Aus der Klinik für Zahnerhaltung, Parodontologie und Präventive Zahnheilkunde,

Universitätsklinikum des Saarlandes, Homburg/Saar

Direktor: Prof. Dr. M. Hannig

Chlorhexidine retention in the oral cavity and the effects of chlorhexidine and octenidine mouth rinsing on the dental biofilm

Dissertation zur Erlangung des Grades eines Doktors der Medizin und der Naturwissenschaften (MD PhD)

der Medizinischen Fakultät

der UNIVERSITÄT DES SAARLANDES

2021

vorgelegt von:

Bashar Reda

geb. am: 02.01.1982 in Aleppo/Syrien

Tag der Promotion: 25.März.2021

Dekan: Prof. Dr. M.D. Menger

Berichterstatter: Prof. Dr. M. Hoth

Prof. Dr. M. Hannig

Dr. A. Beck

Dedication

This thesis is dedicated to the memory of my father

To my family for all the love, encouragement, and support

To my wife, my Soul Mate and Life Partner

To my beautiful homeland Syria and my beloved city Aleppo

Abbreviations:

a.m.	ante meridiem (before midday)
°C	Celsius scale
CHCA	α-cyano-4-hydroxycinnamic acid
CHX	Chlorhexidine
e.g.	exempli gratia
et al.	et alia
etc.	et cetera
FM	Fluorescence Microscopy
HPLC	High-Performance Liquid Chromatography
h	Hour
i.e.	*id est*, in other words
g	Gram
g	Gravitational force
kV	Kilovolt
LDS	LIVE/DEAD staining
MS	Mass Spectrometry
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
µl	Microliter
µm	Micrometre
mg	Milligram
ml	Millilitre

mm	Millimetre
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
min	Minute
nm	Nano meter
ns	Not significant
OCT	Octenidine
ppm	Parts per million
PI	Propidium Iodide
Rel. Int	Relative intensity
s	Second
SLS	Sodium Lauryl Sulphate
SPME	Solid Phase Micro Extraction
M	Molar
mm^2	Square millimetre
SD	Standard Deviation
TEM	Transmission Electron Microscopy

