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# Influence of vinegar on biofilm formation *in situ*

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2. Berichterstatter:

*To my beloved family and friends*

*Meiner geliebten Familie und Meinen*

*Freunden*

# Abbreviations

| | |
|---|---|
| ACD | Allergic contact dermatitis |
| CHX | Chlorhexidine |
| *e.g.* | *exempli gratia*, for example |
| *etc.* | et cetera |
| eDNA | Extracellular DNA |
| EGF | Epidermal growth factor |
| EPS | Extracellular polymeric substances |
| FDA | Food Drug Administration |
| FM | Fluorescence Microscopy |
| GCF | Gingival crevicular fluid |
| H | Hour |
| *i.e.* | *id est*, in other words |
| LB | Left buccal |
| LDS | Live / Dead staining |
| LP | Left palatal |
| max. | Maximum |
| mg | Milligram |
| min | Minute |
| min. | Minimum |
| ml | Millilitre |
| mm | Millimetre |
| $mm^2$ | Square millimetre |
| nm | Nanometre |

| | |
|---|---|
| n.s. | Not significant |
| pH | Portential of hydrogen |
| PI | Propidium iodide |
| RB | Right buccal |
| RP | Right palatal |
| s | Second |
| *S. mitis* | Streptococcus mitis |
| *S. mutans* | Streptococcus mutans |
| SEM | Scanning electron microscopy |
| TEM | Transmission electron microscopy |
| µl | Microlitre |
| µm | Micrometre |

# Content

# 1 Abstract

## 1.1 English


### Influence of vinegar on biofilm formation *in situ*

Objectives: Vinegar has been recognized as a significantly effective antimicrobial for long. The study intended to elucidate the efficacy of commercially available vinegar (distilled vinegar) on *in situ* pellicle formation and biofilms. The aim of part one was to investigate the impact of vinegar on formation of the initial pellicle, and part two was to explore the influence from vinegar on mature biofilm and saliva.

Materials and Methods: The *in situ* biofilm formation was performed intraorally over 3 min and 24 h on bovine enamel slabs mounted in individual splints. The enamel slabs were rinsed with vinegar for 5 s and subsequently with water twice for 30 s. Afterwards, the enamel slabs were removed from the splints or kept exposed in the oral cavity for another 30 min or 120 min. Samples with water rinsing instead of vinegar served as controls. In addition, saliva samples were collected in order to investigate the effect of vinegar rinsing on the salivary microflora. After oral exposure, all the samples were analyzed via *Bac*Light$^{TM}$ viability assay (24h biofilm and saliva), SEM (for all biofilm specimens) and TEM (for all biofilm specimens).

Results: In part one, vinegar caused destruction of the pellicle as detected by SEM and TEM. Compared to the control group, SEM and TEM analyses showed that vinegar rinsing reduced the outer globular layer of the pellicle ($p<0.001$), and resulted in formation of a network-like subsurface pellicle. In part two, the vinegar group revealed a significant reduction in bacterial viability and disruption of the mature biofilm as detected by *Bac*Light$^{TM}$ viability assay, SEM and TEM. Total bacteria amount of saliva samples decreased remarkably ($p <0.001$) after vinegar intervention within 30 min. Reduction of bacterial viability was observed even 120 min after vinegar rinsing in both biofilm and saliva samples ($p <0.001$).

Conclusion: This *in situ* study reveals that rinsing with vinegar for only 5 s alters the pellicle layer resulting in subsurface pellicle formation. Furthermore, vinegar rinsing will destruct mature (24-h) biofilms, and significantly reduces the viability of planktonic microbes in saliva, thereby decreasing biofilm formation. However, the long-term clinical efficacy of vinegar rinsing requires further investigations.

## 1.2 Zusammenfassung


### Einfluss von Essig auf die Biofilmbildung *in situ*

Zielsetzung: Essig ist schon seit langem für seine starke antimikrobielle Wirkung bekannt. Ziel dieser Studie war die Wirksamkeit eines handelsüblichen Essigs (destilliert) auf den oralen Biofilm und die Pellikel zu untersuchen. Dabei wurde im ersten Teil die Wirkung des Essigs auf die Entstehung der initialen Pellikel und im zweiten Teil sein Einfluss auf einen reifen mikrobiellen Biofilm und den Speichel analysiert.

Material und Methoden: Für die Biofilmbildung wurden bovine Schmelzprüfkörper auf individuell gefertigte Schienen montiert und für 3 min bzw. 24 h intraoral exponiert. Die Schmelzprüfkörper wurden zunächst mit Essig für 5 s und danach zwei Mal mit Wasser für 30 s gespült. Anschließend wurden die Schmelzprüfkörper für weitere 30 min bzw. 120 min intraoral exponiert. Für die Kontrolle wurden die Prüfkörper nur mit Wasser gespült. REM und TEM Analysen wurden bei Biofilmproben mit Expositionszeiten von 30 und 120 min sowie 24 h durchgeführt. Darüber hinaus wurde die Zellviabilität mittels B*ac*Light™ -Assay bei allen Biofilmproben aber auch beim Speichel untersucht.

Ergebnisse: Im ersten Teil der Studie zeigten die wiesen REM und TEM Analysen eine durch Essig zerstörte initiale Pellikel auf. Im Vergleich zu den Kontrollproben reduzierte die Essigspülung die äußere globuläre Schicht der Pellikel ($p<0.001$) und führte zu netzwerkartigen Pellikelstrukturen unterhalb der Oberfläche. Im zweiten Teil der Studie zeigten REM, TEM und *Bac*Light™ -Assay eine signifikante Reduktion der bakteriellen Zellviabilität und eine Zerstörung des reifen Biofilmes nach der Behandlung mit Essig. Die Gesamtmenge der Bakterien im Speichel sank erheblich innerhalb von 30 min nach der Spülung ($p<0.001$). Sogar 120 min danach konnte eine Reduktion bakterieller Zellviabilität im Speichel und im Biofilm beobachtet werden ($p<0.001$).

Schlussfolgerung: Diese *in situ* Studie weist auf eine Veränderung der Pellikelschicht und die Bildung von Pellikelstrukturen unterhalb der Oberfläche nach nur 5 s Spülung mit Essig hin. Außerdem zerstört Essig reifen Biofilm, reduziert signifikant die Zellviabilität des maturen (24 h) Biofilms sowie planktonischer Bakterien im Speichel und vermindert dadurch die Biofilmbildung. Die langfristige klinische Wirksamkeit muss jedoch weiter untersucht werden.

# 2 Introduction

Due to demographic changes, oral diseases rose throughout the world [Kassebaum NJ et al., 2017]. Oral diseases include mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss and other disorders that limit the capacity in biting, chewing, smiling, speaking and psychosocial wellbeing [Michiko F and Yamashita Y, 2013]. It has been now accepted that the dysbiosis of the microflora leads to oral diseases due to the increasing proportion of the disease-associated microbes in the oral biofilm [Marsh PD et al., 2015]. Therefore, the management of biofilms plays an important role in prevention and treatment of oral conditions.

Biofilm is a thin film which always covers the tooth surface and mucosa in the oral cavity. Overtime, a large amount of microbes accumulate and adhere to the biofilm, leading to many oral diseases [Marcotte H et al. 1998]. For example, dental caries is related to the decrease of the pH in the local biofilm due to several factors, which leads to enamel demineralization because of the acidic condition [Marsh PD et al., 2015; Theilade E, 1986].

Additionally, the local stimulation of biofilms may cause gingivitis [Meyle J and Chapple I, 2015]. The interaction between the biofilm and immune response cytokines is the key point to periodontitis development [Meyle J and Chapple I, 2015].

So far, antimicrobials are still the main treatment method of oral disease. However, they have side effects as well as poor efficacy for biofilm removal [Hwang G et al., 2017; Singh R et al., 2014]. Thus, there are more and more natural products to be studied.

Vinegar has used widely used for its antibacterial effect for thousands of years. It is common to see vinegar in the market and it appears as an indispensable seasoning in the diet. The diet effects of vinegar have been confirmed, such as significant antibacterial effect, antiathero-scloresis, anti-oxidation and anti-cancer as well as hypotensive activity [Budak et al., 2014]. However, the application of vinegar has not been sufficiently studied in oral field.

In the following, the formation and characteristic of biofilm as well as the efficacy and mechanism of vinegar will be discussed in detail.

## 2.1 Review of literature

### 2.1.1 Oral biofilm

#### 2.1.1.1 Introduction of oral disease

Dental caries and periodontitis are among the most prevalent disease throughout the world. According to the latest study, there are still 3.5 billion people suffering from untreated caries or periodontal disease [Kassebaum NJ et al., 2017]. The 2015 GBD (Global Burden of Disease) report showed that oral diseases have been the main reason (ranked in top 10) of YLDs (Years Lived with Disability) all over the world [GBD 2015 DALYs and HALE Collaborators 2016].

Oral diseases have many clinical manifestations, such as bad breath, bleeding gums, toothache, gomphiasis and loss of tooth. Moreover, plenty of studies prove that oral disease may lead to disease of other sites. Periodontal problems may cause cardiovascular disease [Shaneen J et al., 2010; Ahmed U et al., 2015]. Dysbiosis of the oral microflora maybe an important reason of type-2 diabetes [Shillitoe E et al., 2012]. Therefore, the prevention and further research of biofilm related oral diseases are of considerable significance.

So far, the main reason of most oral diseases is the dental biofilm, which is a complex structure formed by a variety of bacterial interactions.

#### 2.1.1.2 Formation of biofilm

Bacteria enter into the oral cavity from the external environment and rapidly colonize the surfaces, followed by incubation and proliferation in the oral cavity. It has been shown that there are more than 700 bacterial species on the surfaces of teeth and mucosa [Costerton et al., 1995; Kroes et al., 1999].

The formation of biofilms is a dynamic process. Firstly, the enamel surface will acquire a thin pellicle layer after soaking in saliva and gingival crevicular fluid (GCF) for few minutes. The main component of this initial pellicle are salivary proteins, which are particularly enzymes and immune response proteins [Delius et al., 2017]. These salivary proteins undergo conformational changes and cover the enamel surface, which provides a good habitat for the colonization of oral microorganisms. Due to the flow of saliva and mucosal movement, the ambient microorganisms are passively transported to the tooth surface and adhere to the surface of the initial pellicle. In the process of initial contact, the microorganisms are reversibly attached to pellicle by weak physical forces, which are not stable. Afterwards, bacterial adhesins begin to interact with the receptors of the pellicle at specific sites to form a strong adhesion. Early colonization of microbes is affected by a variety of factors, such as breastfeeding [Rautava, 2016; Latuga et al., 2014]. These early colonizers determine the abundance of biofilms in the following formation of biofilm. With the addition of microorganisms accumulating through co-agglutination / co-adhesion, the biofilm becomes more diversified. Over time, due to the metabolism of microorganisms, the internal environment of the biofilm is continuously and dynamically changed, which provides a suitable habitat for other species of microorganisms. Simultaneously, the metabolism level of bacteria in the biofilm changes during the process of microbial succession. More specifically, some of the bacteria enter a dormant state to reduce the nutrient consumption of the metabolic process. The biofilm formed at this stage gets gradually mature [Arciola et al., 2012; Chen et al., 2013].

#### 2.1.1.3 Saliva

Throughout the formation of biofilm, saliva plays an important role as a provider, buffer and transporter.

**Provider**

Salivary proteins are the most important component of the initial pellicle. It has been reported that, there are 72 major salivary proteins identified in the 3-min pellicle [Delius et al., 2017]. The salivary proteins have significant effects on the mineralization of enamel, which can largely determine the structure of the enamel mineralized layers [Dowd, 1999]. The most abundant proteins in saliva are proline-rich peptides, amylase, mucin, secretory IgA, etc. [Marsh et al., 2000].

**Buffer**

Saliva secreted by salivary glands, keeps the mouth wet and lubricated. Many studies have shown that saliva provides a lubricating effect between the hard tissue (tooth) of the mouth and the contact surface of the soft tissue (mucous membrane) [Nordbo et al., 1984; Ranc et al., 2006a; Ranc et al., 2006b; Prinz et al., 2007]. Additionally, as a buffer, saliva can relieve extreme temperature or acidic foods and beverages to reduce the irritation towards the teeth. Salivary proteins protect the enamel from demineralization due to the low pH [Martins et al., 2013]. In the early stage of enamel demineralization, calcium and phosphorus in the saturated concentration will promote the enamel to get mineral supplements, while fluoride in saliva can enhance enamel remineralization and can form fluorapatite, which is resistant to demineralization than hydroxyapatite in enamel [Ten Cate and Arends, 1980]. It has been shown that saliva can remineralize enamel demineralization in the early stage [Amaechi and Higham, 2001; Wetton et al., 2007].

**Transporter**

In the oral micro ecosystem, saliva act as the primary line of defense to prevent the invasion of harmful substances [Woof and Mestecky, 2005]. With the swallowing function, saliva can transport and remove bacteria, shedding cells or food residues to keep the oral homeostasis. Saliva sIgA constitutes a major mucosal immune effector and

provides an important first line of defense for pathogens such as Streptococcus mutans and Porphyromonas gingivalis [Woof and Kerr, 2006]. Additionally, amylase in saliva can digest starch in food, while lysozyme and thiocyanate ions have antimicrobial function. Meanwhile, salivary epidermal growth factor (EGF) can promote the proliferation of mucosal cells. Urea in saliva can be decomposed into ammonia and carbon dioxide by the microbial organismase, which can increase the pH in the biofilm. Inflammatory proteins such as interleukin-1β (IL-1β) and IL-6 in saliva have been shown to be associated with periodontal disease [Kinney et al., 2011; Ebersole et al., 2013; Rathnayake et al., 2013].

### 2.1.1.4 Character of oral biofilm

There is a variety of biofilms in nature, as well as in the human body. According to the location, these biofilms have their own characteristics. Similarly, the oral biofilms have some special features.

**Pellicle**

Typical ultrastructure of the pellicle is chariacterized by three layers as revealed by transmission electron microscopy (TEM) (Fig. 1). The outer layer with a globular structure can be colonised by microbes. The basal layer is a thin electron-dense structure. The subsurface layer is hardly seen under normal conditions, however it will appear quite clear after enamel demineralization.

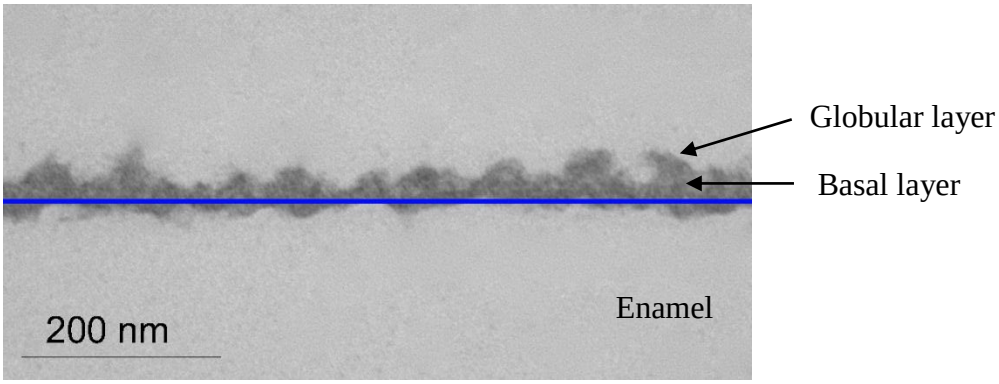



**Fig. 2** TEM micrograph, 3-min pellicle

**Mature biofilm**

The structure of the mature biofilms is much more complicated. The typical spatial structure of the mature biofilm is composed of microbial cells and the extracellular polymeric substances (EPS). In order to maintain the homeostasis of the biofilm, there are some bacterial cells programmed to die while some others are staying in the starving dormant state. At the same time, active nonpathogenic bacteria can produce enzymes that degrade antibiotics, which induce other pathogens in the biofilm to produce antibodies. Compared to planktonic bacteria, the ones in biofilms exhibit greater tolerance to antibiotics [Adil et al., 2014; Zhao et al., 2015]. It has been reported that microbes in biofilms showed greater adhesion to the biofilm [Lagerlöf and Oliveby, 1994], grew more slowly, reduce metabolism, and diminish sensitivity [Wirthlin et al., 2005] compared to planktonic bacteria. EPS is derived from cellular and oral environments and plays an important role in biofilms. The extracellular enzymes in EPS, interacting with extracellular polysaccharides [Wingender et al., 1987], are retained near the cell and activate the matrix enzymatically [Wingender et al., 2002]. Extracellular enzymes are able to digest solid particles in the external environment and provide nutrients to cells [Dohar, 2003; Bjornsdottir et al., 2006], and they degrade apoptotic cells, which are important for self-cleaning and maintaining the balance of biofilm environments. Meanwhile, EPS contains a large proportion of extracellular DNA (eDNA) [Allesen-Holm et al., 2006], and water to prevent the matrix from collapsing [Conner and Kotrola, 1995]. It is worth mentioning that there is a nanoscale microlayer accumulated by the hydrophobic EPS substance at the air-water interface of the biofilm [Gradisar et al., 2007]. This microlayer is a place where large amounts of nutrients accumulate, and also where biofilm conducts gas exchange. External stimuli, such as temperature or pH, have a strong effect on the microlayer.

## 2.1.2 Vinegar

### 2.1.2.1 Introduction of vinegar

From a medical point of view, natural sources of food or food additives have the characteristics of low cost and minimal side effects in the prevention or treatment of diseases [Rutala et al., 2000]. As an important seasoning in daily life, vinegar in the medical field can be traced back to 300 years BC. In ancient China, vinegar was used to treat burns, cellulitis and other diseases. In BC 460, the ancient Greek Hippocrates used vinegar to treat ulcers, coughs and infectious diseases [Johnston et al., 2006]. In recent years, vinegar has been increasingly widely used in the medical field, especially in the field of anti-infective, antioxidant, lipid metabolism and blood glucose control, vinegar plays a unique and significant effect [Budak et al., 2014; Nishidai et al., 2000; Ogawa et al., 2000].

### 2.1.2.2 Antibacterial effect

The most widely used effect of vinegar is its bactericidal property [Hindi, 2013]. In daily life, vinegar is often used as a natural preservative [Brul and Coote, 1999]. Different studies have reported that vinegar can inhibit or remove foodborne pathogens in fruits and vegetables [Sengun and Karapinar, 2004; Chang and Fang, 2007]. By soaking in vinegar for a short time, pathogens such as Salmonella typhimurium are completely removed from vegetables [Sengun et al., 2004]. The bactericidal effect of rice vinegar on E. coli O157: H7 is obvious, which may be related to the possibility that vinegar can enter the microbial cell membrane leading to cell death [Entani et al., 1998; Chang and Fang, 2007; Shen et al., 2016]. Anti-infective studies have shown that vinegar diluted for ear lavage, can be effective in the treatment of ear infections, such as chronic suppurative otitis media [Aminifarshidmehr, 1996], granular myringitis [Jung et al., 2002] and otitis externa [Dohar, 2003]. This may be related to reduction of the pH in the ear canal to inhibit bacterial growth [Dohar, 2003]. Additionally, the undiluted vinegar can effectively remove the bacteria from dentures, and the vinegar remaining on the denture does not cause oral mucosal damage [Shay, 2000; Pires, 2017].

#### 2.1.2.3 Mechanism

Although the antibacterial effect of vinegar is widely used, the specific mechanism is still to be further studied. The antibacterial mechanism of organic acids has three stages. In the first stage, the organic acids interfere with the cell membrane of the bacteria [Freese et al., 1973; Stratford et al., 1998]. Organic acids are fat-soluble and pass through the cell membrane through undissociated forms, resulting in decreased intracellular pH [Ray et al., 1992]. The dissociation of extensive protons may lead to the protonation of the lipopolysaccharide on the cell membrane, resulting in the breakage of transmembrane proton motive force [Brul and Coote, 1999; Brul and Croote, 1999; Bjornsdottir et al., 2006], while dissociation of anions may lead to increased intracellular osmotic pressure in cytoplasmic aggregates, resulting in cell membrane rupture [Alakomi et al., 2000].

### 2.1.3 Application in the oral field

Although vinegar has a dramatical antibacterial effect, there is few research of vinegar applied in the oral field. It has been reported that, the enamel surface would get eroded after incubated in vinegar for four or eight hours under *in vitro* conditions [Willershausen et al., 2014]. However, vinegar commonly stay in oral cavity for only several seconds during the daily feeding process rather than several hours. Also, the ionic composition of saliva could protect the enamel from demineralization [Martins et al., 2013]. Similarly, the oral biofilms could present a short-term stability in low pH value to protect the enamel from erosion effects of acid [Wiegand, 2008]. Moreover, the calcium and phosphate ions in saliva have the efficacy to reduce demineralization of the enamel [Hannig, 2006]. Compared to the other kinds of fatty acids, acetic acid featured the weakest erosive effect at pH2.0 [Hannig et al., 2005]. Therefore, according to the feeding process, short- term rinsing with vinegar would be physiological acceptable.

## 2.2 Aim of this study

The research of vinegar applied in the oral cavity requires to be focused on, especially considering its potential efficacy on oral biofilms which might lead to oral disease. During the process of biofilm development, not only the effect of vinegar on the initial formation of the pellicle should be studied, which is a prerequisite for biofilm growth, but also the effect of vinegar on the mature biofilm, which may be pathogenic. Additionally, the effect of vinegar on saliva should be determined as well. Because of the essential function of saliva in the formation of biofilm, saliva can provide salivary proteins to form the pellicle, but also transport microbes for the development of biofilms to promote biofilm growth and maturation. Therefore, an *in situ* study was performed to investigate the efficacy of vinegar on oral biofilm formation

in three aspects:

1. Effect of 5-s vinegar rinsing on *in situ* fomation of salivary pellicle,
2. Destruction of mature (24-h)biofilms formed *in situ* by 5-s vinegar rinsing
3. Effect of 5-s vinegar rinsing on salivary bacteria

# 3 Materials and Methods

## 3.1 Subjects

Four healthy volunteers, aged between 25 and 35 years, who are staffs from several departments of Saarland University, participated in this study. One experienced dentist performed oral clinical examinations, excluding active dental caries, active periodontal disease, oral mucosal disease, or signs of saliva dysfunction. This check ensured that the participants had good oral conditions, such as physiological saliva flow, healthy teeth or good fillings, no bleeding and periodontal pocket depth of less than 3mm. All participants were instructed to regular diet, no smoking or drinking, and no antibiotics usage within last six months. Before the experiment was carried out, it was made sure that each participant obtained the informed consent of this experiment and signed it. The study protocol had been examined and approved by the Medical Ethics Committee of the Medical Association of Saarland, Germany (# 238/03, 2016).

## 3.2 Production of splints

Individual upper retainers (made of acrylic acid) for holding enamel samples were manufactured for the first and second quadrants of the oral maxilla for all subjects. Firstly, occlusal surfaces were blown off before taking the impression to ensure that there were no impurities which would affect the impression such as food residue. Afterwards, the teeth are ready for the impression. Secondly, a suitable tray, slightly larger than arch, was selected according to the participants. The impression was performed with alginate impression material (Blueprint, Dentsply DeTrey GmbH, Konstanz, Germany). Thirdly, after pouring with vacuum-mixed stone model material, minisplints were made of Duran® (Scheu-dental GmbH, Iserlohn, Germany), covering the maxillary molars and premolars of both sides. For better retention of the mounted specimens, the minisplints were perforated (Fig. 2).

