (a, b). After the rinse with a 5% hydroxyapatite test solution the

accumulation of hydroxyapatite microclusters at the tooth surface could clearly be confirmed (c, d). The spherically shaped aggregates were spread randomly across the investigated enamel slabs, their size varying between around 50 nm (see arrow c, d) to 400 nm. In part, a rather dense concentration of the microclusters was seen. After further 30 min of intraoral slab exposure similar hydroxyapatite deposits could still be detected (e, f) whereas after 120 min only isolated remnants were found at the tooth surface (g, h). A desorption of the accumulated hydroxyapatite aggregates must be concluded.

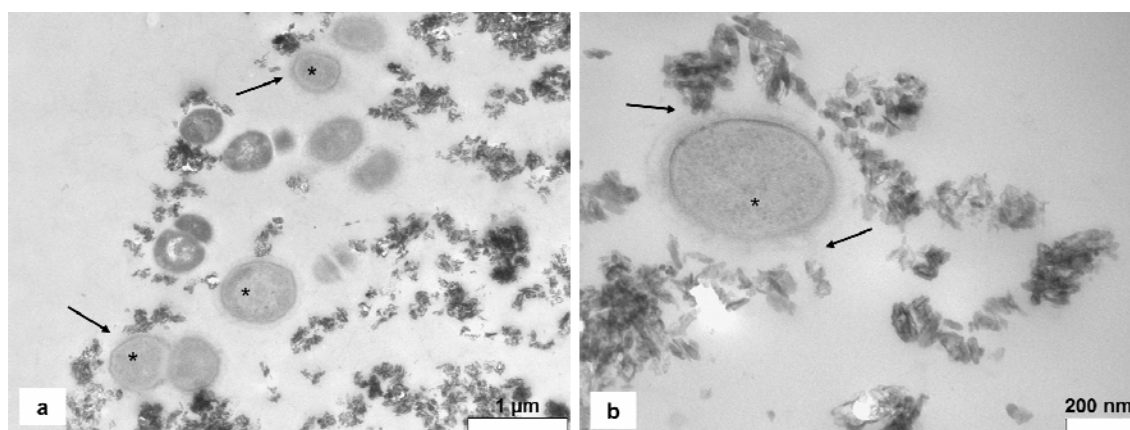


Fig. 6: TEM – analysis of spit-out samples of mouthrinse – saliva mix obtained directly after the 60 s rinse. The solution was enriched with hydroxyapatite microclusters measuring 100 – 500 nm in size and being built of aggregated crystallites. Bacteria originating from the oral cavity were also detected in the representative samples (*). A noticeable accumulation of hydroxyapatite particles could be seen around the present bacteria, possibly derived by binding interactions between hydroxyapatite particles and bacterial fimbriae (➤). Original magnification a: 18,500-fold, b: 68,000-fold