Content

1 ASTRACT…….…….... 01

1.1 English……..……..... 01

1.2 German……..……..... 03

2 INTRODUCTION……..……..……..... 05

2.1 Review of literature……..……..……..... 06

2.1.1 Dental pellicle…….……...……..... 06

2.1.2 Dental biofilm……..……...……... 08

2.1.3 Dental biofilm management…….…..……... 10

2.1.4 Chlorhexidine……...…….……..... 11

2.1.5 Octenidine…….……..... 16

2.1.6 Comparison between chlorhexidine and octenidine…….…..……... 19

2.2 Aim of this work……... 21

3 MATERIALS AND METHODS……..……... 22

3.1 Chlorhexidine retention studies…….……... 22

3.1.1 Studies population…….……..……...……... 22

3.1.2 Studies design……..……..……... 22

3.1.3 Calibration curve of chlorhexidine…….……... 24

3.1.4 MALDI-TOF mass spectrometry……...……... 24

3.1.5 Statistical analysis…….…….……... 25

3.2 Anti-biofilm study…….……..... 26

3.2.1 Study population……... 26

3.2.2 Study design……..……..…….... 26

3.2.3 Specimens and splint preparation…….…….... 27

3.2.4 Vital fluorescence microscopy evaluation - LIVE/DEAD staining……...…….... 28

3.2.5 Transmission electron microscopy evaluation……..…….....…….... 30

3.2.6 Statistical analysis……..…….... 31

4 RESULTS…….....……..... 32

4.1 Retention studies results……..... 32

4.1.1 Chlorhexidine calibration curve…….…….... 32

4.1.2 Chlorhexidine retention in different oral locations after mouth rinsing……..... 34

4.1.3 Chlorhexidine retention after application of different pharmaceutical regimens..... 37

4.2 Anti-biofilm study results……..…….... 42

4.2.1 Chlorhexidine and octenidine effects on biofilm formation……..…….... 42

4.2.2 Chlorhexidine and octenidine disruption effect on mature biofilm…… 51

5 DISCUSSION……...…….... 59

5.1 Discussion of materials and methods……..... 59

5.1.1 Experimental design……..…….... 59

5.1.2 MALDI-TOF evaluation……... 62

5.1.3 Florescence microscopy evaluation after LIVE/DEAD staining…….…….... 63

5.1.4 Electron microscopic investigation……... 65

5.1.5 Statistical analysis……..…….... 65

5.2 Discussion of results.…….…….... 68

5.2.1 Discussion of retention results……..…….... 68

5.2.2 Discussion of anti-biofilm results……..……..... 73

5.3 Conclusions…….……..... 80

5.3.1 Conclusion of retention studies……..…….…….. 80

5.3.2 Conclusion of anti-biofilm study……..…… 80

6 REFERENCES…….……..... 81

7 PUBLICATIONS AND ACKNOWLEDGEMENTS……...……..... **97**

7.1 Publications……...…….…….…….….... 97

7.2 Acknowledgements……...……..... 99

1 Abstract

1.1 English

Introduction: Dental biofilm is the main reason for several oral diseases such as caries and periodontitis. Therefore, there is a high demand for preventative strategies such as the use of chemical agents for the control of dental biofilm. As a consequence, it is always a priority interest and an important research area in the field of dentistry to evaluate and compare the effectivity of different antibacterial agents and to investigate the retention, stability, and efficacy of different treatment regimens.

Objective: This *in situ* study aims to determine the retention of chlorhexidine in different oral locations after the application of various chlorhexidine regimens. Furthermore, it seeks to investigate and compare the anti-biofilm efficacy of chlorhexidine (CHX) and octenidine (OCT) mouth rinses.

Material and Methods: Five volunteers were recruited for this work. In the first retention experiment, the samples were taken with micro-brushes from the interdental area, buccal dental pellicle, anterior labial and posterior buccal mucosa, as well as saliva at five-time points within 24 h after mouth rinsing with 10 ml of 0.2% CHX for 30 s. In the second retention experiment, the volunteers used different CHX treatment regimens from mouth rinses, dental spray, or toothpaste. After application, 2 µl samples were taken from the saliva and buccal mucosa pellicle in addition to the dental pellicle sample formed on a standardized enamel surface. The samples were taken at six-time points within 12 h. Retention of CHX in the aforementioned experiments was measured using Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry.

In the second part of this work, biofilms were formed in the anti-biofilm experiment *in situ* on bovine enamel surfaces fixed to individual acrylic splints. For biofilm formation analysis, the subjects intraorally exposed the splint for 48 h. Mouth rinses were done using 10 ml of 0.1% CHX, 0.2% CHX, 0.1% OCT, or water as control. Each rinsing was performed every 12 h for 30 s. Biofilm colonization and bacterial vitality were investigated at 24 and 48 h. For analysis of biofilm disruption activity, the evaluation of 48 h-mature biofilms was performed before and directly after the first CHX rinse in both concentrations or OCT rinse and repeated 24 h later for the 72 h-mature biofilms. The coverage, vitality, and ultrastructure assessments of biofilm samples were performed by fluorescence microscopy and transmission electron microscopy.

Results: A significant reduction of the intraoral CHX concentration was detected during the first 6 h. Thereafter, CHX retained in the oral cavity at low concentrations of micrograms per millilitre levels for 12 h after application. CHX was even detected 24 h after oral rinsing. CHX retention in the oral mucosa and dental pellicle was significantly higher than the retention in saliva. The retention of CHX after mouth rinsing or spray application was higher compared to the retention after the use of toothpaste.

Rinses with 0.1% CHX, 0.2% CHX, or 0.1% OCT caused a significant reduction in the biofilm formation on enamel surfaces. Bacterial colonization and vitality were both clearly reduced. Moreover,

a remarkable biofilm disruption was observed due to the application of CHX or OCT rinses. Interestingly, only one application of CHX or OCT to a 48-h mature biofilm induced biofilm ultrastructure modification and significantly reduced the biofilm thickness and microbial vitality.

Conclusion: It was found in the present work that the MALDI-TOF technique provides excellent quantification limits for the CHX determination. It allows the detection of CHX without the need for time-consuming sample preparation. The obtained results indicate that dental pellicle and oral mucosa are the essential reservoirs of CHX in the oral cavity. With time, the CHX is desorbed from the oral surfaces toward saliva to produce a persistent bacteriostatic effect in the whole oral cavity. Moreover, the results show that there are significant differences in CHX retention after the application of different CHX regimens; mouth rinses, dental spray, and toothpaste.