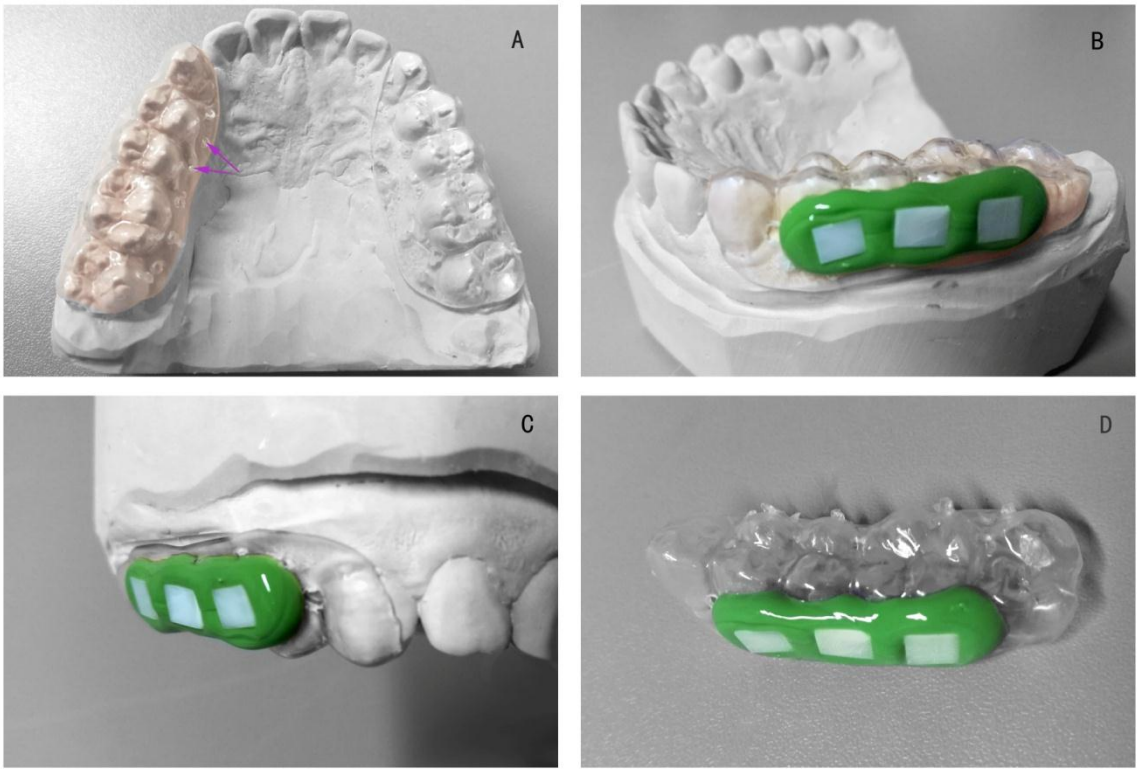



**Fig. 2** splint with mounted specimens. (A) perforation of the splint; (B) fixation of enamel specimens with polyvinyl-siloxane impression material; (C) buccal extension of the splint; (D) final splint before exposing to the oral cavity

## 3.3 Preparation of enamel specimens

### 3.3.1 Extraction and preparation of bovine teeth

Bovine permanent incisors were extracted and selected for this study to be without enamel hypoplasia, defects or cracks [Hannig et al., 2004; Hannig et al., 2005a; Hannig et al., 2008]. The roots were removed from the teeth after extraction by diamond cutting discs (Schott Diamantwerkzeuge GmbH, Stadtoldendorf, Germany) at the boundaries of the crown, while enamel samples were separated under water cooling by a saw (Conrad Apparatebau Clausthal GmbH, Clausthal-Zellerfeld, Germany). Specifications of the enamel specimens had been settled for 4 mm x 4 mm square shape which was used for *Bac*Light™ viability determination and SEM, and 2mm x 2mm square shape which was used for TEM, respectively. The prepared enamel samples were stored in 0.1% thymol solution (pharmacy of the Saarland University Hospital, Homburg, Germany) at 4 °C.

### 3.3.2 Polishing and pretreatment of enamel specimens

In order to standardize the surface of the enamel specimens, polishing was carried out with silicon carbide grinding papers (P600 to P2.500, FEPA-P, waterproof silicon carbide paper, Buehler, Düsseldorf, Germany) by means of a polishing machine (Buehler, Düsseldorf, Germany) under water-cooled conditions. The surface of the enamel specimens was ground by abrasive paper down to P-grit size of '4, 000' (according to Federation of European Producers of Abrasives (FEPA) standard, mean grain size is 5 µm). During polishing procedure, most of the dentin was removed from the specimens to preserve sufficient enamel so that the final thickness of the samples was 1 mm. The surface of each enamel sample was examined by an optical microscope (Motic Deutschland GmbH, Wetzlar, Germany), excluding any samples with discoloration or demineralization at 10-fold magnification.

The principles of the surface treatment of the samples were based on Hannig et al. [Hannig et al., 2005]. Firstly, well-polished enamel specimens were immersed with 3% NaOCl solution (Hedinger, Stuttgart, Germany) for 3 min to remove any residues of the polishing process. Afterwards, slabs were rinsed by distilled water (Ecotainer; B. Braun Melsungen AG, Melsungen, Germany) for 5 times to remove NaOCl solution, and then permeated by 5 min ultrasonication at 4 ℃. Subsequently, the enamel slabs were treated by disinfection in 70% of propanol (Hedinger, Stuttgart, Germany) for 15 min, with the enamel surface up. After rinsed with distilled water twice, the enamel specimens were stored in distilled water at 4 ℃ for 24 hours for rehydration until exposed to the oral cavity [Deimling et al., 2007; Hannig et al., 2008].

### 3.3.3 Mounting of enamel specimens

Well prepared acrylic splints were also disinfected in 70% propanol solution for 3 min followed by rinsing 5 times with distilled water. Then, enamel slabs were mounted to the defined positions on the splints by polyvinyl-siloxane impression material (President light-body, Coltene, Altstätten, Switzerland). The specimens were fixed to the maxillary, which were confirmed to avoid direct contact with the papilla of the parotid duct. The enamel slabs, with margins concealed by a thin layer of impression material, were exposed to the oral cavity (Fig. 3).

## 3.4 Pellicle / Biofilm formation

The study required the formation of two types of biofilms on the enamel slabs. Subjects carried the minisplints for 3 min to obtain the initial pellicle in the first experimental part and for 24 hours to obtain the mature biofilm in the second part of experiments. The uniform time to insert splints into the oral cavity was 9 a.m., and subsequently, splints exposed intraorally for 3 min or 24 h, respectively.

The experiment took place at 9 a.m. All the participants were informed to brush their teeth without toothpaste 24 h beforehand as well as during the whole 24-h experimental period. After the specimens were exposed to the oral cavity for 3 min or 24 h, the volunteers rinsed with 10 ml vinegar (Distilled vinegar, Heuschen & Schrouff OFT B.V. Thailand) for 5 s and then rinsed twice with 10 ml water for 30 s. Immediately, three enamel slabs were dismounted from the splints and analyzed via FM, SEM and TEM. In the following, the remaining enamel specimens were exposed to the oral cavity for another 30 min or 120 min.

## 3.5 *In vivo* rinsing with vinegar

For the *in vivo* rinsing experiments, vinegar (Distilled vinegar, Heuschen & Schrouff OFT B.V. Thailand) was chosen randomly in the market.

### 3.5.1 Part one, 3-min pellicle formation *in situ*

Splints, with 6 enamel slabs fixed on each left and right buccal and palatal sites of the splints, were placed intraorally for 3 min. After rinsing with 10ml of vinegar for 5 s and then twice rinsing with 10ml of distilled water for 30 s each, two slabs were removed from each subject as well as the salivary expectorate was collected. After further exposure in the oral cavity for 30 min or 120 min respectively, another two slabs were removed, meanwhile, salivary samples were collected. The two enamel samples removed from splints at each time were analyzed by SEM and TEM, and the saliva samples were investigated by *Bac*Light$^{TM}$ viability assay. Samples rinsed with water instead of vinegar served as controls. Details of the experimental design are depicted in the flow chart (Fig. 3).

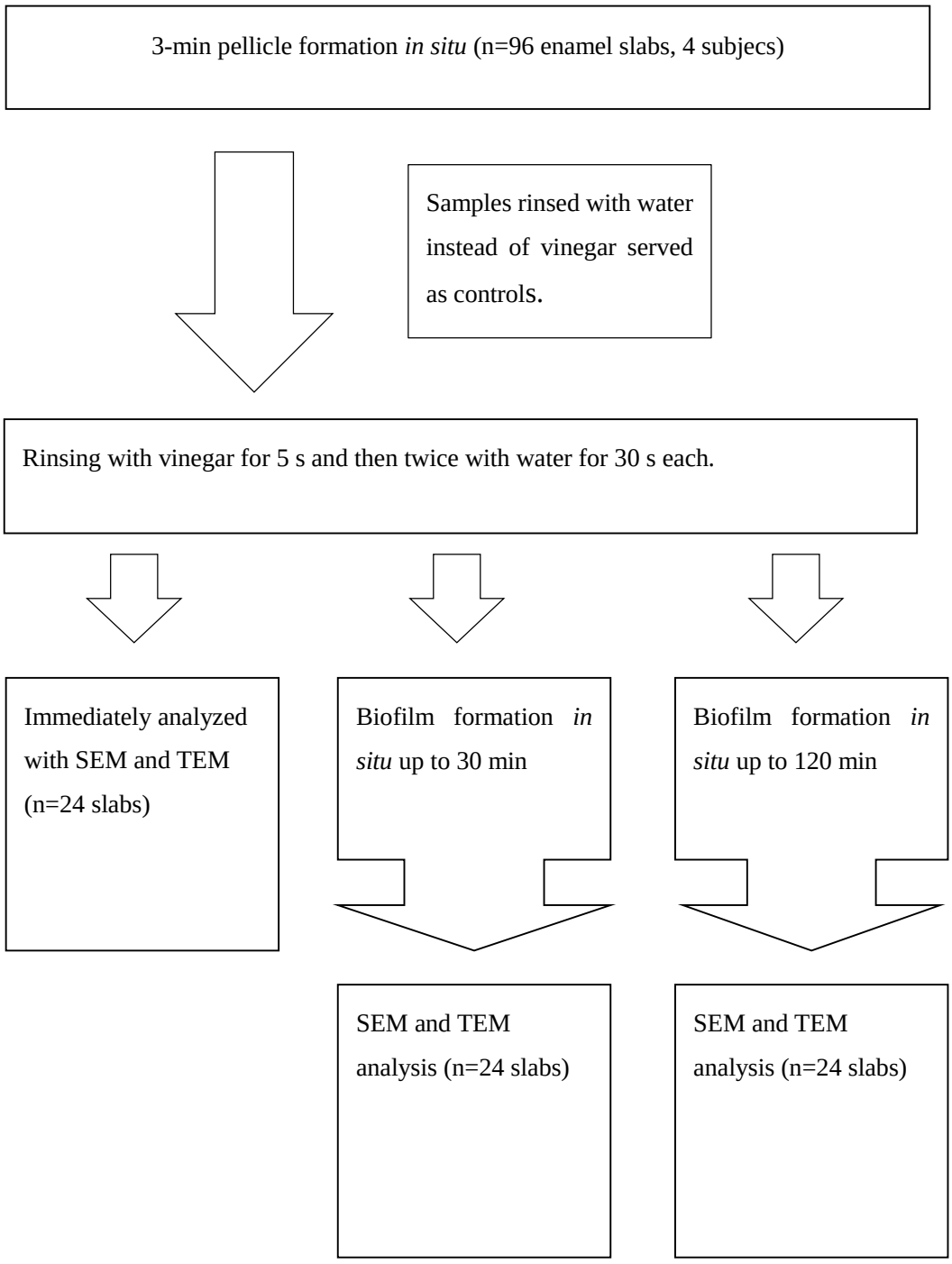



**Fig. 3** Flowchart of part one, formation of 3-min pellicle *in situ*, vinegar or water rinsing, and subsequent analyses

### 3.5.2 Part two, 24-h biofilm formation *in situ*

During the experimental time, all the volunteers were informed to brush their teeth without toothpaste. Twelve enamel specimens were fixed on the splints and then exposed to the oral cavity for 24 hours. Afterwards, three enamel slabs from each side of left and right were removed as control group immediately and analyzed by *Bac*Light™, SEM and TEM. After rinsing with 10ml of vinegar for 5 s and 10ml distilled water twice for 30 s each, three enamel slabs as a group were removed from each side of left and right. Subsequently, the last two groups were removed after exposure time of 30 min or 120 min, respectively. Each group of three slabs were analyzed via *Bac*Light™, SEM and TEM. Details of the experiments were shown in the flow chart (Fig. 4).

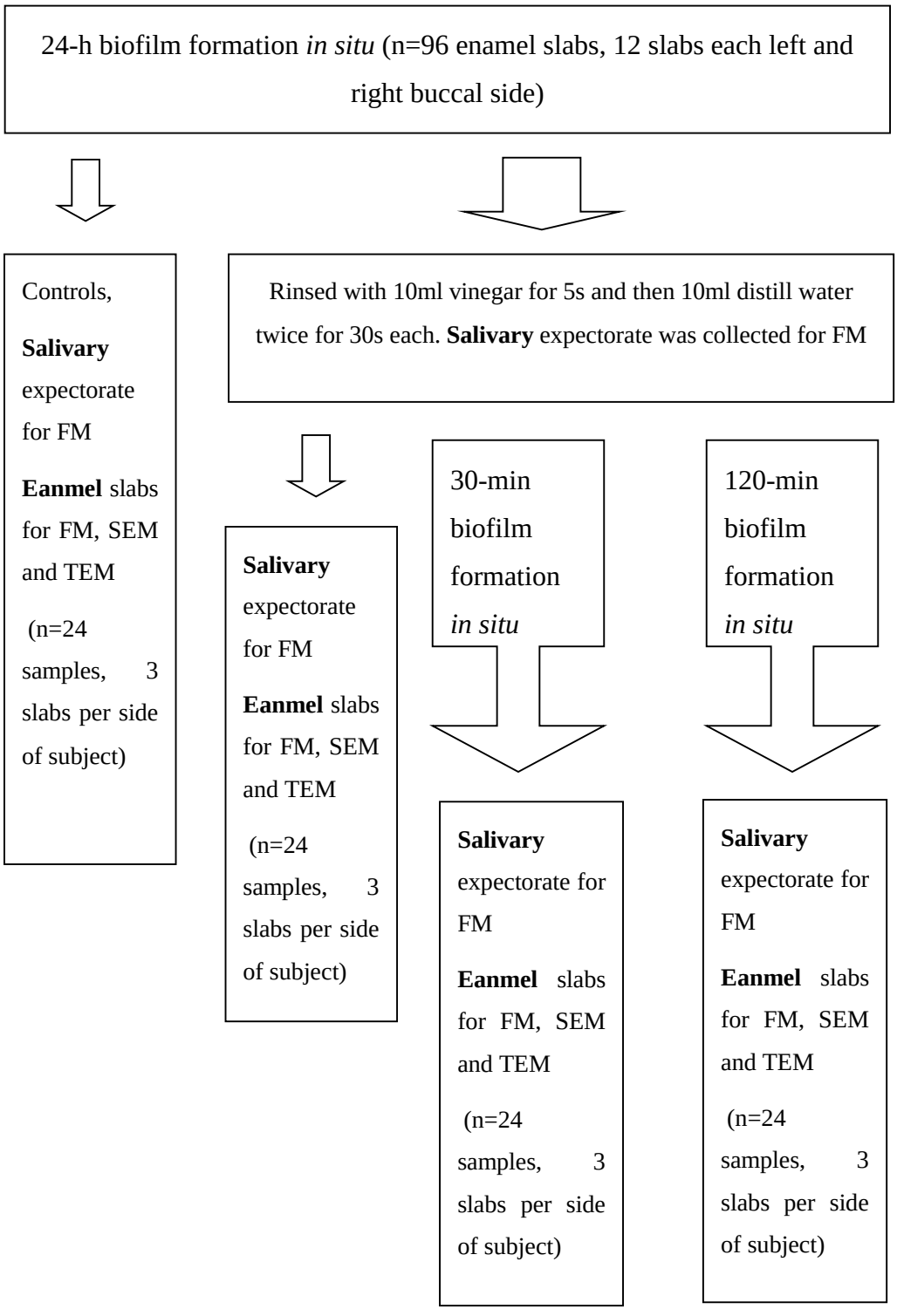



**Fig. 4** Flowchart of part two, formation of 24-h biofilm *in situ*, vinegar rinsing and subsequent analyses.

## 3.6 Collection and preparation of saliva samples

### 3.6.1 Collection and pH test of saliva/expectorate

Saliva samples were obtained in both parts of the experiments. Two milliliters of saliva were collected in a special tube with the technique described by Scully [Scully, 1980]. All of the saliva/expectorate samples were collected between 9 and 12 am with each procedure performed within 5 minutes. The collected saliva samples were diluted to 10 ml with water at room temperature. Following this, the pH of each diluted sample as well as expectorate sample was measured by means of pH test strips (Macherey-Nagel, Carl Roth GmbH +Co, Karlsruhe, Germany).

### 3.6.2 Centrifugation

Expectorates together with diluted saliva samples were placed in the centrifuge (Biofuge primo, Thermo Electron Corporation, Germany). Epithelium cells and other foreign substances were removed by centrifugation 1000 × g for 10 min, the remaining supernatant was placed in new tubes. Subsequently, the supernatant was centrifuged with 10000 × g for 10 min to collect the bacteria which were pressed against the bottom of the tube.

## 3.7 *Bac*Light™ viability assay

### 3.7.1 Mechanism of *Bac*Light™ viability assay

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Art. No. L7012, Invitrogen, Molecular probes, Eugene, Oregon, USA) utilizes mixtures of SYTO® 9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic acid stain to distinguish live and dead bacteria within bacterial populations, based on the intactness of bacterial cell membranes. These two dyes are different in spectral properties, as well as regarding the ability to penetrate healthy bacterial cells. SYTO 9 staining is characterized by labeling all bacteria, including bacteria with intact membranes and incomplete membranes; while propidium iodide can only penetrate bacteria with damaged membranes. When the two are mixed, the SYTO 9 staining fluorescence is reduced by PI, so that the bacteria with the intact membrane are stained with fluorescent green and the bacteria with the damaged membrane are stained with fluorescent red, which can be evaluated before and after the experiment in order to characterize the overall situation and mortality of bacteria [Boulos et al., 1999].

### 3.7.2 Preparation of staining solution

The staining solution was made by 1 µl of SYTO 9, 1 µl of PI, and 1 ml of 0.9% saline solution (B. Braun Melsungen AG, Melsungen, Germany) and then placed in a shading tube. The mixing time was thoroughly for 15 min.

### 3.7.3 Staining of samples

**Enamel specimens**

After exposed in the oral cavity, the enamel slabs were carefully removed from the splints and rinsed with distilled water to remove the saliva on the enamel surface. After washing with physiological saline gently for 5 s, the specimens were stained with 0.1 ml of staining solution for 15 min at room temperature in the dark. The samples were then rinsed in physiological saline to remove the residual staining solution. The dried slabs were fixed on glass slides with double-sided tapes (Leit-Tabs, Art. Nr. G3347, Plano, Wetzlar, Germany), then followed by a drop of *Bac*Light™ oil on the surface.

**Salivary samples**

The bacteria were removed and collected from the salivary samples by centrifugation (see 3.6) and stained by 20 μl of the staining solution for 10 min in the dark. After sufficiently stained, 1 μl of the stained bacteria was transferred on the glass slide.

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

The samples were observed with a fluorescence microscope (Leica DMRB, Leica Mikroskopie & SystemeGmbH, Wetzlar, Germany) and magnified to 1000-fold. Nine microscopic ocular grid fields were selected in each sample according to a defined patterns. Subsequently, nine images were taken from each sample and quantified via Image J (Image J-ij133- jdk15, National Institute of Mental Health). According to the gray value, the fluorescence intensity of bacteria was semi-quantitatively analyzed.

## 3.8 Scanning electron microscopy (SEM)

### 3.8.1 Preparation of enamel samples for SEM analysis

After exposure in the oral cavity, the enamel samples were gently rinsed with sterile distilled water for 5 s and then fixed in 2.5% glutaraldehyde solution at 4 ℃ for 1 hour, followed by washing 5 times with phosphate buffered saline for 10 minutes. Subsequently, the enamel specimens were gradually dehydrated in a series of 50% -100% ethanol solution in an ascending gradient of concentration. Before observed by SEM， the samples were coated by gold-palladium (SC 7640 High Resolution Sputter Coater, Quorum Technologies Ltd., U.K.) in a vacuum state.

### 3.8.2 SEM analysis

Samples were analyzed in a scanning electron microscope XL 30 ESEM FEG (FEI, Eindhoven, Netherlands) at BEAM at 10KV. The settings of the scanning electron microscope were consistent for all the samples. At 30-fold magnification, the samples were divided into nine regions, in which representative pictures were taken at 500 to 20,000 folds magnification.

### 3.8.3 Semi-quantification of SEM results

In each sample, nine photomicrographs of 1000-fold magnification were taken to quantify the grade of the bacterial adherence. In the experimental part one (3-min, 30-min and 2-h pellicle formation), the particulate matter (e.g. bacteria and protein aggregates) on enamel specimens was analyzed via the gray value by Image J. For the samples of 24-h biofilm (experimental part two), the complex coverage state of the enamel surface was described and used as supporting evidence for the *Bac*Light$^{TM}$ viability assay.

## 3.9 Transmission electron microscopy (TEM)

### 3.9.1 Post fixation and dehydration

After the enamel slabs covered with biofilms were removed from the splints, they were washed with distilled water to remove the saliva film, followed by fixing in 2.5% glutaraldehyde solution fixative at 4 ℃ for 1 hour immediately. Afterwards, the samples were washed 5 times in cacodylate buffer for 10 minutes each and stored in the final buffer solution at 4 ℃. Before dehydration, the samples were placed in 2% osmium tetroxide ($O_2SO_4$) for 1h. After incubation with the osmium acid solution, the samples were washed 5 times in phosphate buffer and then dehydrated in ethanol with a rising gradient concentration [Hannig and Balz, 1999].

### 3.9.2 Embedding

Specimens were embedded in Araldite CY212 (Agar Scientific, Stansted, United Kingdom) and propylenoxide (1:1) overnight. Hereafter, they were placed to new Araldite CY212 overnight. And then all the specimens were transferred into the silicone form to be polymerized for 48 h at 65 ℃. After decalcification of the enamel in 1M HCl, specimens were re-embedded in Araldite Cy212.

### 3.9.3 Production of ultrathin-sections

Ultrathin-sections (about 50-80 nm) were cut in an ultramicrotome (Ultracut E, Reichert, Bensheim, Germany) equipped with a diamond knife (Microstar 45 °, Plano GmbH, Wetzlar, Germany). Subsequently, ultrathin sections were mounted on Pioloform-coated coppers grids (Plano GmbH, Wetzlar, Germany) and contrasted with uranyl acetate and lead citrate.

### 3.9.4 TEM analysis

The ultrathin sections were investigated by TEM TECNAI 12 Biotwin (FEI, Eindhoven, Netherlands) under the magnification from 6,800 to 180,000. Representative micrographs were taken at magnifications of 23,000 fold and xy,000 fold.

## 3.10 Statistics

Kruskal-Wallis test and Mann-Whitney U test were used to evaluate the biofilm data originated from SEM. The Kruskal-Wallis test was to check effects of vinegar on 3-min or 24-h biofilms, while the Mann-Whitney U test was utilized for pairwise comparison with calibration by Bonferroni correction. Additionally, the Wilcoxon test was adopted to assess if any disparity occurred in the respective biofilm data derived from live / dead microbe. The standard of statistical significance was $p<0.5$ in Kruskal-Wallis test and Wilcoxon test, while $p<0.01$ was used in Mann-Whitney U test after Bonferroni correction. All the data were analyzed by the SPSS 18 software package (SPSS Inc., Chicago, IL., USA)

# 4 Results

All subjects completed the study without any side effects. After rinsing with vinegar for 5 s, the mortality of biofilm was investigated by *Bac*Light$^{TM}$ viability assay. Moreover, the ultrastructure of biofilm formation was observed by SEM and TEM.

## 4.1 Part one, 3-min pellicle

### 4.1.1 Morphological appearance of the *in situ* pellicle

**SEM**

After exposure in the oral cavity for 3 min, samples rinsed with vinegar showed d istinct morphological difference compared to the control group. Due to vinegar rinsing, the pellicle was removed immediately from the enamel surfaces. While the control samples, comparably, were covered with a thicker globular pellicle over time (Fig. 5). After rinsed with vinegar for 5 s, enamel surface was extremely rarely coverd with large particles, viz., protein aggregates and bacteria (Fig. 6). However, in the control group, a mottled sludge-like pellicle coverage was detected on the enamel surface (Fig. 7), which was unevenly distributed and occasionally appeared with single cocci adhering to the pellicle via fimbriae (Fig. 8).

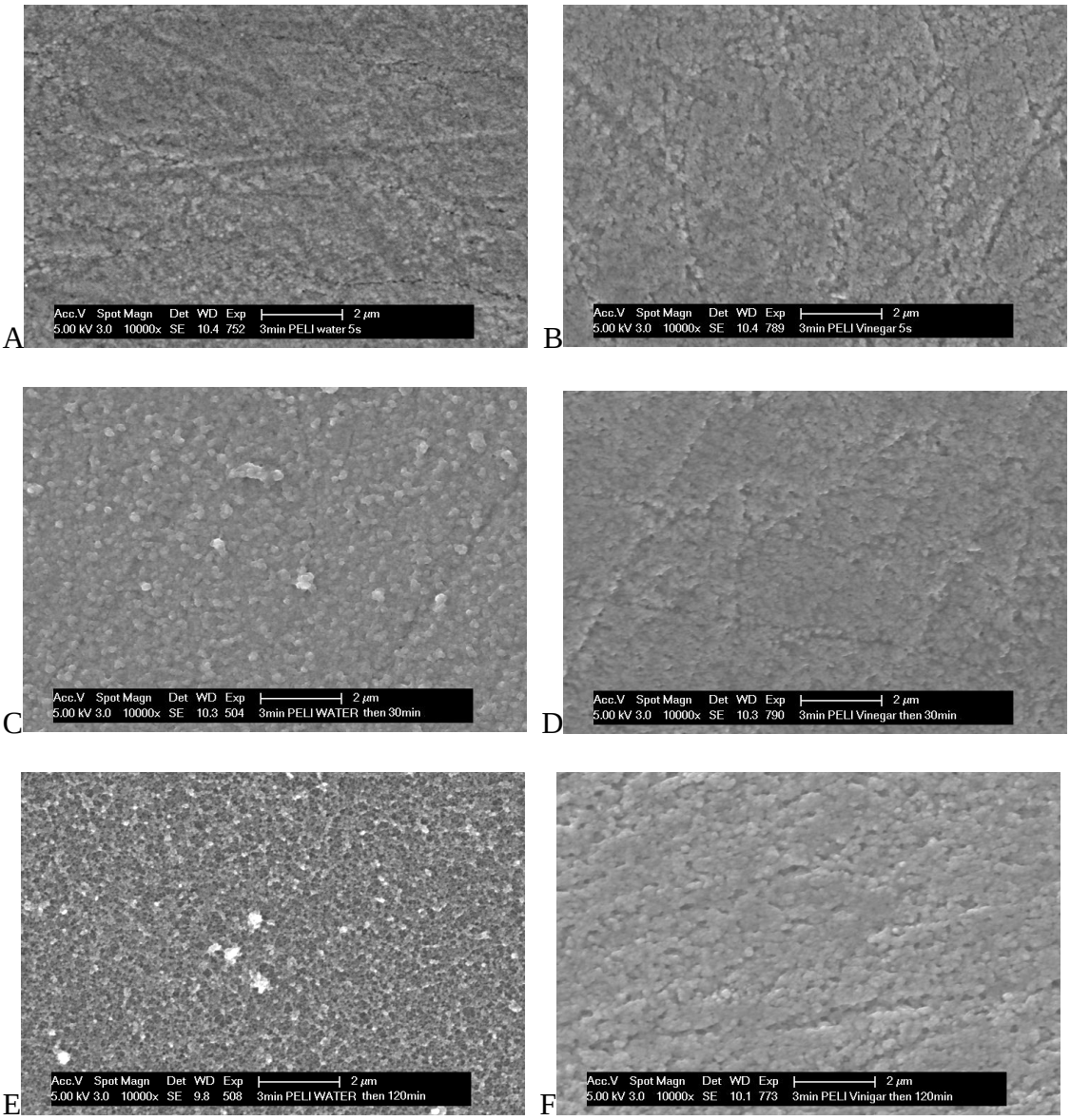



**Fig. 5** SEM micrographs: an overview of 3-min pellicles immediately, 30 min and 120 min after 5-s rinsing with vinegar (B, D and F) or 5-s rinsing with water (A, C and E; control group). In the control group, formation of a globular pellicle layer masking the enamel surface is clearly visible. In the vinegar rinsing group, micro-morphological details (crystallites) of the smooth and clean enamel surface are clearly visible, even 30 and 120 min after rinsing with vinegar. (A and B) immediately after vinegar rinsing; (C and D) 30min after vinegar rinsing; (E and F) 120min after vinegar rinsing. Original magnification: 10,000-fold.

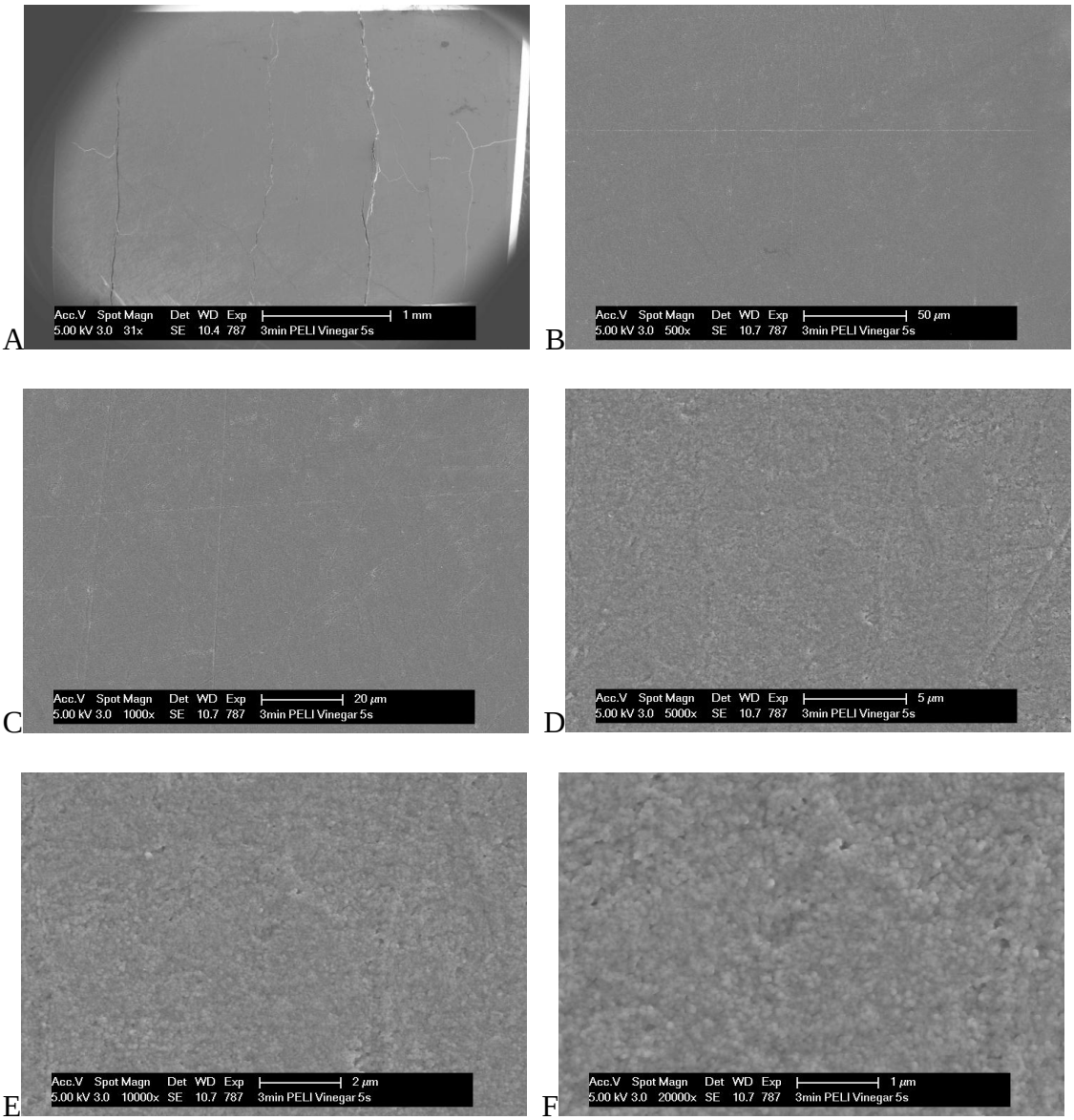



**Fig. 6** Vinegar group SEM micrographs: an overview of 3-min pellicle immediately after rinsed with vinegar for 5 s and then rinsed with water twice for 30s each at different magnification.(A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold (E) 10,000-fold; (F) 20000-fold.

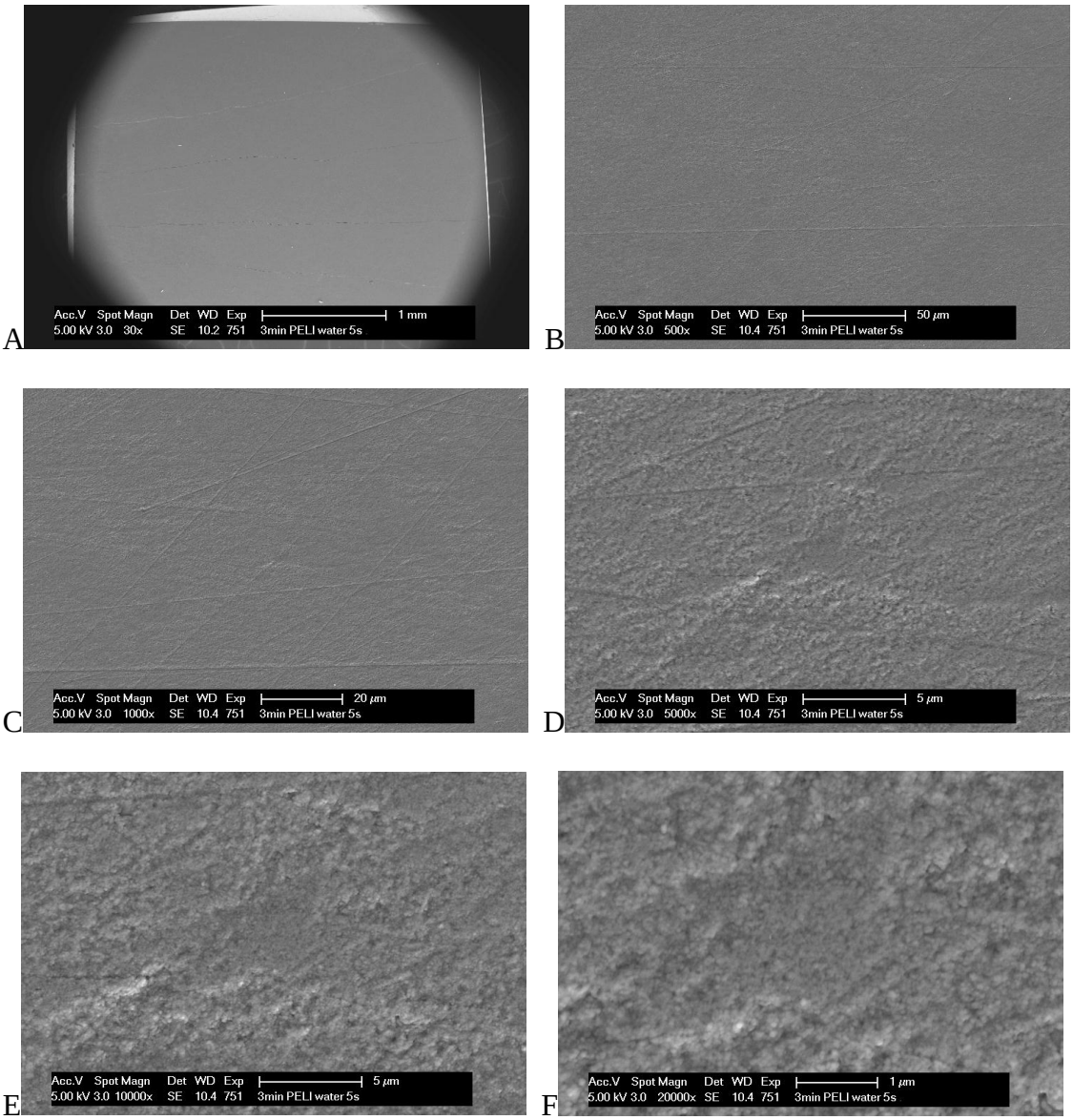



**Fig. 7** Control group SEM micrographs: an overview of 3 min pellicle immediately after rinsing with water for 5 s and then water rinsing twice for 30s each at different magnification.(A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold (E) 10,000-fold; (F) 20,000-fold.

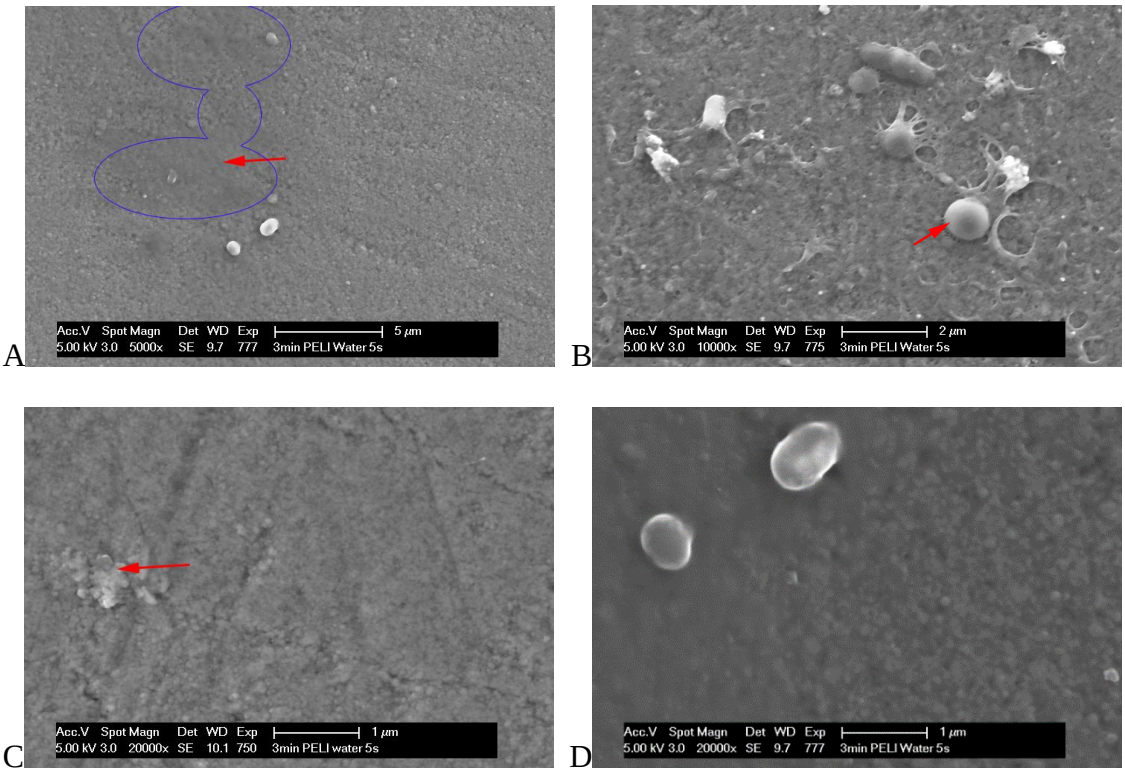



**Fig. 8** Control group SEM micrographs: 3-min pellicle immediately after water rinsing for 5 s and then water rinsing twice for 30s each. Enamel surface was covered by a mottled sludge-like coverage (A); single cocci adherent to the pellicle layer (B); protein aggregate (C); mottled sludge-like coverage on the enamel surface as well as bacteria. Original magnification:（A） 5,000-fold; (B) 10,000-fold; (C）20,000-fold; (D）20,000-fold.

At 30 min after rinsing with vinegar for 5 s and subsequent water rinsing twice for 30 s each, nearly no globular pellicle layer was detectable on the enamel surface. Compared with 30 min before (Fig. 6), there was basically a slight difference in the surface morphology (Fig. 9). In contrast, the enamel surfaces of the control group were covered by thicker pellicles with bacteria as well as protein agglomerates attached (Fig. 10). Simultaneously, the diversity of adherent bacteria microbes increased, since not only coccoid but also rod-shaped bacteria could be detected (Fig. 11).

Then, 120 min after rinsing with vinegar, the enamel surface were still clean with slight pellicle coverage, and little difference compared to the previous time points (Fig.12). Nevertheless, enamel specimens of the control group revealed a clearly detectable globularly shaped pellicle layer, which were interleaved into complex meshes (Fig. 13).

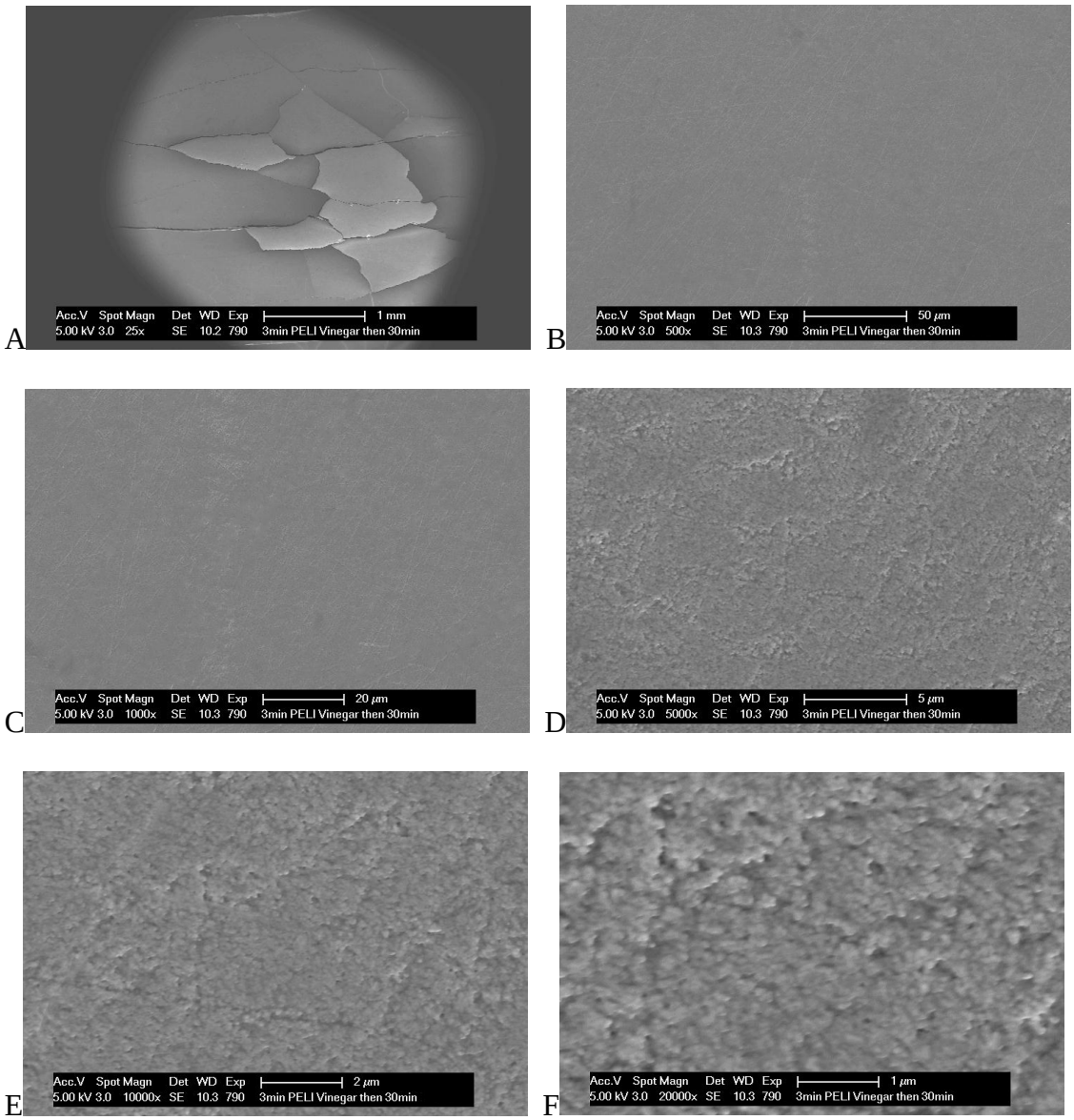



**Fig. 9** Vinegar group SEM micrographs: an overview of 3-min pellicle 30 min after application of vinegar at different magnifications. (A) 25-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

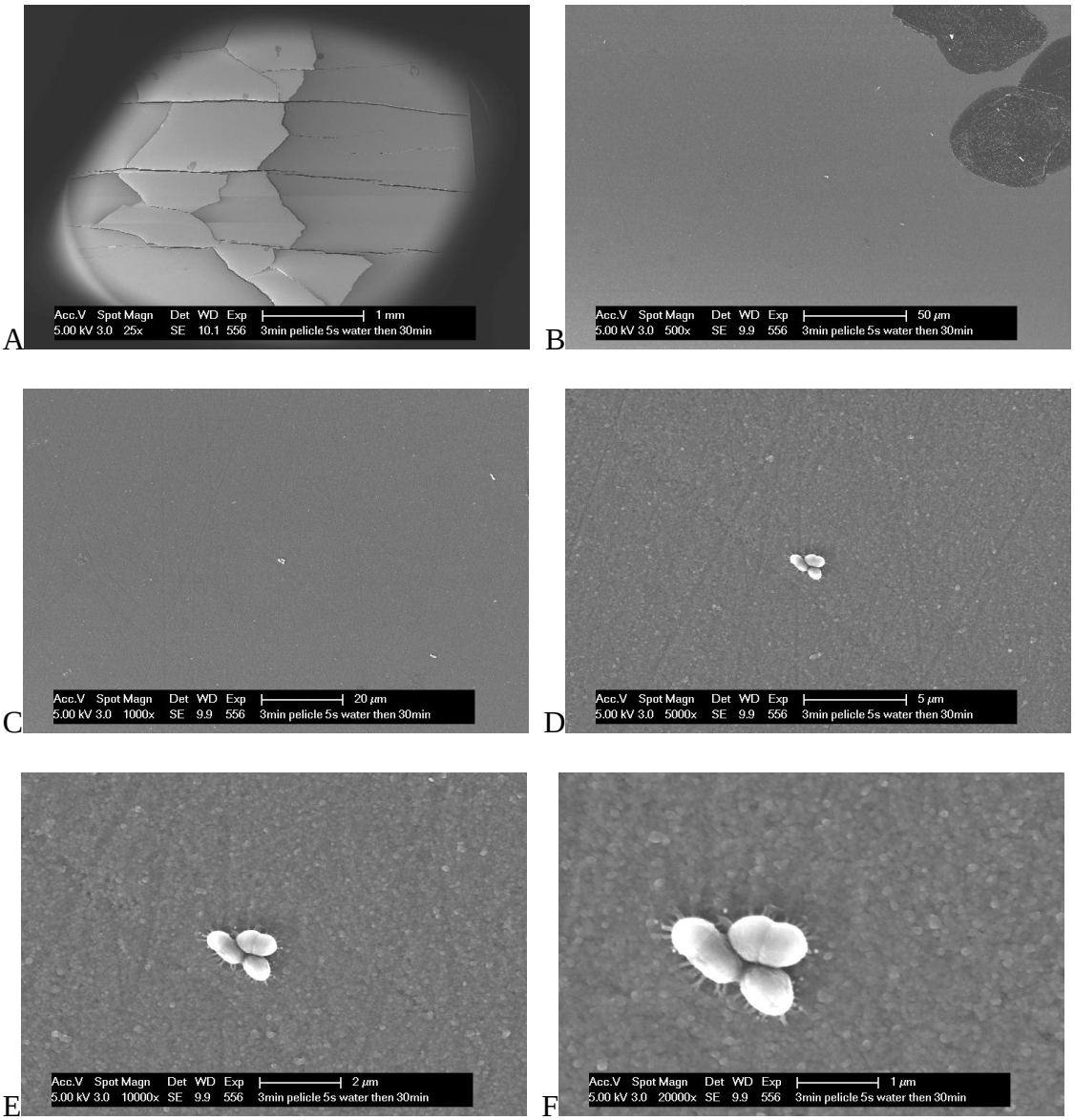



**Fig. 10** Control group SEM micrographs: an overview of 3-min pellicle 30 min after application of water, at different magnifications. (A) 25-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

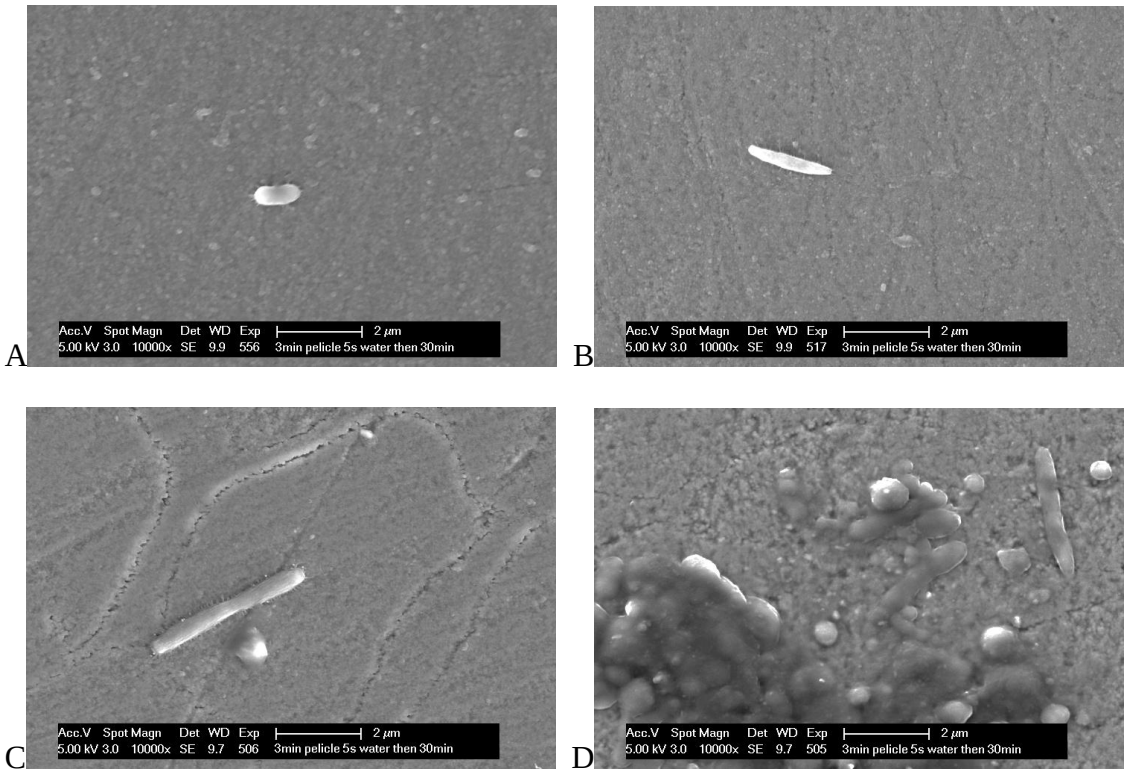



**Fig. 11** Control group SEM micrographs: 3-min pellicle 30 min after rinsing with water. Different species of bacteria were observed. Original magnification: 10,000-fold.