It also was concluded in this work that the mouth rinses based on CHX in both concentrations 0.1% and 0.2% as well as 0.1% OCT have a significant effect on reducing biofilm formation confirmed through the reduction of the oral bacterial colonization and biofilm vitality on native enamel specimens. Additionally, this study demonstrates an important biofilm disruption effect of the CHX and OCT rinsing, since they were effective to dislodge pre-existing biofilms, even in the total absence of mechanical biofilm management. However, 0.1% OCT showed significantly better results in anti-biofilm activity in comparison with CHX at the same concentration.

1.2 German

Einleitung: Der bakterielle Biofilm ist die häufigste Ursache oraler Erkrankungen wie Karies und Parodontitis. Präventive Strategien zur Kontrolle des Biofilms beinhalten neben der mechanischen Plaqueentfernung insbesondere die Anwendung chemischer Substanzen. Ein besonderes Interesse der zahnmedizinischen Forschung liegt in der Bewertung und dem Vergleich der Wirksamkeit, der Retention und der Stabilität verschiedener antimikrobieller Wirkstoffe.

Ziel: Diese *in situ*-Studie hat zum Ziel, die Retention von Chlorhexidin in verschiedenen oralen Regionen nach Anwendung unterschiedlicher Behandlungsschemata zu bestimmen. Darüber hinaus soll die Anti-Biofilm-Aktivität von Chlorhexidin (CHX) und Octenidin (OCT)- Mundspülungen untersucht und miteinander verglichen werden.

Material und Methoden: An dieser Studie haben fünf Probanden teilgenommen. Für das erste Retentionsexperiment haben die Probanden 10 ml 0,2% CHX als Mundspüllösung intraoral für 30 s appliziert. Anschließend wurden Proben aus dem Interdentalbereich, der bukkalen Dentalpellikel, der vorderen labialen und hinteren bukkalen Mukosa sowie dem Speichel mit einer Mikrobürste an fünf verschiedenen Zeitpunkten innerhalb von 24 h entnommen. Im zweiten Retentionsexperiment verwendeten die Probanden verschiedene CHX-Applikationsformen wie Mundspülungen, Mundspray und Zahnpasta. Nach der Anwendung wurden Proben an sechs Zeitpunkten innerhalt von 12 h gesammelt. Zusätzlich zu der Dentalpellikel auf Schmelzprüfkörpern wurden je 2 µl Probe vom Speichel und der bukkalen Mukosa entnommen. Die Retention von CHX wurde in den oben genannten Experimenten mittels Matrix-unterstützter Laser-Desorption/Ionisation-Flugzeit (MALDI-TOF) Massenspektrometrie gemessen.

Im zweiten Teil dieser Studie, einem Anti-Biofilm-Experiment, erfolgte die Biofilmbildung *in situ* auf bovinen Schmelzprüfkörpern. Diese Prüfkörper wurden an Kunststoffschienen fixiert und für 48 h intraoral exponiert. Für Mundspülungen wurden je 10 ml 0,1% und 0,2% CHX sowie 0,1% OCT oder Wasser als Kontrolle verwendet. Die Applikation der Testlösung fand alle 12 h für 30 s statt. Die Biofilmbildung und die bakterielle Vitalität wurden nach 24 h und 48 h evaluiert. Um den Einfluss der CHX- und OCT-Mundspülung auf den reifen Biofilm zu beurteilen, wurde der Biofilm auf bovinen Schmelzproben für 48 h gebildet. Danach wurde die Mundspüllösungen 0,1% und 0,2% CHX oder 0,1% OCT intraoral appliziert. Die zweite Applikation der getesteten Substanzen erfolgte nach weiteren 12 h. Die Biofilm-Ultrastruktur und die bakterielle Vitalität wurden nach 48 h direkt vor und nach dem Spülen sowie nach 72-h intraorale Expositionszeit evaluiert. Die Vitalität des Biofilms wurde nach der Vitalfärbung fluoreszenzmikroskopisch analysiert. Zusätzlich wurde die Ultrastruktur des Biofilms mittels Transmissionselektronenmikroskopie untersucht.

Ergebnisse: Die stärkste Abnahme von CHX erfolgte während der ersten 6 h, danach sank die Konzentration in geringerem Maße ab. CHX war in der Konzentration von einem Mikrogramm pro Milliliter für mindestens 12 h nachweisbar und konnte sogar 24 h nach der Anwendung nachgewiesen

werden. Die CHX-Retention in der Mukosa und in der Dental pellikel war signifikant höher als im Speichel. Die CHX-Retention nach der Mundspülung oder der Sprayapplikation war wesentlich höher als nach der Verwendung der Zahnpasta.

Spülungen mit 0,1% CHX, 0,2% CHX oder 0,1% OCT führten zu einer deutlichen Reduktion der Biofilmbildung auf dem Zahnschmelz. Sowohl die Besiedlung als auch die Viabilität der Bakterien waren signifikant beeinträchtigt. Darüber hinaus wurde eine starke Disruption des Biofilms durch die CHX- oder OCT-Spülungen beobachtet. Bemerkenswerterweise bewirkt bereits eine einmalige Anwendung von CHX oder OCT bei einen 48 h alten Biofilm eine substantielle Veränderung der Ultrastruktur und induziert eine Reduktion der Biofilmdicke und der bakteriellen Vitalität.

Schlussfolgerung: Die vorliegende Studie zeigte, dass die verwendete MALDI-TOF Methode zur CHX-Bestimmung hervorragende Quantifizierungsgrenzen bietet und eine schnell durchführbare CHX-Quantifizierung ohne zeitaufwendige Probenvorbereitung erlaubt. Die Ergebnisse weisen darauf hin, dass die Mukosa und die Pellikel als das Hauptreservoir für CHX fungieren. Durch eine kontinuierliche Freisetzung von CHX in den Speichel können diese Reservoirs den bakteriostatischen Effekt von CHX in der Mundhöhle verlängern. Jedoch gibt es signifikante Unterschiede in der CHX-Retention zwischen der Mundspülung, dem Mundspray und der Zahnpasta.

Im Rahmen dieser Studie wurde auch gezeigt, dass das Spülen mit CHX in beiden Konzentrationen 0,1% und 0,2% sowie mit 0,1% OCT die Entstehung des bakteriellen Biofilms stark hemmt. Darüber hinaus wurde ein wichtiger Disruptionseffekt der CHX- und OCT-Spülungen auf den reifen Biofilm demonstriert. Allerdings zeigte 0,1% OCT signifikant bessere Ergebnisse bei der Anti-Biofilm-Aktivität im Vergleich zu CHX bei gleicher Konzentration.

2 Introduction

Dental biofilm is the main cause of several oral diseases, such as caries (TAKAHASHI, NYVAD, 2008; COLOMBO, TANNER, 2019) and periodontal diseases (LÖE et al., 1965; LARSEN, FREIHN, 2017). These diseases are common in the world and still a public health issue for a large part of the world's population (BURT, 1998). Thus, there is a strong need for primary preventive strategies. The keystone of these strategies is the control of dental biofilm. The most widely used method for dental biofilm control is mechanical cleaning. The standard tools used for mechanical cleaning are the toothbrush (manual or electric), floss, and interdental brushes. However, mechanical cleaning is efficient only in places, where access to biofilm sediments is possible. Additionally, the majority of the patients do not have the degree of motivation and skills needed even for the regular oral hygiene procedures (LINDHE, KOCH, 1967). Therefore, acceptable biofilm management by mechanical measures is hard to be achieved by most persons, especially by individuals with periodontitis, who have deep periodontal pockets, and disabled persons, for whom appropriate oral hygiene is a non-realistic goal (LOUISE et al., 2017). Therefore, supportive treatment for biofilm management would be required.

In this context, antibacterial products are often described in situations where oral hygiene measures are difficult, compromised, or impossible (ADDY, MORAN, 1997). In other words, the use of chemically active substances can be a valuable tool for biofilm control when the manual procedures are not used long enough or on a regular daily basis to achieve the appropriate elimination of dental biofilm (MANDEL, 1988; TAKENAKA et al., 2019). The major goal of the antiseptics is to preserve, enhance, or even replace both preventive and therapeutic mechanical measures (TEN CATE, MARSH, 1994).

To enhance the use of these agents, several studies have been done and techniques have been used to evaluate different antiseptics. These studies either determine the agents' retention and stability after application or to evaluate the antimicrobial effects on dental biofilm. Additionally, experimental researches were performed to obtain more information about the behaviour and mechanism of action behind the efficacy of oral antiseptics.