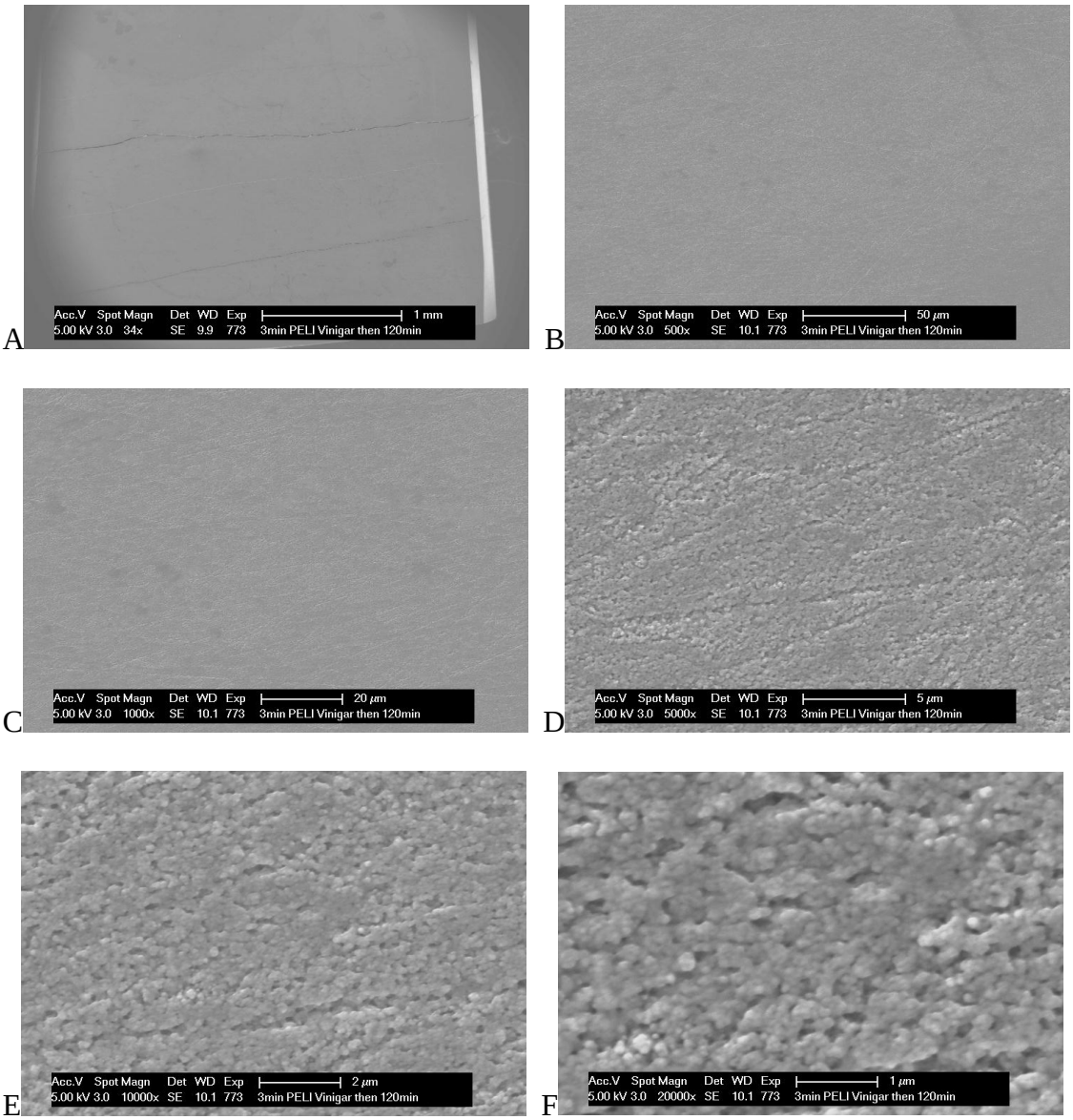



**Fig. 12** Vinegar group SEM micrographs: an overview of 3-min pellicle 120 min after 5-s rinsing with vinegar, at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

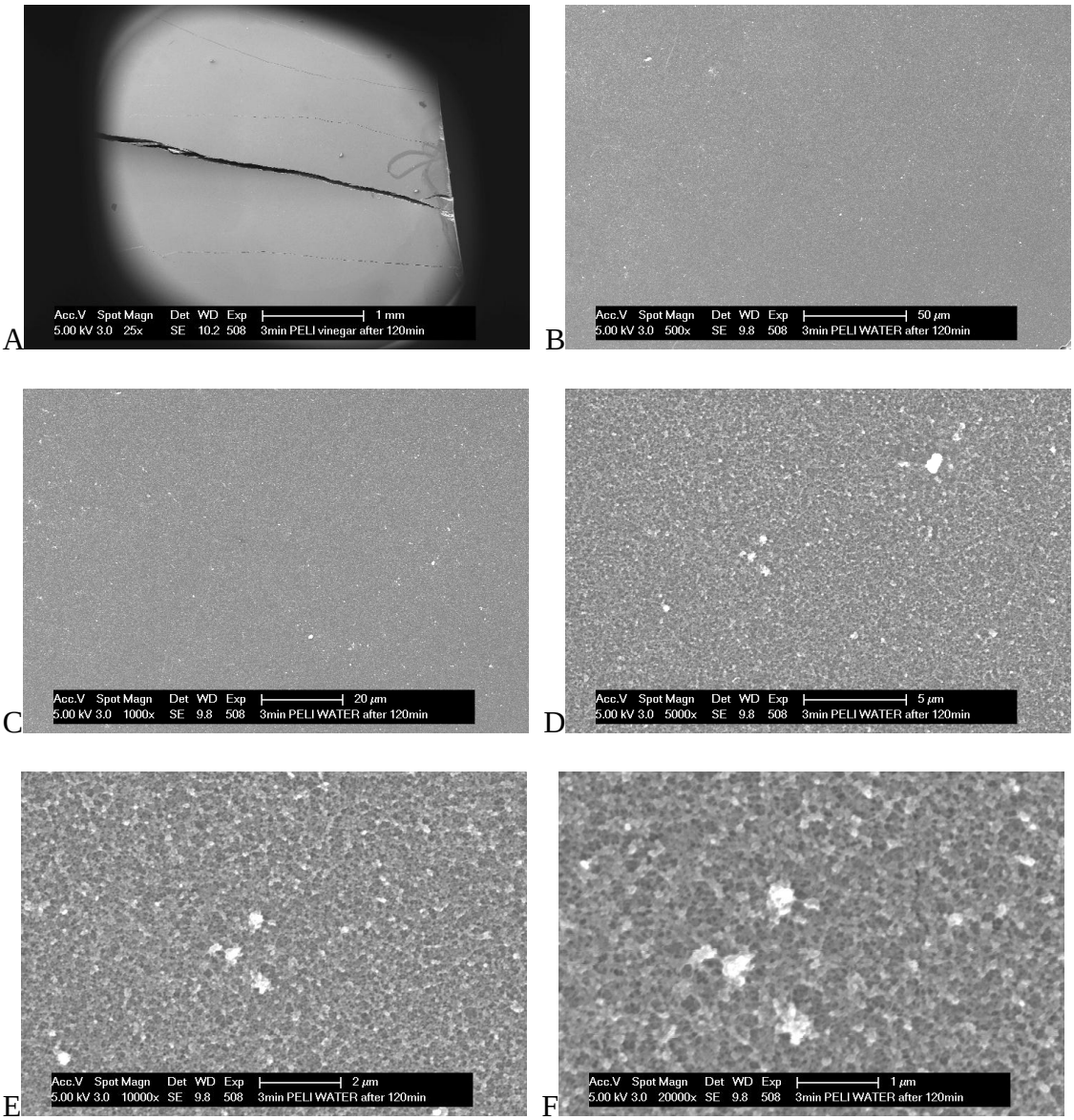



**Fig. 13** Control group SEM micrographs: an overview of 3-min pellicle 120 min after rinsing with water, at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

According to the chronological comparison of the control groups, the pellicle developed from 3 min as a fine granular layer to 30 min as granular layers with globularly shaped protein clusters, then to 120 min as network globular structure. With increasing formation time, the structure of the pellicle got more complex (Fig. 14). On the other hand, the adherence of bacteria has also changed. At 3 min, single bacteria attached to the pellicle were occasionally observed, and then after 30 min, some bacteria gathering together as small groups could be detected. Finally, at 120 min, SEM analysis of the enamel surfaces revealed aggregation of bacteria assembled in orderly matrix and exhibited strong proliferative activities (Fig. 14). Simultaneously, the quantity and diversity of bacterial species also increased rapidly (Fig. 15). In contrast, the vinegar rinsed samples showed a distinctly smooth surface with a very thin pellicle that did not mask the enamel surface micromorphology, and it was difficult to find any attached particles or bacteria (Fig. 16).

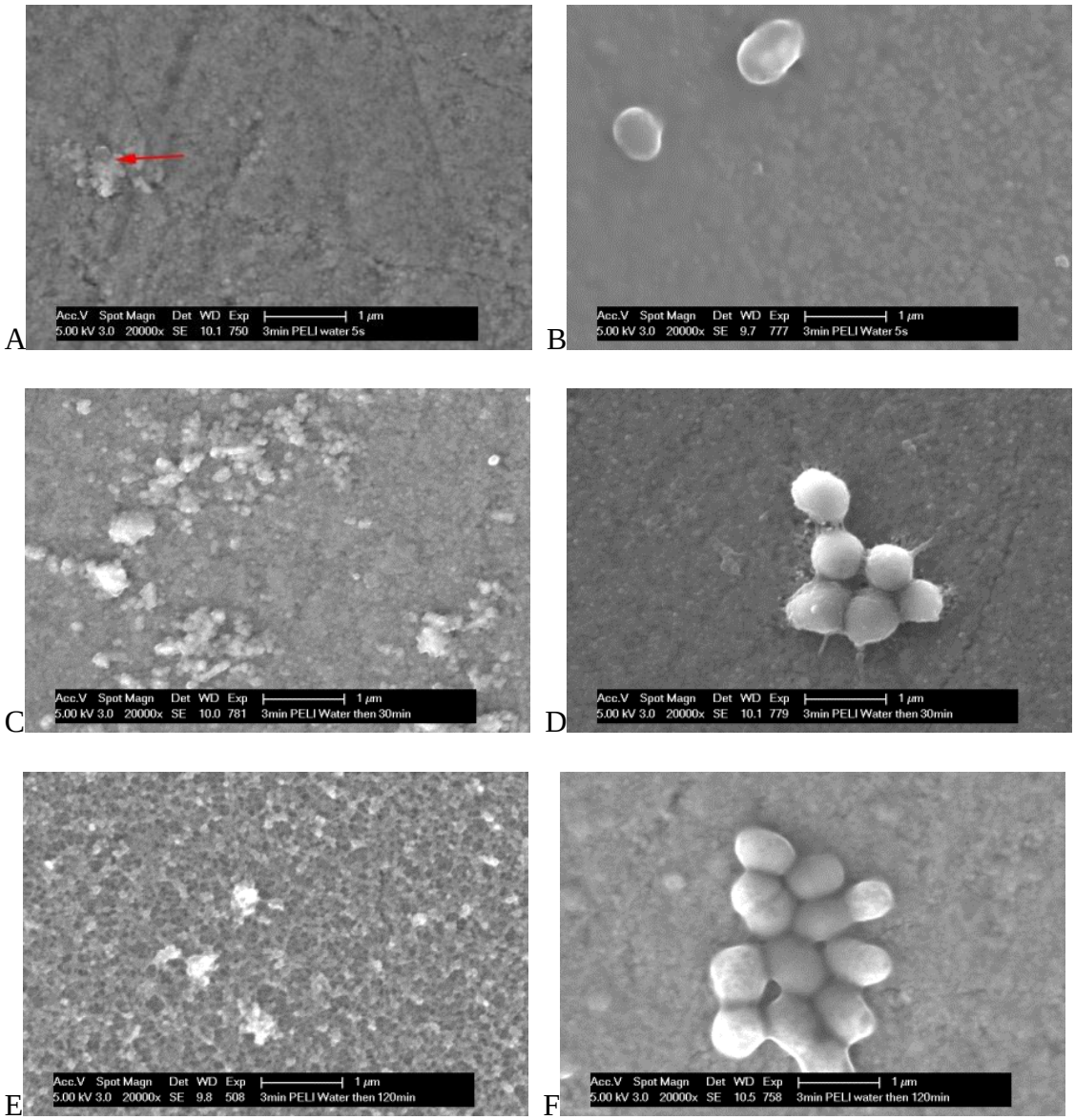



**Fig. 14** Control group SEM micrographs: biofilm formation within 120 min. (A, C and E) structural pattern of pellicle formation. (A) 3 min: sludge-like substances onto granular pellicle; (B) 30 min: clusters of globular protein aggregates; (C) 120 min: network structure of globular agglomerates. (B, D and F) bacterial aggregation during biofilm formation. (B) 3 min: single bacteria; (D) 30 min: bacterial aggregate; (F) 120 min: bacterial colony.Original magnification: 20,000-fold.

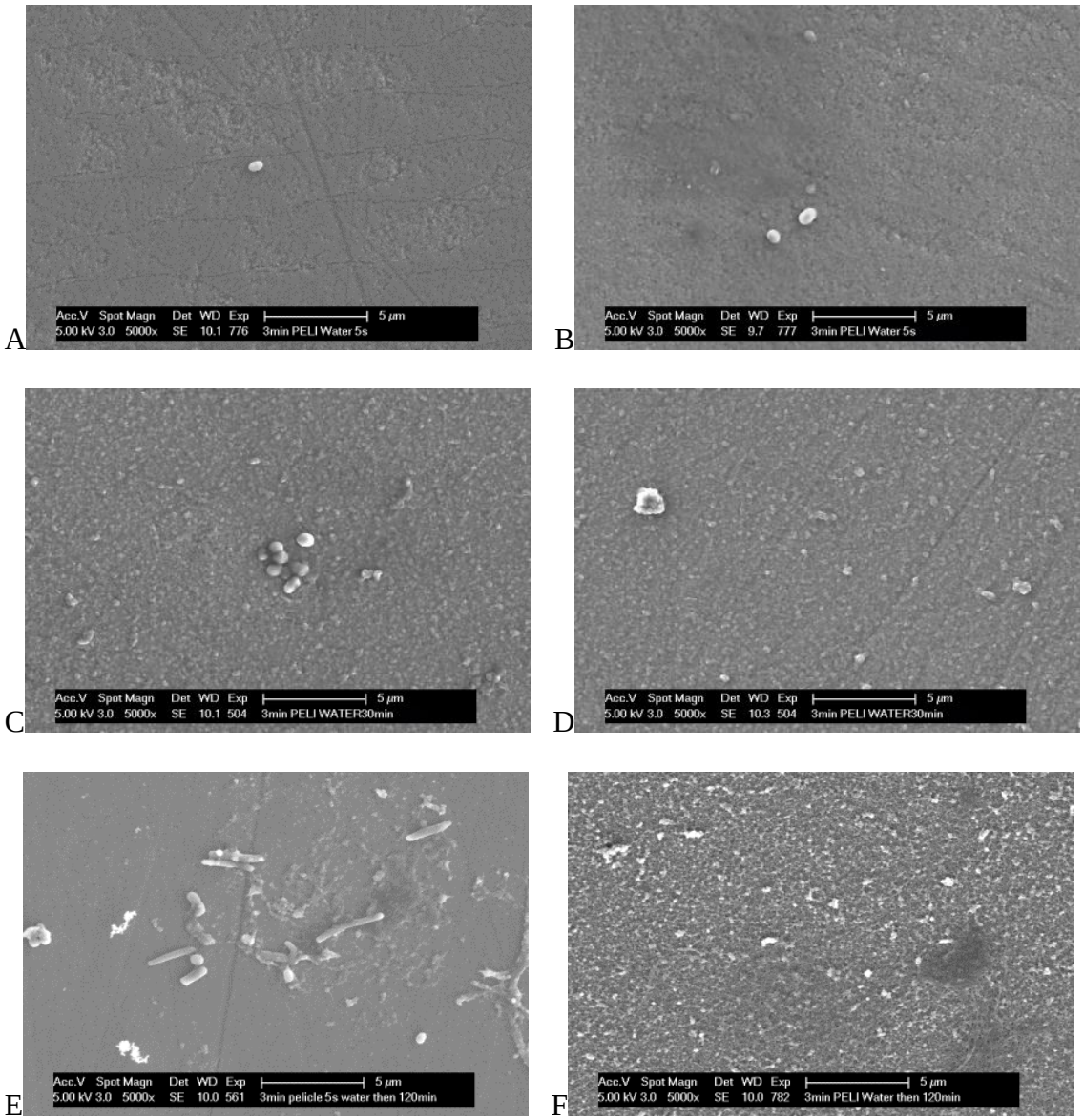



**Fig. 15** Control group SEM micrographs: increasing quantity of particles within 120 min of biofilm formation. (A) 3 min: single cocci; (B) 30 min: groups of cocci; (C) 120 min: cocci and rod-shaped bacteria; (D, E and F) increasing number of protein aggregates from 3-min to 120-min pellicle formation time. Original magnification: 5,000-fold.

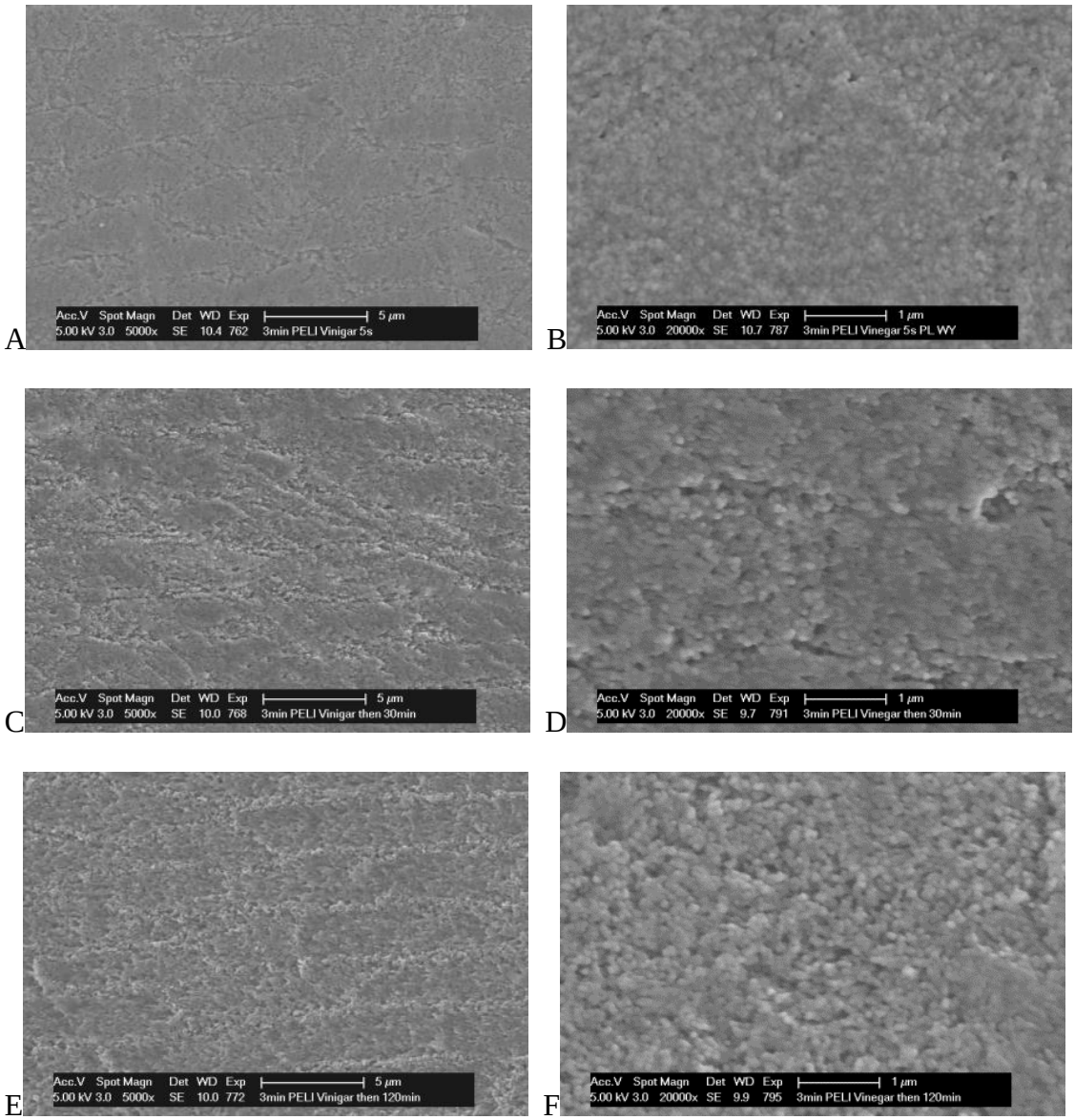



**Fig. 16** Vinegar group SEM micrographs: ‘clean‘ enamel surfaces even 120 min after rinsing with vinegar. 3 min (A and B), 30 min (C and D) and 120 min (E and F); with 5,000-fold magnification (A, B and C) and 20,000-fold magnification (D, E and F).

## TEM

The ultrastructure of the pellicle was clearly revealed by TEM. The micrographs of the control group showed that the enamel slabs were covered by an outer globular layer and an electron-dense basal pellicle layer after exposed intraorally for 3 min and rinsed with water for 5 s. Within 120 min, the outer globular layer got thicker while the sublayer kept steady thin electron-dense over time. Simultaneously, the enamel surface was covered with separate globular particles within 120 min after water rinsing.

The pellicles formed in the vinegar group were characterized by only an less dense not continuous basal layer without an outer layer. However, there was a subsurface appeared after vinegar rinsing. The outer globular layer was only detectable 120 min after vinegar rinsing (Table. 1 and Fig. 17).