Therefore, the aim of this work was to determine the retention of the gold standard chlorhexidine in different oral locations, especially after the application of different chlorhexidine formulations. Additionally, we aimed to evaluate and compare the anti-biofilm efficacy and biofilm disruption activity of two antimicrobial agents, chlorhexidine and octenidine.

2.1 Review of literature

2.1.1 Dental pellicle

2.1.1.1 Formation of the pellicle

After tooth surface cleaning, by way of tooth brushing or professional prophylaxis, salivary macromolecules begin to adsorb at the dental surface immediately. This adsorption will form a conditioning film which termed the acquired pellicle (HANNIG, 1999; LANG et al., 2008). The selective adsorption of salivary proteins on the tooth surface plays an essential role in building the thin film of an acquired pellicle (LENDENMANN et al., 2000). Previously, it was believed that the pellicle film is basically from the embryologic origin (NASMYTH, 1839). However, it was discovered in the middle of the 20th century, that the pellicle is acquired after tooth eruption. Since then, it has been stated that the embryologic membrane is lost after the eruption of the teeth and is replaced by an acquired coating that mainly consists of positively charged groups of salivary proteins (TRAUTMANN et al., 2020).

2.1.1.2 Chemical and ultrastructural composition of the pellicle

The experimental studies which are investigating the pellicle composition are hampered due to the small amount of dental pellicle which can be collected and analysed (HANNIG, JOINER, 2006). The early obtained results allowed concluding that the dental pellicle is mainly conducted from salivary glycoproteins (mucins). Later, improved techniques for harvesting dental pellicle have been introduced. These methods combined both mechanical and chemical harvesting of the pellicle which permitted the determination of a lot of salivary and fewer non-salivary elements as ingredients of the dental pellicle (Al-HASHIMI, LEVINE, 1989; CARLEN et al., 1998; TRAUTMANN et al., 2019).

Furthermore, Carlen and co-workers showed that there is a high similarity in the protein profile between the dental pellicles from different parts of the oral cavity and the locally available salivary biopolymers (CARLEN et al., 1998). These results indicate that the locally salivary protein patterns play an essential role in the formation and composition of the dental pellicle layer. However, it was found also that there are some differences in the fatty acid profile between saliva and the dental pellicle. These findings indicate that the formation of the dental pellicle is a highly selective process and lipids available in saliva are not adsorbed equivalently to the pellicle layer (REICH et al., 2013).

Regarding the chemical composition, the pellicle samples consist of 46% amino acids, 14% total carbohydrates, and 2.7% hexosamines in addition to salivary α-amylase, lysozyme, immunoglobulins, and mucins (ARMSTRONG, 1967; AL-HASHIMI, LEVINE, 1989). Recently, a total of 1188 different proteins were identified on only 3 min old initial human pellicle (TRAUTMANN et al., 2019). Furthermore, with time, there are significant qualitative and quantitative proteome changes during the acquired enamel pellicle formation. In the end, the acquired pellicle will play an important role in the formation of dental biofilms (LEE et al., 2013).

Little information is available about lipids in the dental pellicle although they formed almost 22% of the pellicle's dry weight (HANNIG, JOINER, 2006). In general, glycolipids are the main lipid portion of the pellicle which in turn consider as the source for pellicle glucose (KENSCHE et al., 2013). Phospholipids were also found in the pellicle such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (SLOMIANY et al., 1986). The existence of phospholipids is proposed to have an important role in the consistency of the pellicle (SLOMIANY et al., 1990).

Electron microscopy is used to examine the structural composition of the dental pellicle. It was reported in previous studies that the pellicle thickness ranges between 100 and 1300 nm on enamel surfaces. The pellicle thickness varies upon the location of the surface in the oral cavity, the age, and the maturity of the pellicle (TINANOFF et al., 1976; HANNIG, 1999).

Previous studies suggested that pellicle formation reaches equilibrium between adsorption and desorption of salivary proteins within almost 2 h. Consequently, no additional increase in amino acid amount was detected after 120 min (SONJU, RÖLLA, 1973; LAMKIN et al., 1996). In contrast, another study showed that pellicle formation is not complete after 2 h because of further accumulation of salivary biopolymers that do not reach a plateau of adsorption on the dental surfaces after a 2-h period (HANNIG, 1999). Based on the morphologic criteria, the dental pellicles consist of two layers:

1) A direct electron-dense basal layer as a result of the direct, initial phase of salivary protein adsorption onto the tooth surface.