**Table. 1** *In situ* formed pellicle layer and ultrastructural findings (appearance of the residual pellicle layer) after rinsing with water (control) and vinegar, respectively.

| | **Control groups** | | **Vinegar groups** | | |
|---|---|---|---|---|---|
| **Time of rinsing** | Ultrastructural appearance of the outer layer | Ultrastructural appearance of the basal layer | Ultrastructural appearance of the outer layer | Ultrastructural appearance of the basal layer | Ultrastuctural appearance of the subsurface |
| 0 min after rinsing | Globular particles, 20-40 nm thick | Electron dense, 10-20 nm thick | Not detectable (completely removal) | Residues of the sublayer, | Pellicle network up to 300 nm thick |
| 30 min after rinsing | Globular particles, 100-170 nm thick | Electron dense, 10-20 nm thick | Electron dense layer, up to 40 nm thick | Residues of the sublayer, | Pellicle network up to 300 nm thick |
| 120 min after rinsing | Globular particles, 100-400 nm thick | Electron dense, 10-20 nm thick | Globular particle, up to 100 nm thick | Residues of the sublayer, | Pellicle network up to 300 nm thick |

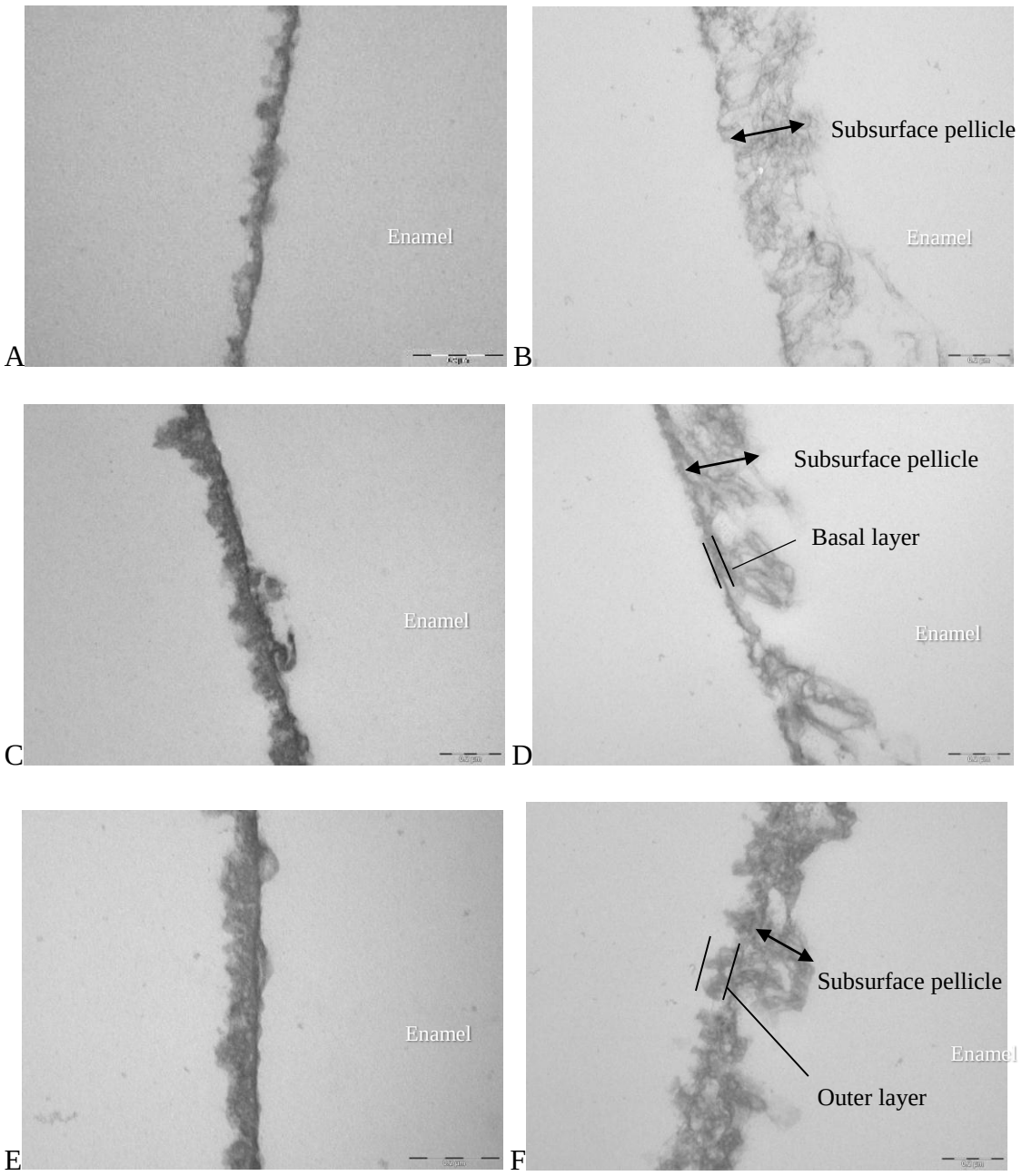



**Fig. 17** TEM micrographs: gallery of representative pellicle layers after rinsing with water (left) and vinegar (right). (A, C and E) Control group, the outer globular layer increased over time, also, the electron-dense basal layer was clearly observed; (B, D and F) Vinegar group, the outer pellicle layer is removed due to vinegar rinsing and the subsurface is formed; (A and B) immediately after vinegar rinsing; (C and D) 30 min after vinegar rinsing; (E and F) 120 min after vinegar rinsing. Original magnification: 68,000-fold.

### 4.1.2 Semi-quantification of particles in the *in situ* pellicle

After 3 min of oral exposure, large particles on enamel surfaces (including bacteria and protein aggregates) were observed by SEM and counted with Image J to quantify the arrangement of the outer pellicle layer so as to determine the interference of the vinegar in the pellicle formation process. Table. 2 summarizes the median and range of all samples. Pellicle formation in the control group had a significant difference between the three time points (immediately, 30 min and 120 min after water rinsing) (Kruskal-Wallis test, $p<0.01$), while there was little difference between the three time points in the vinegar rinsing group (Kruskal-Wallis test, $p>0.05$). A further comparison by Mann-Whitney U-test found that pellicle particles increased significantly from 3 min to 30 min to 120 min in the control group (Mann-Whitney U test, $p <0.001$). However, in the vinegar rinsing group, pellicle particles did not increase with time (Mann-Whitney U test, $p> 0.05$). At all three times of pellicle formation, compared to the control group, the adsorption of particles onto the enamel slabs rinsed with vinegar was significantly inhibited (Kruskal-Wallis test, $p>0.05$) (Fig. 18).

**Table. 2** Quantification of pellicle particles without or following rinsing with vinegar evaluated by SEM. Exposition of the enamel specimens for 3 min. Median numbers of pellicle particles according to Image J (Median(Q1,Q3)). A significant difference between vinegar rinsing and water rinsing was shown immediately after rinsing procedure ($p<0.05$), 30 min ($p<0.001$) and 120 min ($p<0.001$) after rinsing. (Mann-Whitney U test). Within the 120-min experimental period, the use of water resulted in a significant increase of particles (Mann-Whitney U test, $p < 0.01$), while after the use of vinegar, no difference between 3-min, 30-min and 120-min specimens were detected (Mann-Whitney U test, $p > 0.05$). Numbers of particles per 11261 $\mu m^2$ of surface area.

| Groups / Exposure time | immediately after rinsing | 30min after rinsing | 120min after rinsing |
|---|---|---|---|
| Control (water) | 3.5(1,7) | 16(8.75,29.75) | 45.5(24.75,124.25) |
| Vinegar | 0.5(0,2) | 1.5(0,2) | 0.5(0,3) |

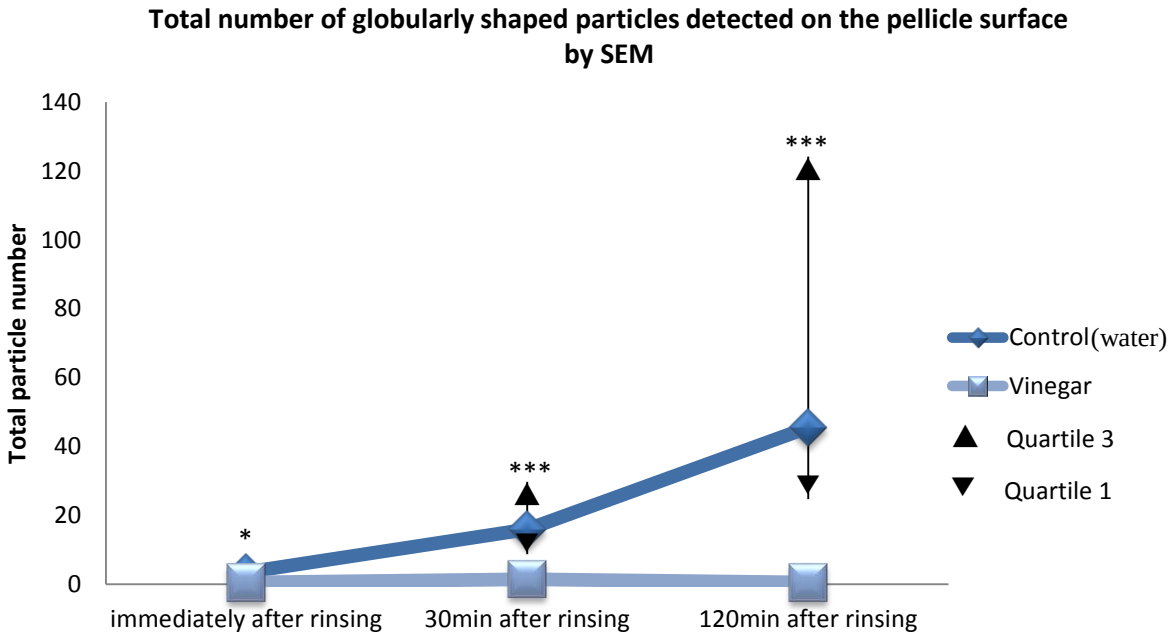



**Fig. 18** Total number of globular particles detected in the pellicle surface by SEM. Control group showed typicle formation of pellicle with a significant increase of globular particles over time (Mann-Whitney U test, $p<0.001$). The enamel surfaces of the vinegar rinsing group kept quite "clean" after rinsed with vinegar over the 120-min observation time. After rinsing with vinegar, there were great differences immediately (Mann-Whitney U test, $p<0.01$), 30 min (Mann-Whitney U test, $p<0.001$) and 120 min (Mann-Whitney U test, $p<0.001$) after rinsing compared to the control group, mean ± SD. *: $p<0.05$; ***: $p<0.001$

## 4.2 Part two, 24-h biofilm

### 4.2.1 Visualization of adherent bacteria in the *in situ* biofilm

#### 4.2.1.1 *Bac*Light™ viability assay

In the 24-h biofilms, live microbial cells (stained green) and membrane ruptured microbial cells (stained red) could be clearly distinguished by *Bac*Light™ viability assay (Fig. 19). After exposure in the oral environment for 24 h, enamel slabs were also analyzed regarding the biofilm structure. It was observed that the bacteria in the samples were predominantly cocci and rods, in which the cocci showed regular chain and dispersive forms. Occasionally, epithelial cells were found in the biofilm. (Fig. 20). Thickness of the biofilms varied with bacteria in some areas extremely dense arranged by overlapping clusters, while in some areas no bacteria could be detected (Fig. 21).

In the control group, live bacteria were exceedingly abundant in 24-h biofilms, especially in dense colonies, while dead bacteria had less quantity and mainly concentrated in the bottom part of the biofilm (Fig. 22).

After application of vinegar for 5 s, there was a little change in thick clusters of colonies in quantity. Most bacteria exposed at the surface were stained red after membrane rupture. However, thick colonies were detected staining red on the edge circle, while the central areas were still stained bright green (Fig. 23). The proportion of dead bacteria increased over time. The red stained bacteria were rapidly spreading from the edge circle of the colony to the central surface, whereas the green stained bacteria faded gradually, and only a small amount of healthy bacteria could be observed 30 min after vinegar rinsing (Fig. 24). 30 min after vinegar rinsing, the proportion of dense colonies decreased overall, but bacteria arranged as monolayer had a decreasing trend. With the red stained bacteria spreading further, green stained bacteria were still detected in the colony center (Fig. 25). 120 min after vinegar rinsing, the biofilms appeared smaller and thinner overall in some samples without dense colonies or monolayer areas (Fig. 26). However, there were still visible green stained cocci and rods, whereas other species of bacteria were completely dead (Fig. 27).

Throughout the vinegar rinsing process, there were two impressive characteristics. The first one was that the dense layer of bacterial cells in the biofilm could still remain viable, while the majority of the residual biofilms revealed a scattered distribution of dead bacteria (Fig. 28). The second was that the surviving bacteria were of two major shapes: cocci and rods.

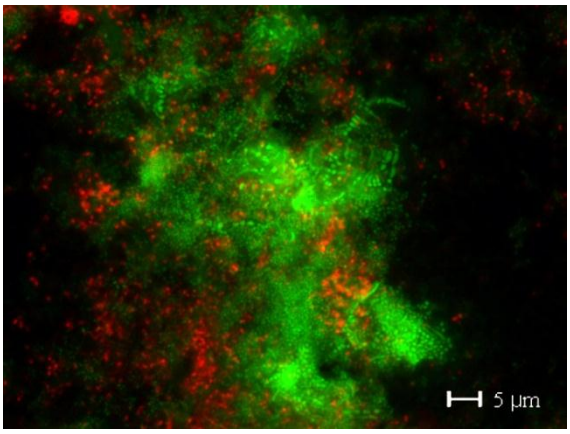
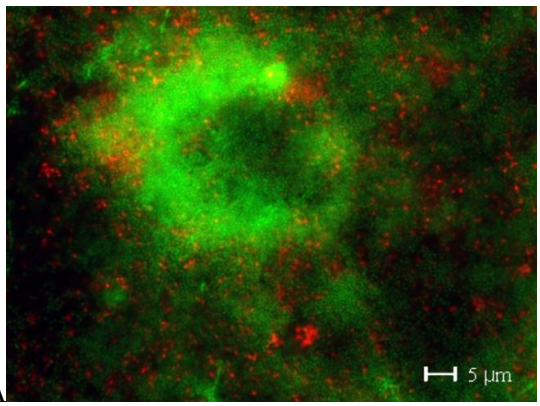



**Fig. 19** *Bac*Light™ viability assay: Clear differentiation of live (green) and dead (red) bacteria. Control sample exposed to oral cavity for 24 h. Green: live; Red: dead. Original magnification: 1,000-fold.

A 

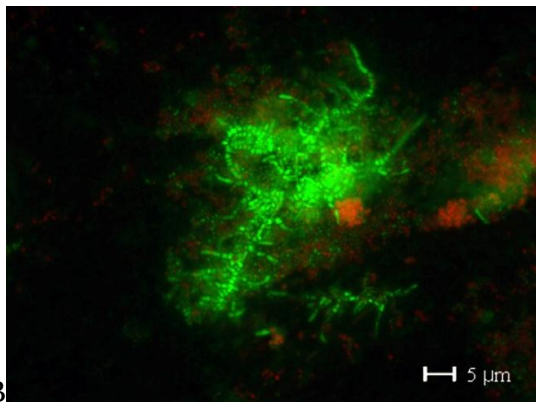

B 


**Fig. 20** *Bac*Light™ viability assay: two typical aggregations of bacteria presented in the 24-h biofilm of control samples. (A) dispersive distribution; (B) chain-like distribution. Green: live; Red: dead.Original magnification: 1,000-fold.

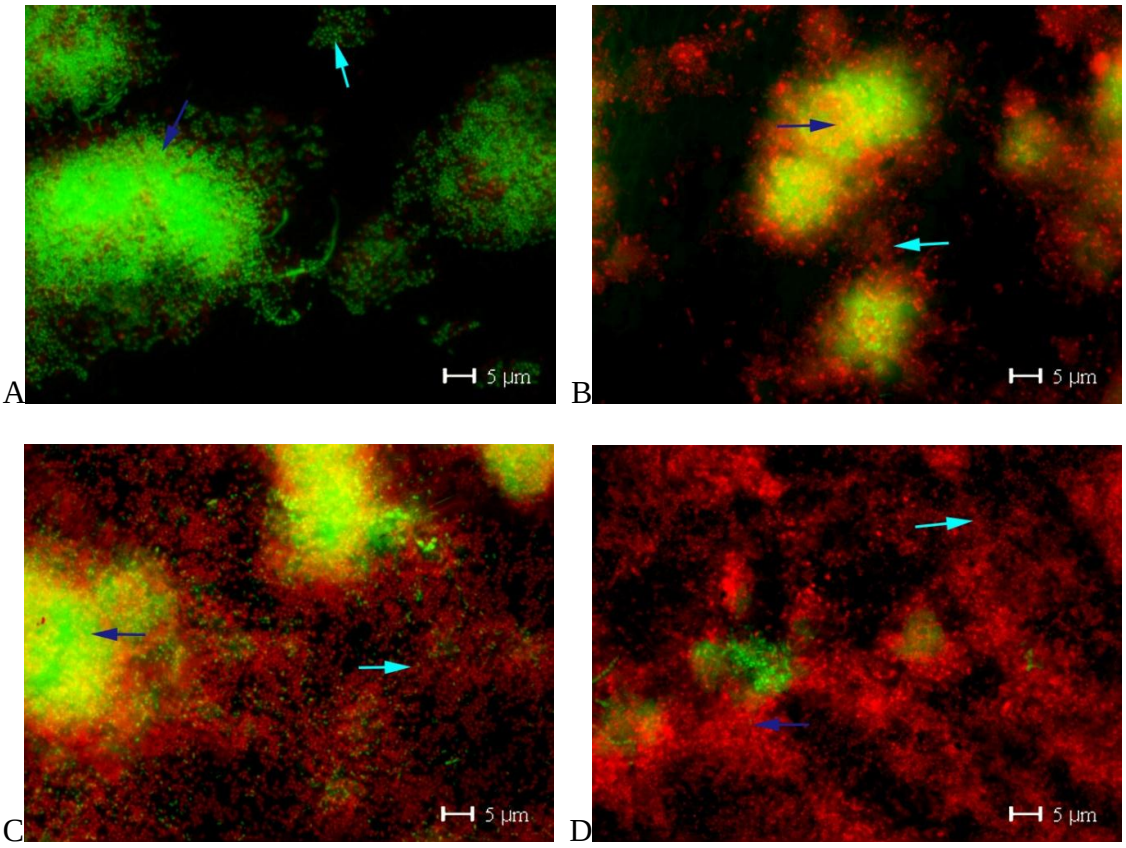
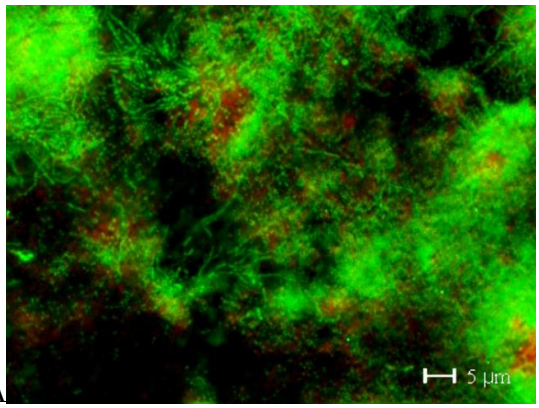



**Fig. 21** *Bac*Light™ viability assay of 24-h biofilm: uneven thickness of microflora, with dense areas (Dark blue arrows) and loosely arranged (Light blue arrows). (A) control group; (B) immediately after vinegar rinsing; (C) 30 min after vinegar rinsing; (D) 120 min after vinegar rinsing. Green: live; Red: dead. Original magnification: 1,000-fold.

A 

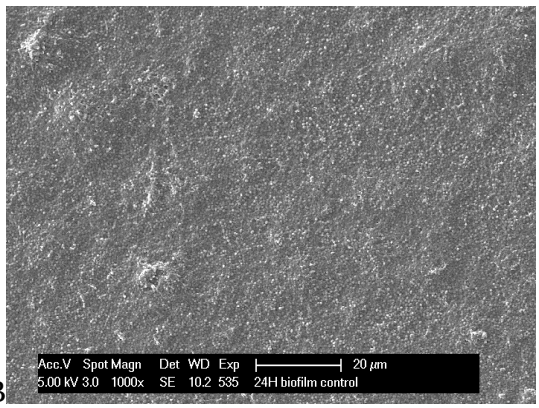
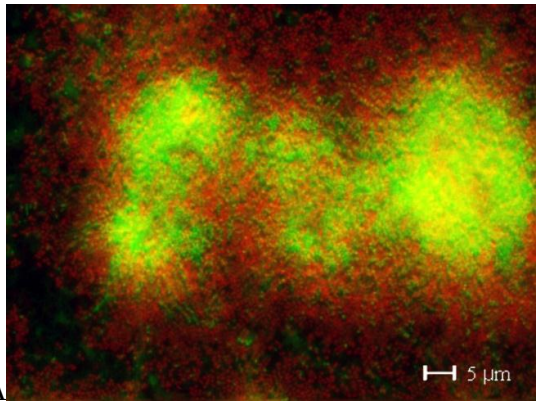
B 


**Fig. 22** Visualization of living/ dead bacteria of a 24-h biofilm (control group). (A) *Bac*Light™ viability assay. Green: live; Red: dead; (B) corresponding SEM micrograph. Original magnification: 1,000-fold.

A 

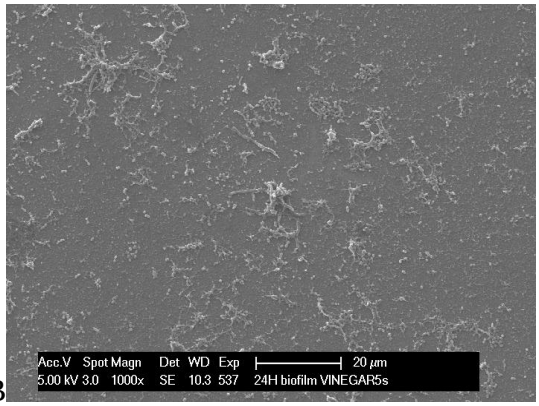
B 


**Fig. 23** Visualization of living/ dead bacteria of a 24-h biofilm (immediately after vinegar rinsing for 5 s). (A) *Bac*Light™ viability assay. Green: live; Red: dead; (B) corresponding SEM micrograph. Original magnification: 1,000-fold.

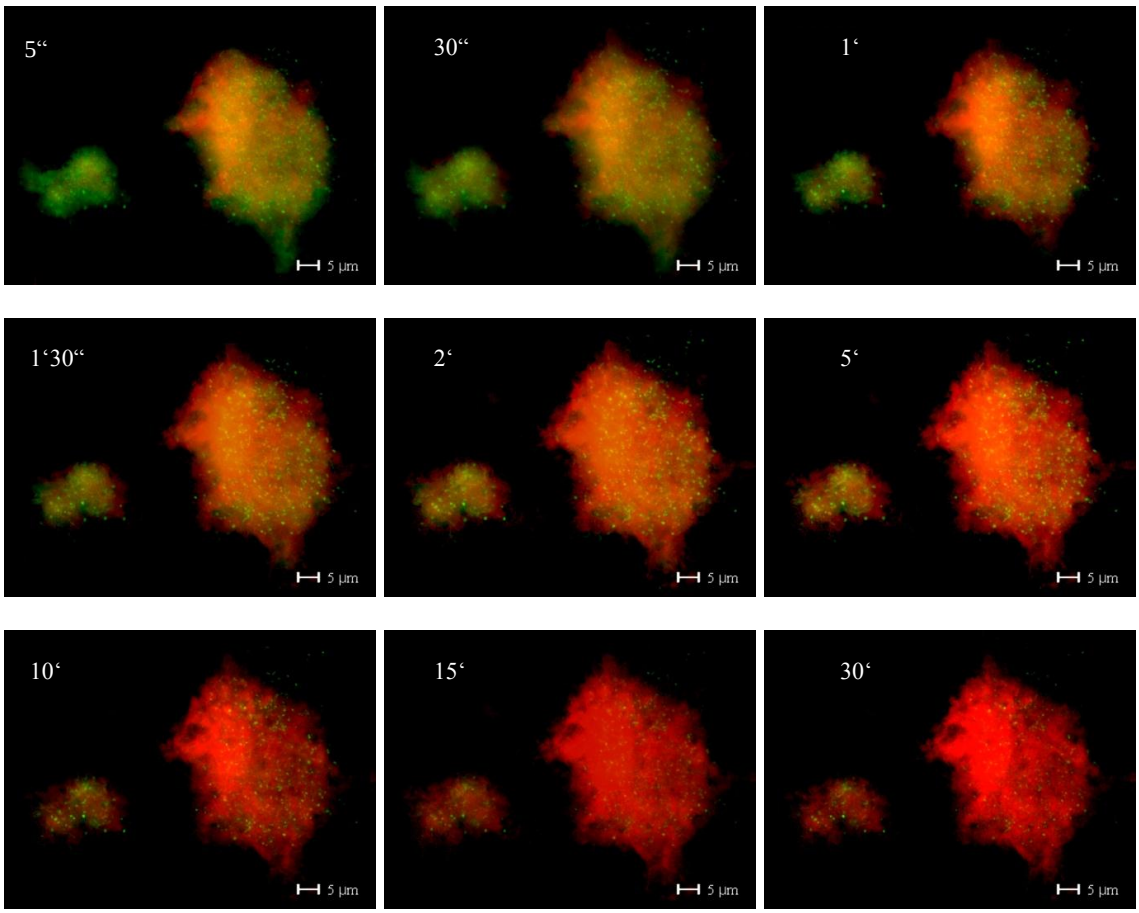



**Fig. 24** *Bac*Light™ viability assay of a 24-h biofilm dynamic effect: increasing proportion of dead bacteria (red) within 30 min after application of vinegar. Green: live; Red: dead. Original magnification: 1,000-fold.

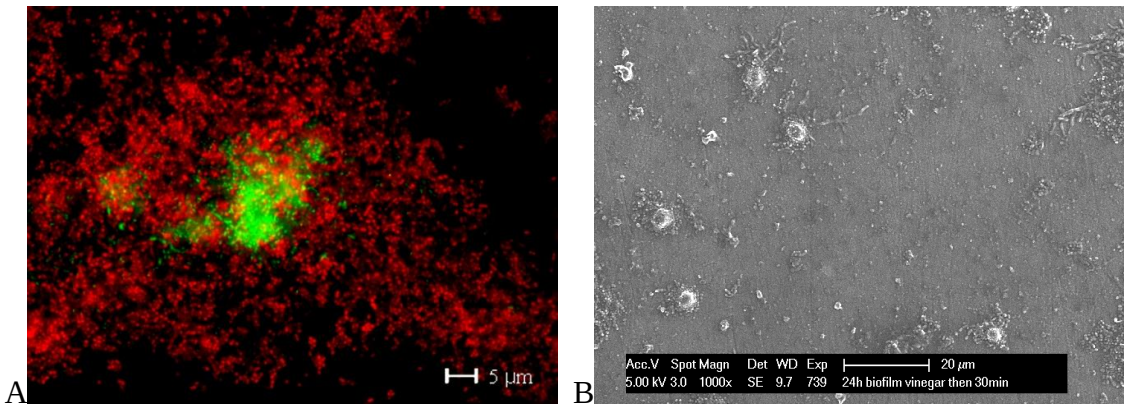



**Fig. 25** Visualization of living/ dead bacteria of a 24-h biofilm 30 min after vinegar rinsing. (A) *Bac*Light™ viability assay, Green: live; Red: dead; (B) corresponding SEM micrograph. Original magnification: 1,000-fold.

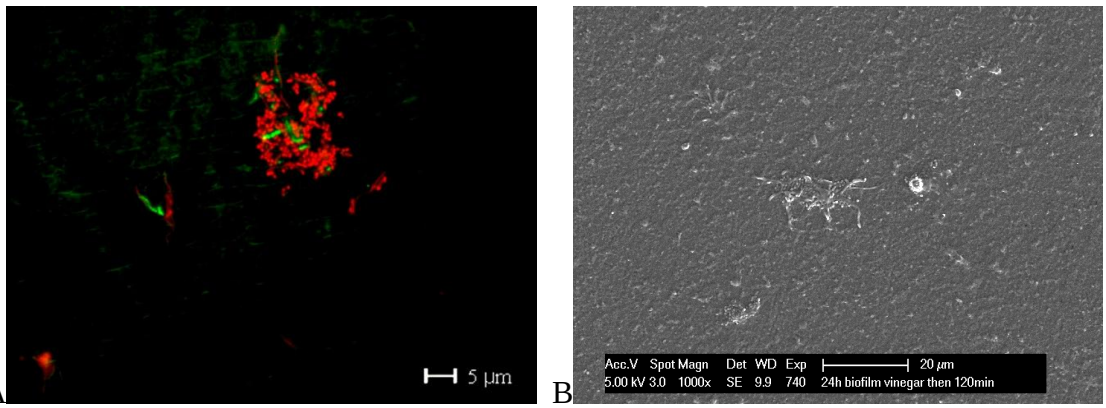



**Fig. 26** Visualization of living/ dead bacteria of a 24-h biofilm in 120 min after vinegar rinsing. (A) *Bac*Light™ viability assay, Green: live; Red: dead; (B) corresponding SEM micrograph. Original magnification: 1,000-fold.

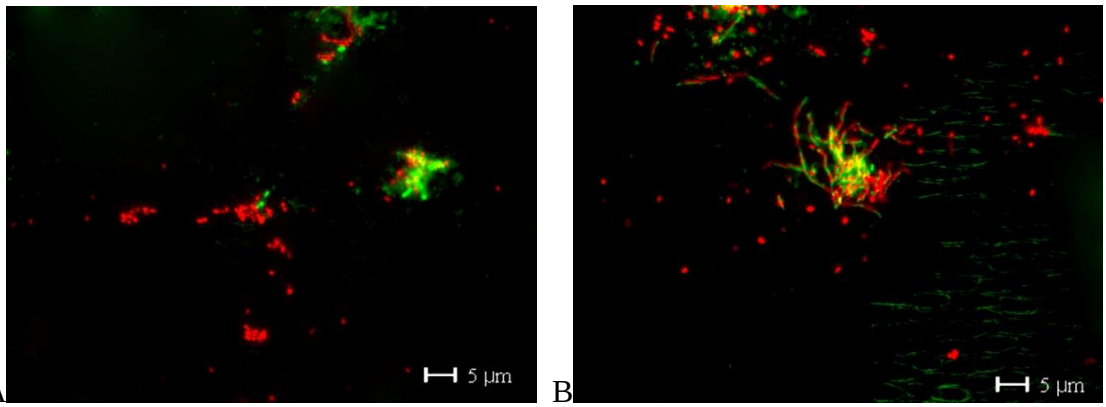



**Fig. 27** *Bac*Light™ viability assay: 120 min after vinegar rinsing, two major shapes of bacteria were observed surviving in 24 h biofilm. (A) cocci; (B) rods. Green: live; Red: dead. Original magnification: 1,000-fold.

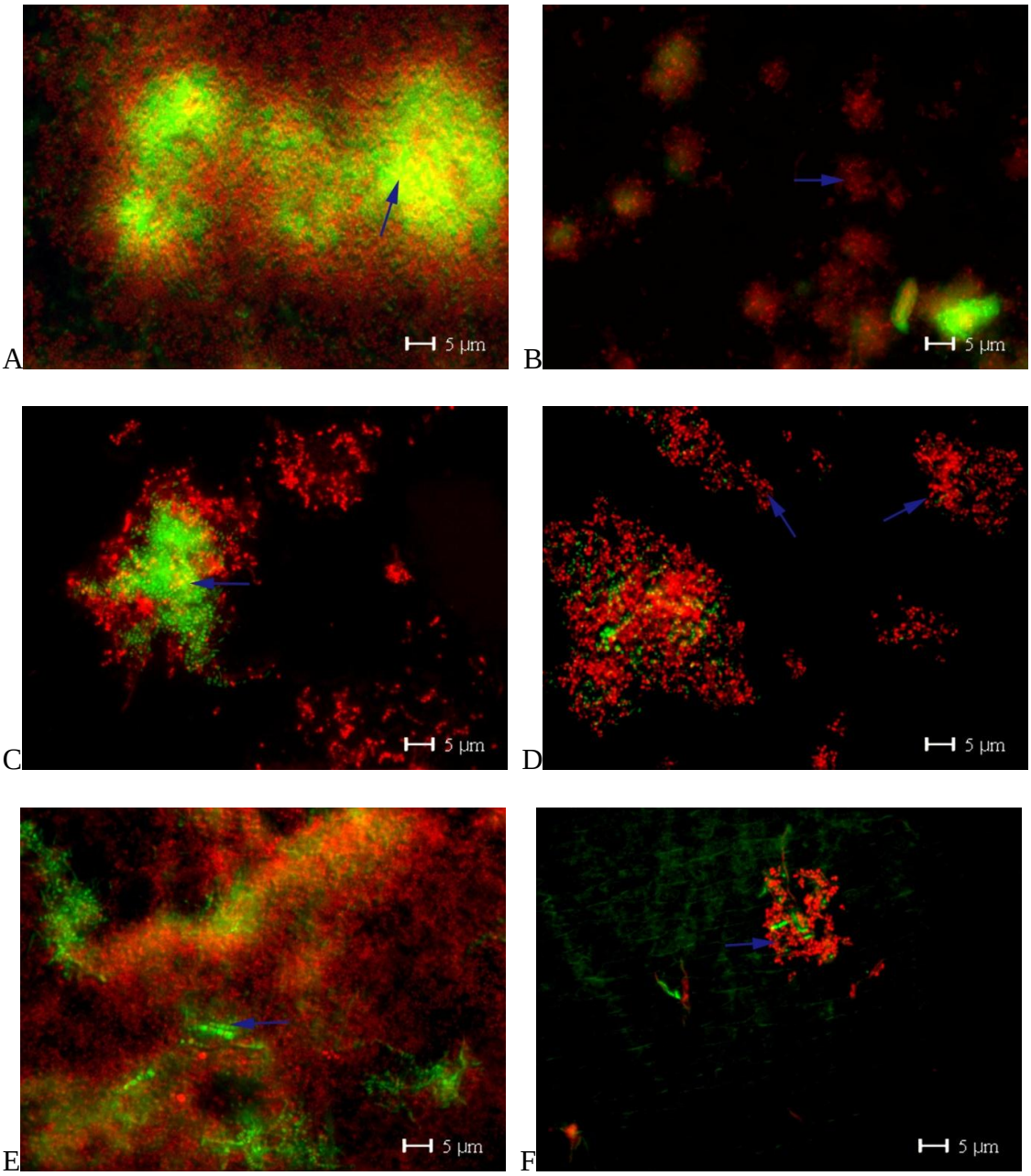



**Fig. 28** *Bac*Light™ viability assay: 24-h biofilm after vinegar rinsing kept viable (green) in dense bacterial layers and red in scattered distributed bacterial layers. (A and B) immediately after vinegar rinsing for 5 s; (C and D) 30 min after vinegar rinsing; (E and F) 120 min after vinegar rinsing; (A, C and D) dense layers of biofilms; (B, E and F) scattered distribution of bacteria. Green: live; Red: dead. Original magnification: 1,000-fold.

#### 4.2.1.2 SEM

SEM facilitates morphological description of the bacterial appearance in the *in situ* 24-h biofilm, mainly in the form of chain-like cocci and rod-shaped bacteria. Uneven distributed colonies could be observed more intuitively by SEM (Fig. 29). In the 24-h biofilm, the surface of the whole biofilm was characterized by exposed bacteria, some of which were connected firmly while others were relatively loosely arranged with mono-layer bacteria covering the enamel surface (Fig. 30).

The biofilm was observed to be unevenly arranged and distributed throughout the enamel surface at low magnification, while different shapes of bacteria closely connected together were detected in high magnification (Fig. 31).

After vinegar rinsing for 5 s, biofilm residues covered the enamel surface. Observation in high magnification revealed that numerous of damaged bacteria existed in the margin of the colonies, while lots of bacteria in the dividing state were found in the center areas (Fig. 32). 30 min after vinegar rinsing, the biofilm surface showed many large gaps, and the surface of the bacteria presented a rough texture. The bacteria at the margin of the colonies lose their normal form and structure, and also in the center of the colonies, a regular shape of bacteria could be hardly detected. The whole biofilm was covered by a layer of residues with no typical bacteria structure (Fig. 33). 120min after vinegar rinsing, biofilms were disintegrated into loose colonies as observed under low magnification. With the enlargement of magnification, the junction of colonies were found to be extremely loose, and the connection between bacteria got thinner. Interestingly, most of bacteria in the dividing state were observed only in chain-like arranged cocci (Fig. 34). There were many cell residues detected in biofilm disintegrating areas. The surface of the biofilm was destroyed after vinegar rinsing by losing intact bacterial cell structure (Fig. 35) and figure 21 recorded the process of this change. During the vinegar rinsing process, the connection of the surface bacteria in the 24-h biofilm was destroyed from dense to larger voids, and then the bacteria shed from the biofilm.

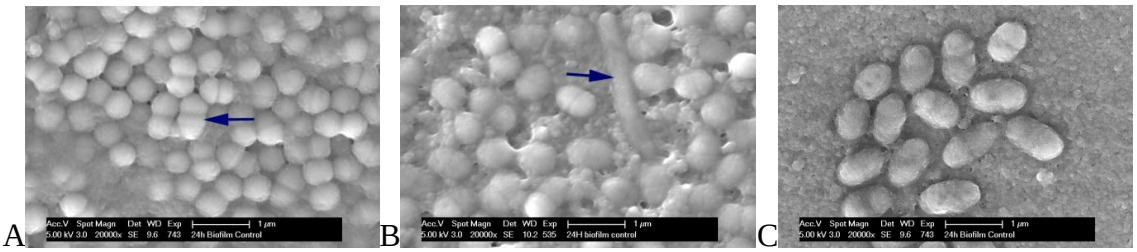



**Fig. 29** SEM micrographs: characteristic shapes of bacteria adherent to the 24 h biofilm in the control group. (A) cocci; (B) rods; (C) cocci in the state of division. Original magnification: 20,000-fold.

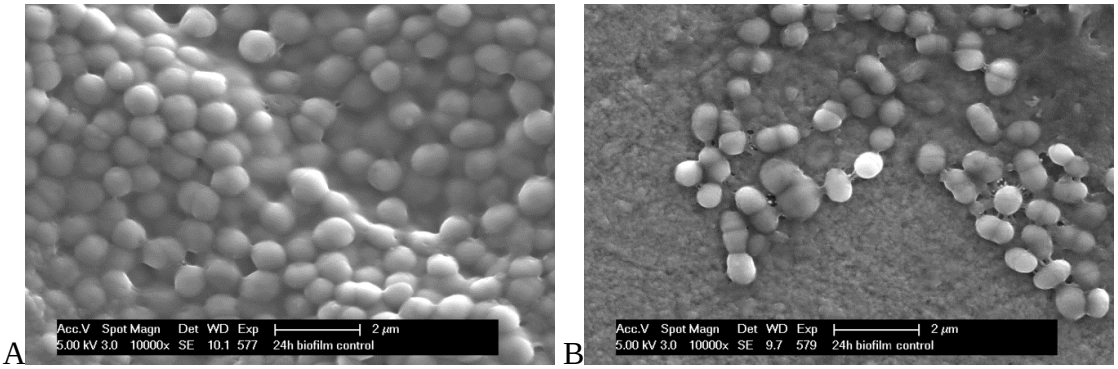



**Fig. 30** SEM micrographs: 24-h biofilm revealing different thickness of the adherent microflora in the control group. (A) dense bacterial layers; (B) loose connection of bacteria with forming a monolayer. Original magnification: 10,000-fold.

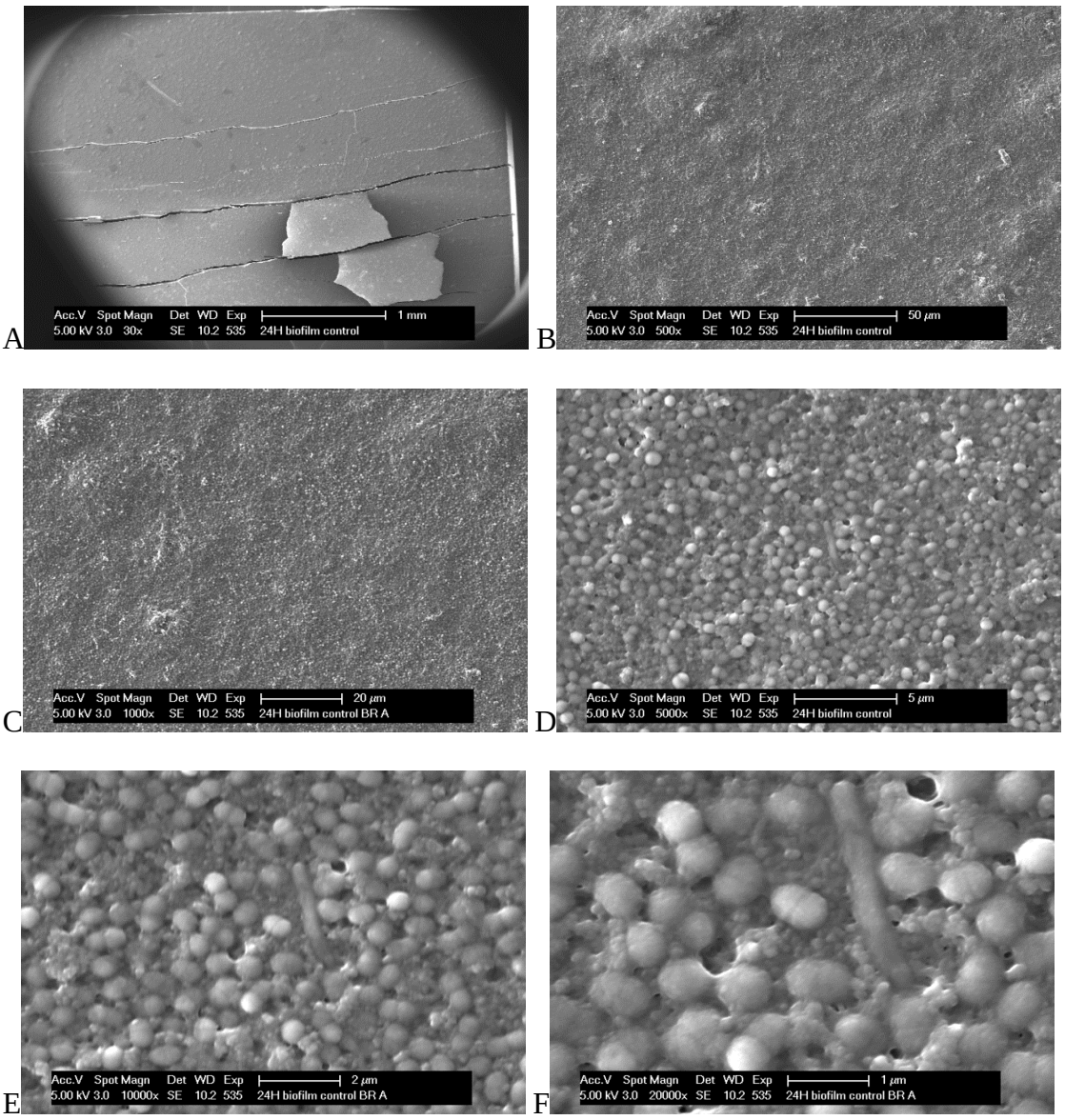



**Fig. 31** SEM micrographs of the control group: an overview of the 24-h biofilm at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

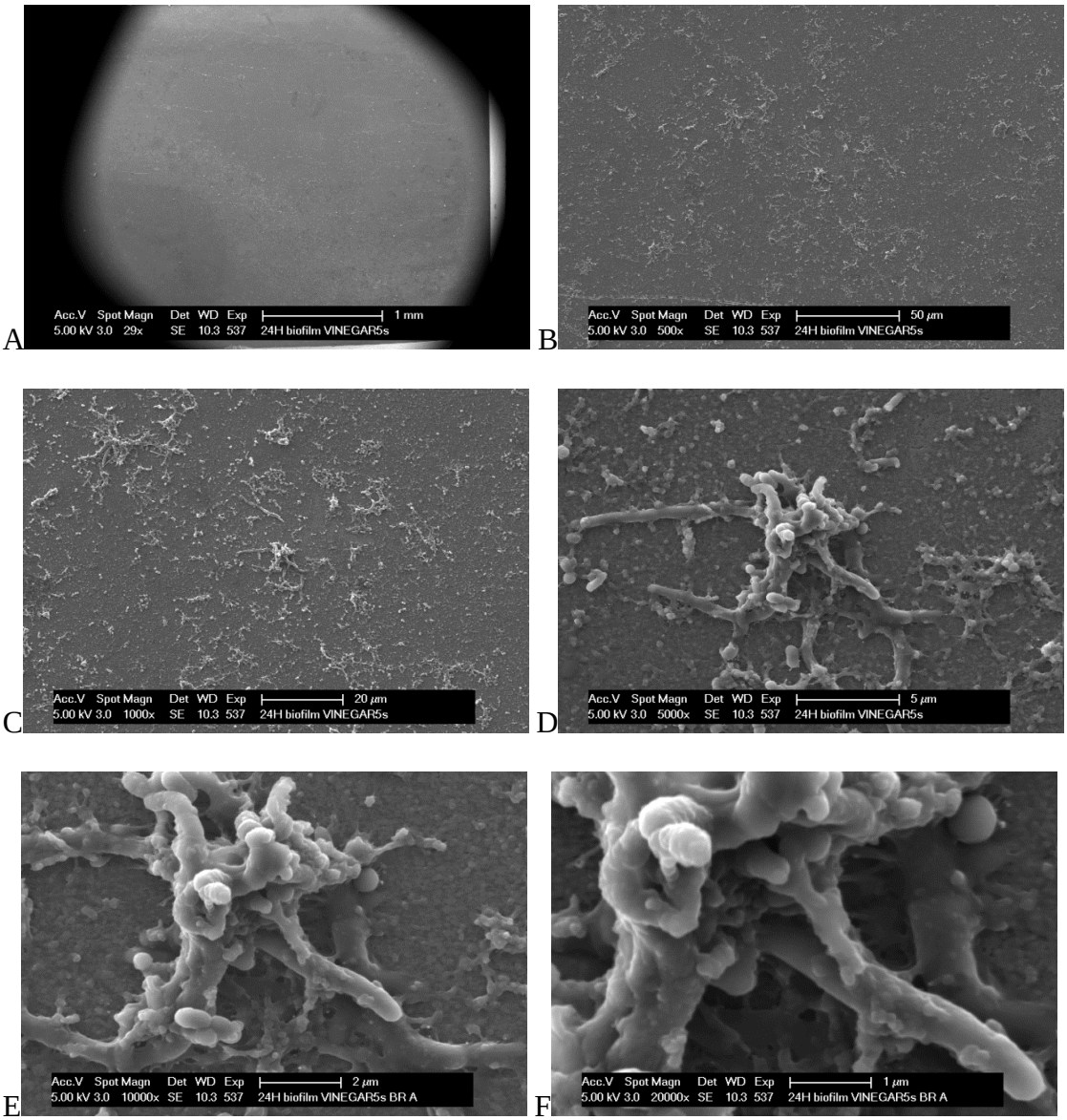



**Fig. 32** SEM micrographs of the vinegar group: an overview of the 24-h biofilm immediately after rinsing with vinegar at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

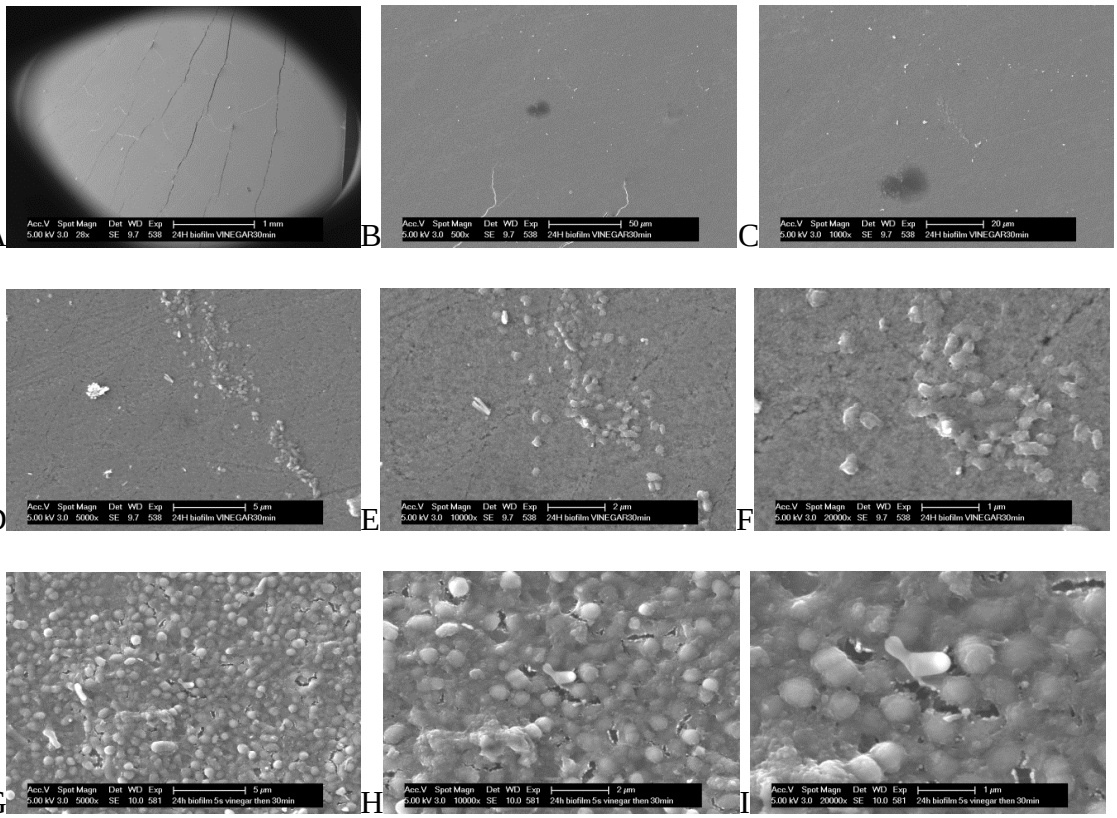



**Fig. 33** SEM micrographs of the vinegar group: an overview of the 24-h biofilm 30 min after vinegar rinsing at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) loose bacterial layer observed in 5,000-fold; (E) loose bacterial layer observed in 10,000-fold; (F) loose bacterial layer observed in 20,000-fold; (G) dense bacterial layer observed in 5,000-fold; (H) dense bacterial layer observed in 10,000-fold; (I) dense bacterial layer observed in 20,000-fold.

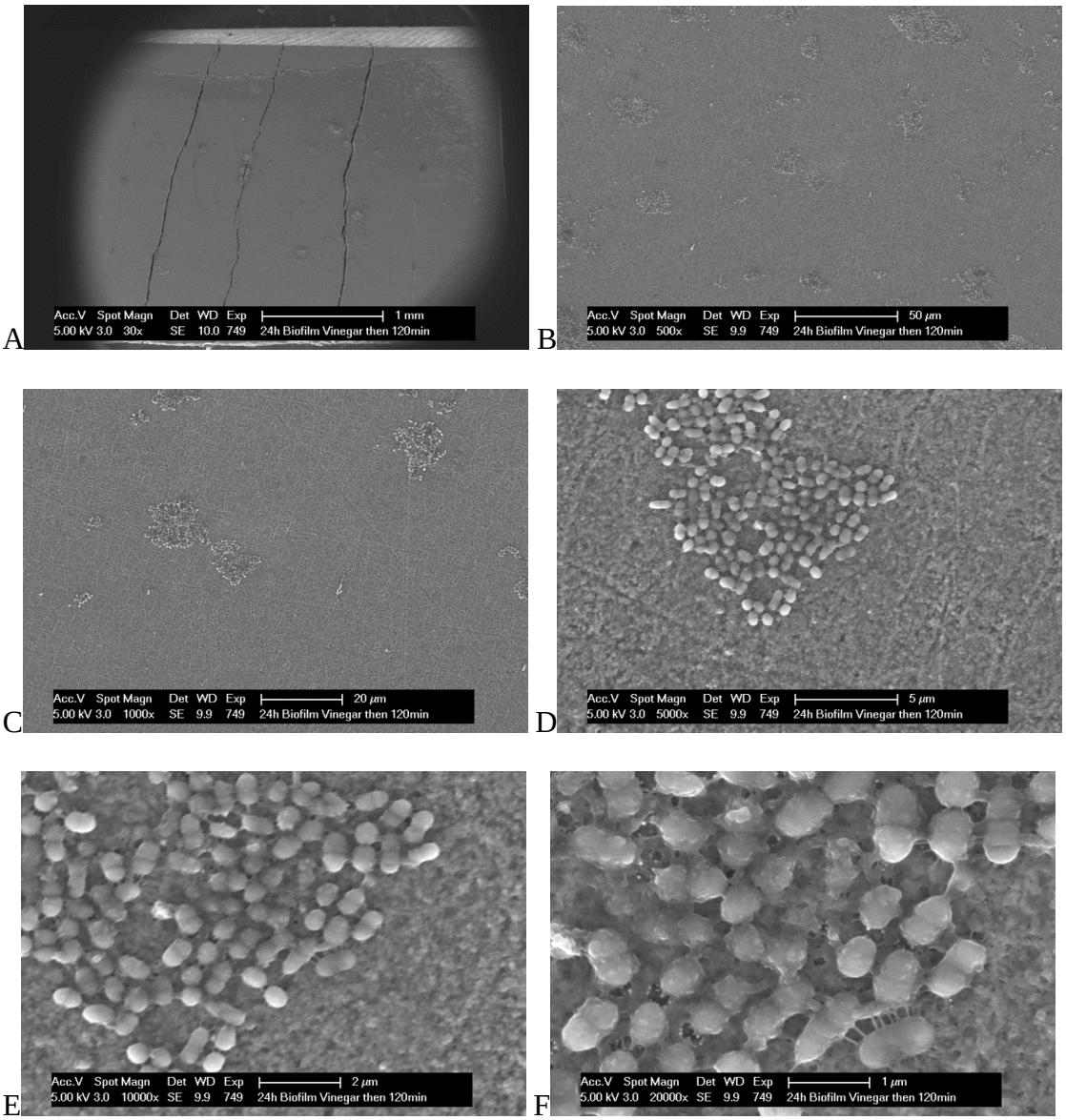



**Fig. 34** SEM micrographs of the vinegar group: an overview of the 24-h biofilm 120 min after vinegar rinsing at different magnifications. (A) 30-fold; (B) 500-fold; (C) 1,000-fold; (D) 5,000-fold; (E) 10,000-fold; (F) 20,000-fold.

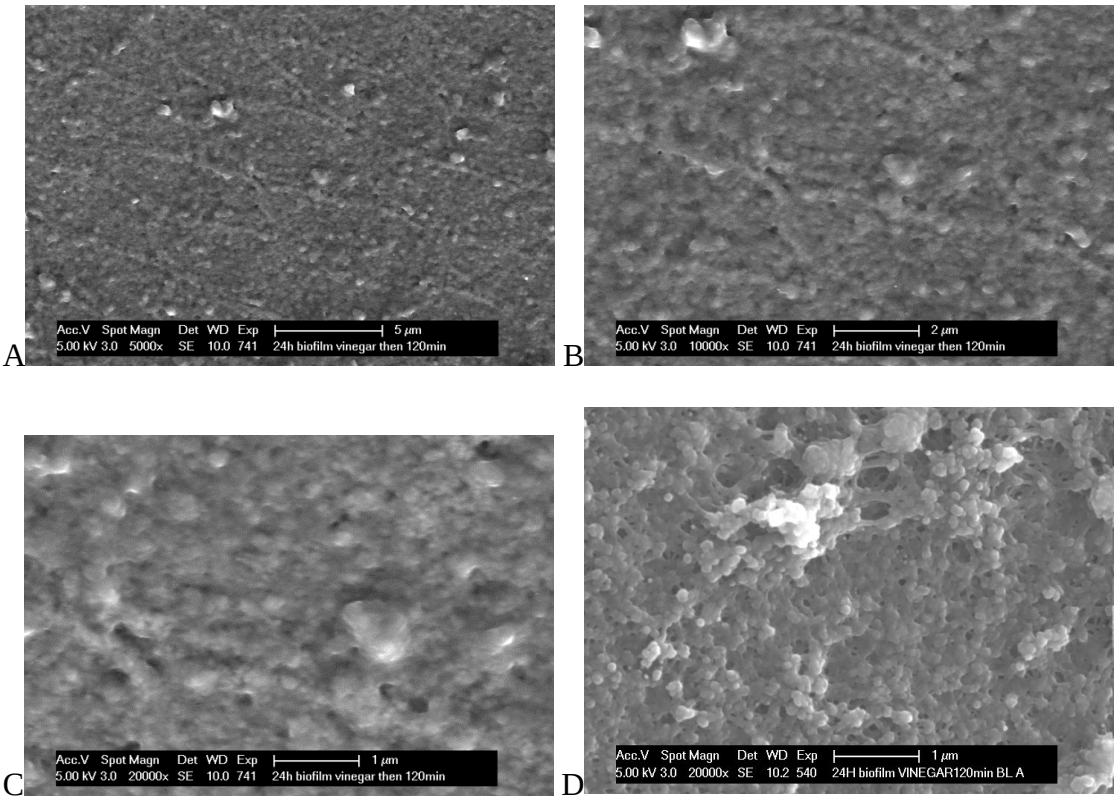



**Fig. 35** SEM micrographs of the vinegar group: no bacterial structures cover the surface of the 24-h biofilm 120 min after vinegar rinsing. (A) 5,000-fold; (B) 10,000-fold; (C) single layer in 20,000-fold; (D) dense layer in 20,000-fold.

### 4.2.1.4 TEM

The ultrastructure of the 24-h biofilm was clearly shown by TEM investigation. The control group showed that the surface of the enamel slabs was covered by thick biofilms after 24 hours exposure in the oral cavity. There was a wide range of microbes in the biofilm. After vinegar rinsing, the total amount of bacteria was reduced. In addition, many bacterial cell membranes were damaged and parts of the biofilm matrix were dissolved (Fig. 36)

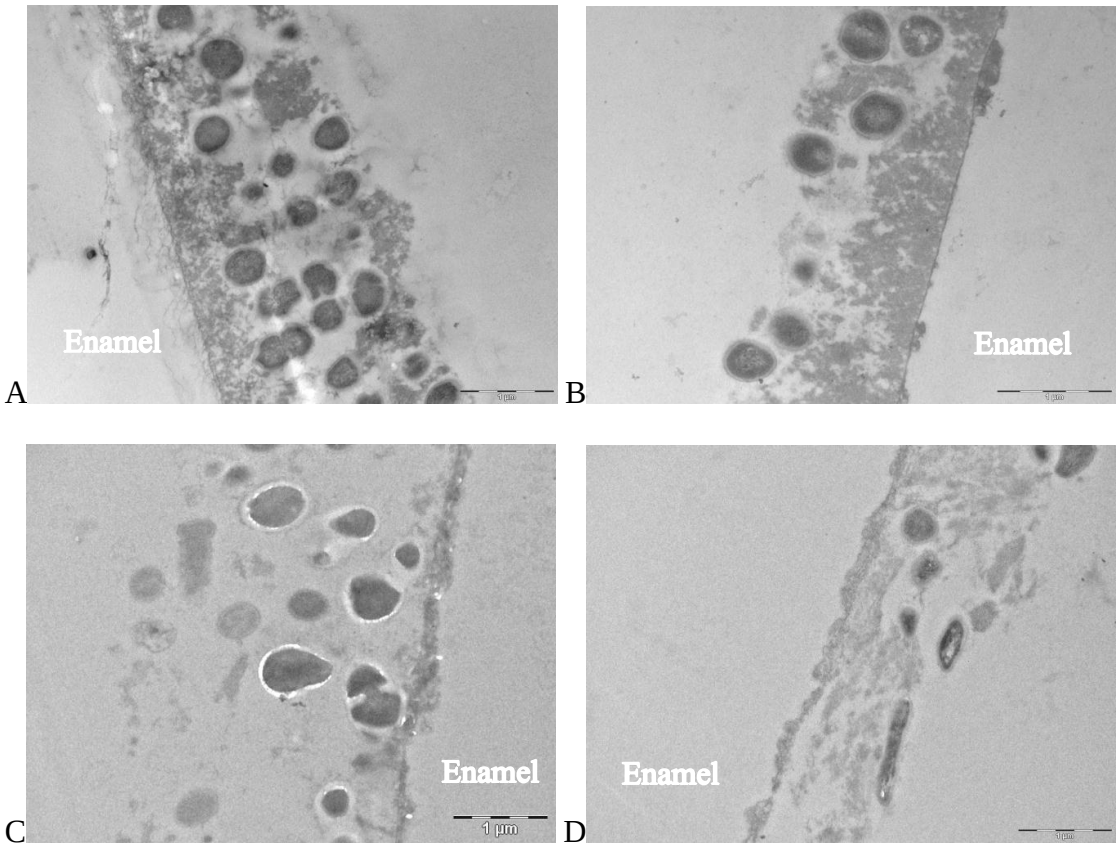



**Fig. 36** TEM micrographs: gallery of representative 24 h biofilm at immediately, 30min and 120min after rinsing with vinegar. (A) Control group; (B) After invention immediately; (C) After invention 30min; (D) After invention 120min. Original magnification: 23,000-fold.

### 4.2.2 Visualization of planktonic bacteria in saliva

The *Bac*Light™ viability assay showed that the quantity of bacteria in the saliva changed significantly after rinsing with vinegar (Fig. 37). Saliva samples of control groups contained large amounts of bacteria shapes such as spherical, rod, chain and rod and so on. Compared to the cocci, which dominated the 24-h biofilm on enamel, the bacteria in the saliva were dominated by rods or short rods like. The distribution of these bacteria was loose in saliva and relatively concentrated in the mucus due to uneven viscosity. Most of the bacteria were stained in green, while a small part of the bacteria was stained in red at 1,000 times magnification (Fig. 38). In the expectorate after vinegar rinsing, the amount of bacteria was drastically reduced either stained green or red. Saliva samples 30 min after vinegar rinsing showed that the number of bacteria was slightly higher than before, but mainly revealed red stained bacteria. Randomly, there were green colonies in the center of the cell mass. 120 min after vinegar rinsing, the total number of bacteria slightly increased compared to the 30-min sample, but was less than in the control group.

### 4.2.3 Measurement of salivary pH

The pH of the saliva samples was measured with pH test paper (Carl Roth GmbH +Co, Karlsruhe, Germany). The pH of the vinegar itself in this experiment was assessed to be 2.0, and the pH of the expectorate after vinegar rinsing was 2.3. 30 min after rinsing with vinegar, the oral pH was in the range of 5.5, and 120 min after rinsing with vinegar, the pH of the saliva was close to the physiological state (pH =6).

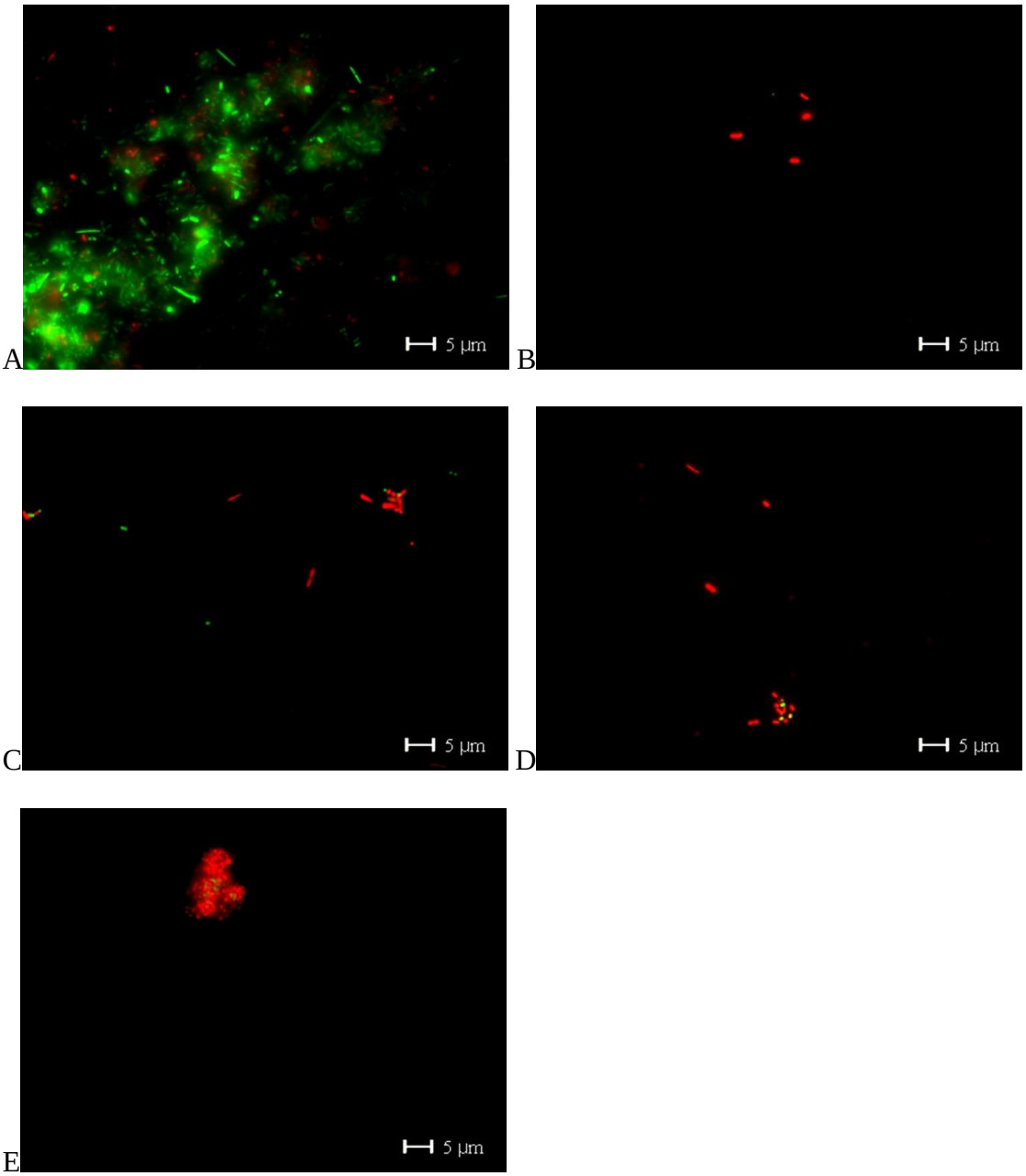
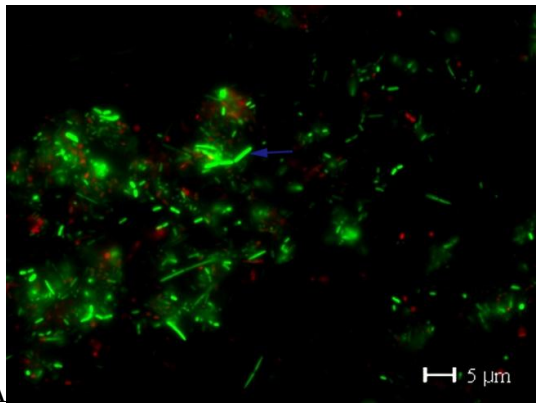



**Fig. 37** *Bac*Light™ viability assay: bacterial quantity in saliva changed significantly after vinegar rinsing. (A) control; (B) immediately after vinegar rinsing for 5 s without subsequent water rinsing; (C) expectorate of the second water rinsing after vinegar rinsing for 5 s; (D) 30 min after vinegar rinsing; (E) 120 min after vinegar rinsing. Original magnification: 1,000-fold.

A 

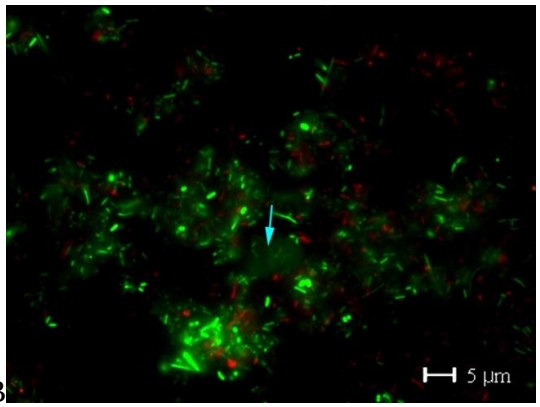
B 


**Fig. 38** *Bac*Light™ viability assay: an overview of saliva from the control group. (A) cocci and rods (dark blue arrow); (B) bacteria concentrated in the mucus (light blue arrow). Original magnification: 1,000-fold.

### 4.2.4 Semi-quantification of adherent bacteria in the *in situ* biofilm

#### 4.2.3.1 Total amount of bacteria

Salivary samples were stained with the *Bac*Light™ viability assay and measured regarding the gray value of G/R channel with Image J.

The total bacterial amount of the salivary samples was significantly different. Compared to the control group, expectorate immediately after vinegar rinsing for 5 s showed total bacterial amount reduced sharply (Mann-Whitney U test, $p < 0.01$), as well as 30min after rinsing, the total microbial amount in saliva were significantly reduced (Mann-Whitney U test, $p < 0.01$). While 120 min after vinegar rinsing, the total count of planktonic bacteria basically returned to the physiological level (Mann-Whitney U test, $p > 0.05$) (Fig. 39).

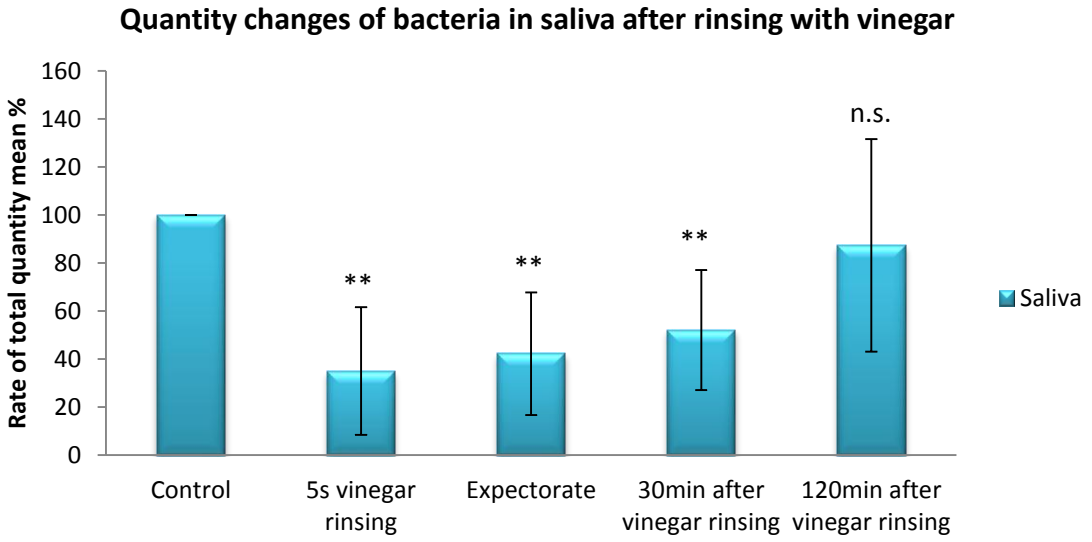



**Fig. 39** *Bac*Light™ viability assay for detection of the total relation amount of bacteria in saliva after vinegar rinsing at different times. Compared to controls set as 100%, a significant reduction of bacteria was shown over a period of 30 min after vinegar rinsing (Mann-Whitney U test, $p<0.01$). However, 120 after vinegar rinsing, there is almost the same quantity of bacteria as in the control group, mean ± SD. n.s.: not significant; **: significant for $p<0.01$.

#### 4.2.3.2 The live / dead bacterial colonization

Measured by *Bac*Light™ viability assay, it was possible to assess the influence of vinegar rinsing on the oral microecology of live/ dead adherent bacteria in the *in situ* biofilm and planktonic bacteria in saliva, respectively. Compared with the control group, the mortality of bacteria in the biofilm was significantly increased within 120 min after vinegar rinsing (Kruskal-Wallis test, $p<0.001$). Moreover, the killing rate of the bacteria was remarkably increased within 30 min after vinegar rinsing (Mann-Whitney U test, $p<0.05$). However, there was no significant difference in bacterial mortality between the last two time points (30 min after vinegar rinsing and 120 min after vinegar rinsing) (Mann-Whitney U test, $p>0.05$) (Fig. 40). Saliva samples also showed very significant mortality after vinegar rinsing (Mann-Whitney U test, $p<0.001$). Especially, directly after vinegar rinsing, the mortality immediately increased, and 30 min and 120 min after vinegar rinsing, the mortality remained at a high proportion, with no significant difference (Mann-Whitney U test , $p>0.05$) (Fig. 41).

After rinsing with vinegar, planktonic bacteria in saliva showed different sensitivities to the vinegar compared with biofilm bacteria. Figure 42 shows that the survival rate of bacteria in the biofilm was not significantly different from that of the planktonic bacteria in control groups (Mann-Whitney U test, $p> 0.05$), whereas in the vinegar groups the difference between planktonic bacteria and biofilm bacteria was remarkably significant (Mann-Whitney U test, $p <0.01$).

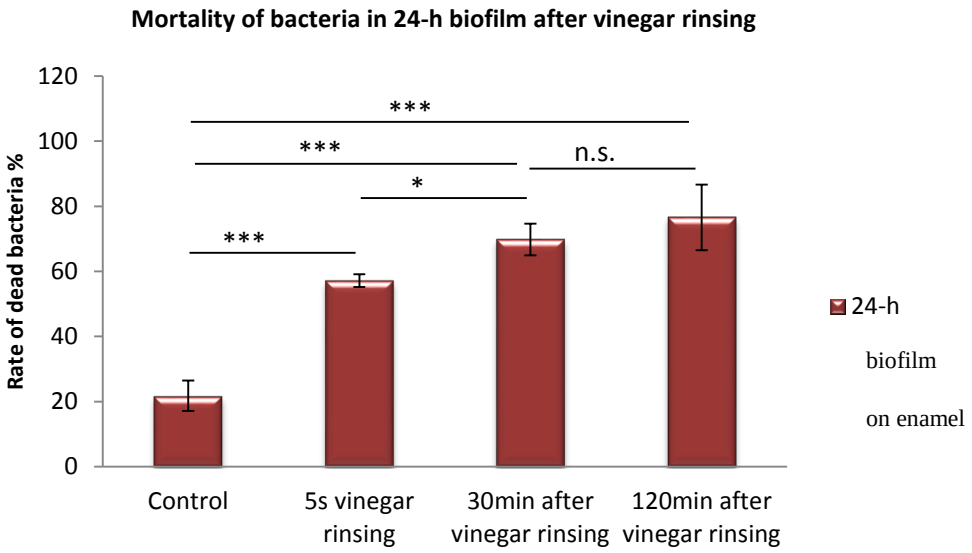



**Fig. 40** *Bac*Light™ viability assay for determination of bacterial mortality in 24 h biofilm before and after vinegar rinsing at different times. Enamel specimens were exposed for 24 hours. A significant increase of bacteria mortality was shown after vinegar rinsing for 5 s (Wilcoxon test, $p < 0.001$). Within 30 min after vinegar rinsing, the mortality of bacteria continued to increase significantly (Mann-Whitney U test, $p<0.05$).n.s.: not significant; *$p<0.05$; ***$p<0.001$

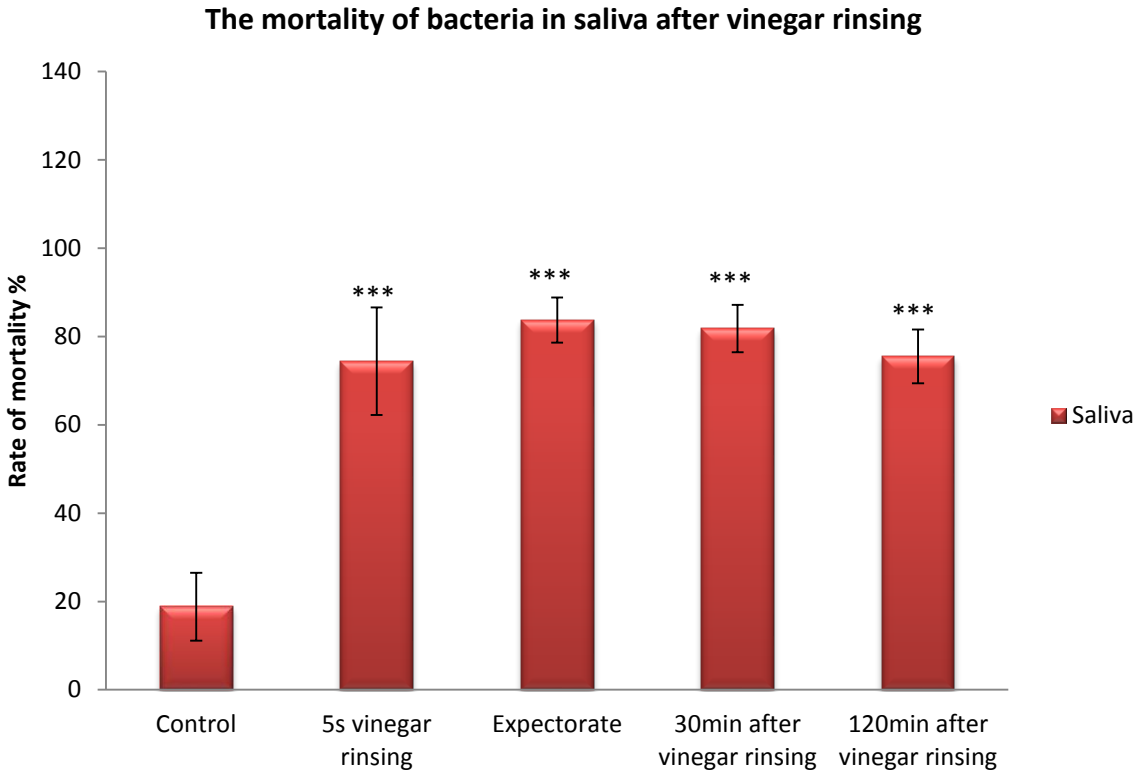



**Fig. 41** *Bac*Light™ viability assay for determination of bacteria mortality in saliva before and after vinegar rinsing at different times. Bacteria gray value was calculated by Image J. Statistical evaluation of bacterial death rate showed a significant increase in saliva (Mann-Whitney U test, $p<0.001$) after vinegar rinsing, mean ± SD.***$p<0.001$

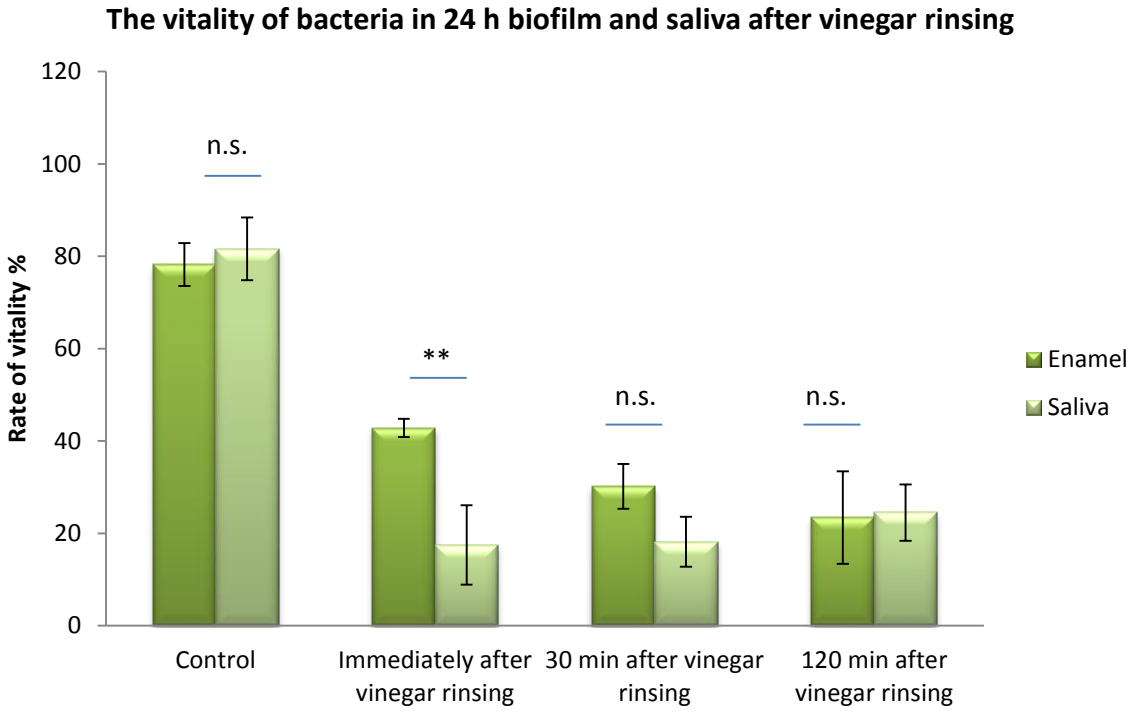



**Fig. 42** *Bac*Light™ viability assay for comparison of bacteria vitality rate between 24-h biofilm and saliva before and after vinegar rinsing. Bacteria gray value was calculated by Image J. A significant difference between biofilms on enamel and saliva was apparent immediately after vinegar rinsing (Mann-Whitney U test, $p<0.01$). n.s.: not significant; **: significant for $p<0.01$.

# 5 Discussion

## 5.1 Discussion of materials and methods

### 5.1.1 Adoption of bovine teeth

As an alternate to human teeth, bovine teeth were commonly used in biofilm studies [Hannig M et al., 2003; Hannig C et al., 2005a]. Bovine teeth are easy to obtain and similar to the physical and chemical properties of human teeth so that these are considered as an appropriate substrate for *in situ* experiments on oral biofilm formation [Hannig et al., 2007; Jung et al., 2010].

In the present experiments, bovine incisor enamel was selected as substrate in the *in situ* experiments to form the 3-min pellicle and 24-h biofilm.

### 5.1.2 Experimental design

#### 5.1.2.1 *In situ* biofilm model

Bioadhesion taking place in the oral cavity is difficult to simulate *in vitro*, which makes *in situ* experiments an irreplaceable role in studing oral biofilm formation [Hannig C and Hannig M, 2009]. Studying the effect of vinegar on the dynamic balance of the oral environment, in particular the salivary microflora requires consideration of a variety of factors. Thus, *in situ* experiments can reflect the actual situation of the oral environment, and allow investigation of the effects of vinegar under conditions closely related to clinical application.

#### 5.1.2.2 Removable minisplints

Minisplints are an appropriate tool for performing oral *in situ* experiments with fixed enamel specimens. Due to the complexity of the oral cavity, formation of biofilms at

different locations is different. However, minisplints can reproduce the position of enamel specimens in experiments at different stages, which makes the experiment more accurate [Hannig, 1997; Hannig M and Balz, 1999; Hannig, 1999].

#### 5.1.2.3 Experimental conditions

In the first part of this study, 3-min pellicle was selected as a model to investigate the effect of vinegar rinsing on the formation of initial biofilms.

In the second part, 24-h mature biofilms were selected as a model to investigate the influence of vinegar rinsing on mature existent biofilms.

### 5.1.3 *Bac*Light™ viability assay

Cellular membrane toxicity of fluorochromes stains bacteria to distinguish living cells or dead ones, which provides a quantitative visual impression of the proliferation or viability of the microflora [Auschill et al., 2001; Netuschil et al., 1998]. LDS was able to rapidly identify cell viability and is relatively simple to operate, which was used in many studies [Hannig et al., 2013a; Hannig et al., 2013c]. Therefore, the *Bac*Light ™ viability assay was used to observe the activity of bacteria in the biofilm and saliva after rinsing with vinegar.

### 5.1.4 Electron microscopic investigations

Scanning electron microscopes are ideal tools for observing the appearance and ultrastructure of biofilms [Hannig and Joiner, 2006]. SEM can provide a top view of the enamel surface to describe the status and distribution of bacteria at the biofilm surface. With the increase of magnification, the integrity and morphological changes of bacteria can be observed in detail. TEM provides a perfect method for analysis of ultrastructural and internal structures of biofilms. After the application of the vinegar, biofilm internal changes, thickness changes, etc., can be detected by TEM.

## 5.2 Discussion of results

### 5.2.1 Chronological sequence:

There are few investigations of vinegar application in the oral field. Most of the studies used acetic acid applied on the enamel surface for much longer times than 5 s; many of them are *in vitro* studies [de Castro RD et al., 2015; da Silva FC et al., 2008; Willershausen I et al., 2014; Meurman JH et al., 1996]. However, as a whole micro-ecological biotopes, many factors in the oral environment are mutually by rinsing affected solutions. The results of *in vitro* study have distinct differences as compared with *in situ* study. The present *in situ* experiments found that vinegar has the potential to inhibit the formation of biofilm in multiple ways.

The development of biofilms is a dynamic process [Marsh PD et al., 2009]. After formation of the pellicle, early bacterial colonizers will be transported by saliva to attach to the pellicle surface. With more and more bacterial adherence, the mature biofilm formed. The effect of vinegar on distinct stages in biofilm formation has been analysed in the present study.

Firstly, after contact with saliva and gingival crevicular fluid, the pellicle is formed on the cleansed tooth surface. The main composition of the pellicle are salivary glycoproteins, carbohydrates and lipid which have been examined by analytical techniques [Hannig M and Joiner A, 2006]. In the 3-min pellicle experiment, the control group presented the normal process of pellicle formation. The thickness of the globular layer increased uponthe 120-min experimental trial ($p<0.001$), which proves former research on pellicle formation [Hannig M, 1999; Hannig M and Joiner A, 2006]. In comparison, these particles layers were removed immediately due to vinegar rinsing, and were sparsely formed during the next 120 min after vinegar rinsing, which suggests that the formation of the pellicle was strongly reduced at least for 120 min. Therefore, vinegar presents substantial inhibition in the initial biofilm formation.

Second, after formation of the pellicle, the early bacterial will adhere to the pellicle surface [Marsh PD et al., 2009]. Early microbes (such as streptococci) attach to the pellicle surface by specific adhesins [Heller D et al., 2016; Gibbons RJ et al., 1991; Abeygunawardana C et al., 1991; Scannapieco FA et al., 1995; Murray PA et al., 1992; Ruhl S et al., 2004]. In the present experiments, bacteria can already be observed in the control group at 30 min and 120 min after initial 3-min pellicle formation via SEM and TEM, with many fimbriae firmly fixed on the pellicle surface, which proved the adherence of early bacterial colonies. Additionally, the morphological variations of bacteria are quite few with coccoids and rod-shaped bacteria dominating, which is consistent with the morphological characteristics of the early colonizers described in the literature [Heller D et al., 2016]. Moreover, the quantity of protein particles increased further after the formation of the pellicle for 30 min ($p<0.001$) as well as the frequancy of single microorganism adhering to the surface in the control group. However, in the vinegar rinsing group only the enamel surface covered by a thin pellicle with rarely visible protein particles or microbial attachment was observed. Additionally, the vast majority of microorganism in saliva was strongly affected by vinegar rinsing ($p<0.001$), which results in delay of the early microbial colonization process, thus hindering the formation of biofilm. Therefore, the vinegar altered the bacteria in both initial biofilm and saliva, leading to the inhibition of early biofilm growth.

Third, after colonized by early microbes, biofilms gradually accumulate a wide variety of other species of bacteria, which promotes growth of the biofilm. It has been reported that bacteria embedded in mature biofilm presented more tolerance to antibiotic than planktonic cells [Blanc V et al., 2014]. Therefore, these biofilm are difficult to be removed by antibiotic [Hwang G et al., 2017]. In the present study, vinegar presented a stronger antibacterial effect to planktonic bacteria in saliva than bacteria embedded in 24-h biofilm ($p<0.01$). This result proved that the mature biofilm could functionally protect the microbes better than saliva. Moreover, in the 24-h biofilm experiment, vinegar destroyed the biofilm structure significantly. Especially 30 min and 120 min after vinegar rinsing, the biofilm was almost wiped out, even the matrix was in part disrupted.

Actually, there are many studies of rinsing solutions used for oral biofilm management and removal, such as chlorhexidine, cetylpyridinium chloride and plant solutions [Dabholkar CS et al., 2016; Santos GOD et al., 2017; Pitten FA et al., 2001; Sreenivasan PK et al., 2013]. Among them, chlorhexidine is recognized as a golden standard [Jones CG, 1997]. However, more and more side effects were reported after widely clinical application of chlorhexidine rinsing, such as tooth staining, taste disturbance and even serious allergic reactions [Flotra L, 1973; Bahal S et al., 2017]. In comparison, vinegar present better advantages in clinical experience. As it is commonly to see in daily diet, vinegar is of higher safety than antibiotics, not to mention that vinegar could avoid the bacterial resistance. Therefore, vinegar illustrates a significant efficacy to manage and inhibit biofilm formation.

### 5.2.2 Spatial sequence

#### 5.2.2.1 Enamel

Enamel is the outer thick layer of the tooth crown mainly consisting of hydroxyapatite [Ichijo T et al., 1993]. It would be taken for granted that vinegar with extremely low pH value might destroy the enamel. Some *in vitro* study also revealed that applying vinegar for a couple of hours may cause dental erosion [Willershausen I et al., 2014; Meurman JH et al., 1996]. According to the daily diet habits, vinegar will not stay in the oral cavity for several minutes due to the deglutition. It has been reported that even the 3-min pellicle could protect the enamel from acid erosion for 60 s [Hannig M et al., 2004]. Therefore, short-term application of acid proved safety due to the protective effect of oral biofilm [Hannig C et al., 2009]. On the other hand, pure enamel is not exposed intraorally but always covered by biofilm and soaking in saliva in the oral cavity. So *in situ* experiments have better advantage in studying erosive proceses than *in vitro* studies [Wake N et al., 2016; Wiegand A et al., 2008].

In the present 24-h biofilm *in situ* study, the enamel showed scarcely any change after vinegar rinsing for 5 s due to the thick coverage and buffering effects of mature biofilm. This result proved that short period usage of acids will cause no damage of the enamel

surface [Hannig M et al, 2003]. In the 3-min pellicle *in situ* study, the subsurface pellicle layer below the enamel surface appeared immediately after vinegar rinsing for 5 s with less dense electron appearance as revealed by TEM. This indicated that vinegar could substantially promote the formation of the subsurface pellicle layer, which could prevent further demineralization [Hannig M and Hannig C, 2014; Hannig C et al., 2009]. As recent studies of Hannig et al. showed that even the 3-min pellicle could protect the enamel surface from 60-s acid effects, and even after 5-min acid rinsing, pellicle residue still could be detected [Hannig M et al., 2004; Hannig M et al., 1999a].

The enamel demineralization happens frequently due to many factors, i.e., the physical and chemical structure, the composition and pH as well as the chemical balance between enamel and solution [Nancollas, 2005; Dorozhkin, 2012]. In the process of enamel erosion, early-stage surface interactions would soften the enamel to a few micrometers [Imfeld, 1996; Finke et al., 2000; Addy and Shellis, 2006; Cheng et al., 2009], and the permanent loss of the enamel structure would happen after longer erosive conditions [Addy and Shellis, 2006; Cheng et al., 2009]. In the oral cavity, saliva as well as the biofilm have buffer capacity for extreme pH values [Hannig M et al., 2014; Garc á-Godoy F et al., 2008; Martins C et al., 2013]. For these reason, vinegar which can reach the enamel surface through saliva and biofilm has been diluted and will cause only scarce erosive effects, as indicated in the present study.

Moreover, the subsurface pellicle layer turned to appear more shallow 120 min after vinegar rinsing as well as the electron density presented much higher, which indicates that additional adsorption of salivary proteins and remineralization happened. According to previous studies, the remineralization period is 1-4 h in consciousness time and 6-8 h in sleeping time [Lippert F et al., 2004], which may be related to a decrease in saliva secretion during sleep leading to the reduction of remineralization efficacy [Schneyer LH et al., 1956]. Remineralization will be established within 2 h, yielding no difference as compared to 24-h remineralization time [Eisenburger M et al., 2001].

Therefore, 5-s vinegar rinsing caused only very minimal damage to the enamel surface, promoting formation of the protective subsurface pellicle layer that protects the enamel against further demineralization [Hannig M and Hannig C, 2014]

#### 5.2.2.2 Saliva

As an important component of the oral micro ecology, saliva plays a substantial role in forming oral biofilms and maintaining tooth integrity. Saliva contributes to the remineralization, supersaturated with calcium, phosphate and fluoride [Buzalaf MAR et al., 2012; Rios D et al., 2006]. Also, saliva contains more than a thousand of different proteins resulting in the formation of the pellicle on enamel surface [Denny P et al., 2008; Hannig and Joiner, 2006]. Afterwards, saliva transfers microbes to the pellicle and promotes the development of biofilms [Jakubovics NS, 2015]. In the 3-min pellicle experiment, the *in situ* pellicle developed with bacterial adherence 120 min after water rinsing. However, there was no bacterial attachment 120 min after vinegar rinsing. In the 24-h biofilm experiment, the thickness of the biofilm reduced gradually as well as the bacteria in saliva were reduced after vinegar rinsing over a period of 120 min, which proved that some functions of saliva could be influenced by vinegar such as bacterial reservoir and contribution to biofilm formation.

Additionally, saliva is a good buffer in the oral cavity due to bicarbonate concentration and salivary flow rate [Bardow A et al., 2000; Wikner S et al., 1994]. This buffer capability takes an important part to maintain the pH value of saliva [Bardow A et al., 2000]. In the present experiment, the characteristic of the expectorate is of great difference immediately after vinegar rinsing (pH=2.3) compared with the control saliva (pH=7.0). The control saliva was a very mucus-rich secretion, while the vinegar expectorate was only a watery fluid essentially devoid of mucus. This suggested that vinegar might change the characteristic of saliva. However, vinegar causes inhabitation of biofilm formation as well as biofilm removal, which means that there are some more influencing factors beside the pH value. It has been supposed that salivary flow rate will be increased already before the acid rinsing, which improves the buffer capacity

resulting in a protective effect due to the effective dilution of acids [Hara AT et al., 2014].

#### 5.2.2.3 Extracellular polymeric substances (EPS)

EPS plays an important role in maintaining the balance of biofilms [Wingender J, 2002]. Ectoenzymes and extrinsic protein in EPS can provide nutrients for embedded bacteria in the biofilm to improve its survival rate, which degrade unhealthy cells towards inside of biofilm and digest particle foreign matter towards the outside[Rice AR et al., 2003; Stoodley P et al., 2001]. Moreover, EPS can maintain the mechanical stability of the biofilm and generate a gradient of pH and oxidation reduction potential, which ensures the survival of bacteria under harsh conditions [Flemming HC, 2011]. After vinegar rinsing, however, the biofilm matrix was destroyed, while the connection between the bacteria was still visible under the surface, which indicates that EPS can buffer the low pH environment, in order to preserve the embedded microorganisms in the biofilm. To sum up, this damage of the mechanical integrity of the biofilm can lead to a decrease in the bond strength or tensile strength of the biofilm, resulting in the dissolution of the densely connected bacterial community into small colonies, even into single bacteria, in order to achieve an antimicrobial effect.

#### 5.2.2.4 Microbiota

So far, there are over 700 species of bacteria detected in the healthy oral cavity with high diversity and subject specificity [Aas JA et al., 2005; Kolenbrander PE, 2000]. In the oral cavity, bacteria exist in two modes, first is the planktonic mode in saliva, and second is the embedded mode in the biofilm coating formed on all kinds of oral surfaces, such as teeth and mucosal surfaces [Simón-Soro A et al., 2013]. Normally, bacteria in saliva derive from the detachment of oral biofilms [Marsh PD et al., 2006]. However, most oral bacteria exist in biofilms, with more complex characteristics [Peterson SN et al., 2014]. It has been reported that the bacterial species in saliva present significant distribution within the biofilm [Mager DL et al., 2003]. In the present experiment, the salivary bacteria were almost completely killed and removed immediately after vinegar rinsing, while in the next 120 min, the amount of bacteria (basically all dead) were

increased. This might be explained by the fact that bacteria were detached and mobilized from biofilm after vinegar rinsing, increasing the number of bacteria in the oral fluid.

In order to overcome oral diseases associated with inflammation, antibacterial effects are the main direction, with antibiotics as the prevalent route [Pitten FA et al., 2001; Sreenivasan PK et al., 2013; Santos GOD et al., 2017]. Chlorhexidine was used as a gold standard considering antibacterial effects [Jones CG, 1997], which has been proved by the bactericidal effect and biofilm removal [Malhotra R et al., 2011; Santos GOD et al., 2017]. However, as other microbicidal agents do, chlorhexidine has many side effect with the worst case being fatal allergy [Flotra L, 1973; Bahal S et al., 2017]. In comparison, vinegar has a significant bactericidal effect as well as biofilm removal properties which has been proved by the present experiments. In the 24-h biofilm study, both bacteria embedded in the biofilm and planktonic microbes in saliva were killed after vinegar rinsing for 5 s. Moreover, this antibacterial efficacy is not transient but long lasting at least for 120 min. Most of the research on the antimicrobial efficacy of chlorhexidine was applied for 30-s to 60-s rinsing [Johnson NR et al., 2015; Lakade LS et al., 2014; Malhotra R et al., 2011]. By contrast, vinegar presents sufficient antibacterial effect by rinsing for only 5 s, which indicates that vinegar has a wide potential for further application in prevention or treatment of bacteria- caused oral diseases.

As a natural product, vinegar has higher safety than antibiotics [Budak NH et al., 2014]. Compared to vinegar, antibiotics have much more side effects [Singh R et al., 2014; Alzoubi K et al., 2013]. Additionally, long-term use of antibiotics can cause drug resistance resulting in protracted course of disease [Kubicek-Sutherland JZ et al., 2015; Vranakis I et al., 2014; Chait R et al., 2016]. For example, periodontitis reveals a high tare of recurrence even after treatment by periodontal maintenance therapy which includes surgical or non-surgical procedures plus antibiotics [Renvert S et al., 2004; Axelsson P et al., 2004; Lorentz TCM et al., 2009; Costa FO et al., 2014; Costa FO et al., 2011]. Yet, it has been reported that the recurrence of periodontitis is related to the application of antibiotics [Serino G et al., 2001]. Therefore, due to its safety and antimicrobial effects, vinegar might be used in the chronic inflammatory disease treatment, such as recurrence of periodontitis.

In the present study, some of the bacterial cell membranes were destructed after vinegar rinsing as seen in TEM figures. One explanation might be the low pH value (vinegar 5%; vinegar expectorate pH 2.3) after vinegar rinsing exceeding the tolerance limit of most oral bacteria [Huang CB et al., 2011]. Some other researches believed that the underlying mechanism might be undissociated acetic acid rather than the acidity of the vinegar [Halstead FD et al., 2015]. Non-ionised acetic acid might more readily cross the cell membranes leading to collapse of the proton gradients [Salmond C V et al., 1984; Walter A et al., 1984]. Due to the internal pH of the bacterial cell (around pH7.6) is higher than that of acetic acid outside the cell, the internalized acid will dissociate and damage the bacterial cell membrane and DNA [Hirshfield IN et al., 2003; Slonczewski JL et al., 1981; Slonczewski JL et al., 2009; Lund P et al., 2014].

On the other hand, the composition of vinegar is complex including acetic acid, gallic acid, catechin, etc. [Budak NH et al., 2014]. Apart from acetic acid, gallic acid has antimicrobial effect as well [Lee DS et al., 2014; Lu J et al., 2016]. Likewise, catechin also was reported as an active agent due to its antibacterial effects [Zhang H et al., 2016; Miklasińska M et al., 2016]. Therefore, the significant bactericidal efficacy of vinegar could be a multi-causal effect.

Additionally, more and more studies pointed out that bacteria embedded in the biofilm have stronger resistance to antibiotics [Penesyan A et al., 2015; Van Acker H et al., 2014], which could be proved in the 24-h biofilm experiment. The mortality rate of bacteria in the mature biofilm was lower than that in saliva ($p<0.001$). In saliva, there are almost no bacteria (no matter alive or dead) detectable immediately after vinegar rinsing for 5 s, while in the 24-h biofilm, only the outer layer of attached bacteria was dead. However, this antimicrobial effect was lasting for at least 120 min, which indicated that vinegar might be applied in clinical treatment of chronic inflammation.

Furthermore, fluoride toothpastes are widely used nowadays, and vinegar rinsing might provide a good combination with fluoride toothpastes. The short-term acidic condition due to vinegar rinsing could promote the effect of fluoride on the enamel surface [Kondo KY et al., 2016; Cardoso CA et al., 2015]. At low pH, the fluoride can produce lipophilic

HF, which can easily pass through the bacterial cell membrane. Due to self-protective mechanism, the internal pH of bacterial cells is higher than the extracellular pH value. After entering into the cell, HF will be dissociated into $H^+$, which can inhibit the metabolism of bacterial cells, and break down the pH gradient so as to inhibit the bacterial activity. On the other hand, $F^-$ can reduce the glycolysis by inhibiting the enolase, and inhibit intracellular glycogen synthesis as well [Kondo KY et al., 2016; Rošin-Grget K et al., 2013]. Although tooth brushing was prohibited in the present experiment, vinegar rinsing would probably double the protective effect of fluoride on the enamel surface.

Therefore, the impact of vinegar on the oral environment is not simply an antimicrobial effect, but also regulation of the proportion of bacteria to reconstruct the homeostasis of the oral micro-ecosystem, which might be of high significance for the future research on oral disease prevention and control.

## 5.3 Conclusion

In conclusion, this *in situ* study has demonstrated the potential of vinegar rinsing for management of biofilm formation in the oral cavity. Interestingly, pellicle formation is strongly affected due to vinegar rinsing by reduction of the outer globular layer. Vinegar has the potential to kill most of the bacteria in saliva, contributing to postponement of biofilm formation. Although the effects of vinegar rinsing were significant in this study, the long-term clinical efficacy required further investigations.

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