2) The outer layer that forms as a consequence of additional adsorption of salivary proteins which resulted in the formation of a less dense loosely arranged pellicle layer (HANNIG, 1999). This outer layer varies in more than one complex ultrastructural pattern. One of the patterns is the globular pellicle, which is characterized by a consistent presence of globules of different sizes and configurations. These globules are distinctly different from microorganisms because of the dimensions, contour, and absence of cell membrane. Another pattern is the fibrillar micelle-like structure pellicle, which consists of 3- to 7-nm wide fibrils and often includes some finely globular particles. The last pattern is the granular structure layer, which is usually seen after 12 h with a relatively smoother surface. This granular pellicle does not exhibit globular or fibrillary structures, but it instead contains distinct laminations. Apparently, the variation on pellicle morphology is a reflection of its time-dependent, complex chemical composition, oral location effect, and the influence of shearing forces in this location (LIE, 1977; HANNIG, 1999).

2.1.1.3 Functions of the pellicle

Two important aspects have been studied in regards to the acquired pellicle functions:

1) The acquired pellicle plays an important role in enamel protection in two fields: First, by resisting the acid-induced demineralization. Several previous studies proved that salivary pellicle considerably reduces enamel erosion and mineral loss by acidic drinks (MEURMAN, FRANK, 1991) or orange juice

(AMAECHI et al, 1999). The acquired pellicle possesses this protective property because of its chemical components. For example, the proteins inside even a 3-min initial pellicle are resistant to removal by an acidic attack. Moreover, 120-min pellicles possess more protective proteins that are resistant to removal which can suggest an increase in protection against acid attacks with the time of pellicle formation (HEGDE, SAJNANI, 2017; TAIRA et al., 2018). Dental surface demineralization is rarely seen because of the protective effects of the acquired pellicle in spite of the continuous consumption of acidic drinks. Secondly, it is also believed that acquired pellicle plays an important role in enamel protection by reducing friction and abrasion between teeth, it also protects between teeth and the oral mucosa, especially during mastication and parafunctional habits (LENDENMANN et al., 2000). Furthermore, it was demonstrated that acquired pellicle can significantly reduce daily toothpaste abrasion (JOINER et al., 2008). Interestingly, it was reported that the protective role of the dental pellicle is partly lost after using abrasive toothpaste because the pellicle is partly or completely removed with this rigorous oral hygiene practice. Therefore, aggressive hygiene procedures should be avoided to preserve the protective functions of the dental pellicle (KUROIWA et al., 1992).

2) The acquired pellicle plays a big role in bacterial adherence and biofilm formation. At first, the pellicle film alters the charge and free energy of the dental surface and replaces it with the positively charged pellicle proteins. This then attracts the negatively charged microorganisms by electrostatic forces and initiates the bacterial adhesion process. Afterward, this pellicle provides several receptors for facilitating additional bacterial adhesion. The bacteria, in turn, have special surface constructions such as fimbriae and fibrils that assist in the adherent to tooth surfaces. In time, these bacteria will form dental microbial biofilm or dental plaque (SOCRANSKY, HAFFAJEE, 2002).

2.1.2 Dental biofilm

2.1.2.1 Dental biofilm definition

Dental biofilm has been defined as a specialized biofilm that consists of a diverse community of microorganisms formed on the dental surface (SOCRANSKY, HAFFAJEE, 2002; MARSH, 2004). More than 6000 species-level phylotype microorganisms have been identified in this biofilm (KEIJSER et al., 2008). They are non-randomly embedded in an extracellular slime matrix, which consists of biopolymers of host and bacterial origins, such as polysaccharides, proteins, glycolipids, and DNA (MARSH, 2004; FLEMMING et al., 2007).

2.1.2.2 Ultrastructural composition of the dental biofilm

As mentioned before, the oral bacteria will use the electrostatic forces and their surface structures to attach the tooth surfaces (SOCRANSKY, HAFFAJEE, 2002). At first, the microorganisms will establish a thin basal layer on the dental surface. This basis bacterial layer is in contact or occasionally penetrating the dental pellicle. While one set of bacterial surface structures, proteins, and extracellular polysaccharide mediate the attachment to solid surfaces, a second set of these agents mediate cell-to-cell relation in building a 3-dimensional form of the dental biofilm (VALM, 2019). With time, this

3-dimensional biofilm structure resembles towers or mushrooms towards the lumen of the solution. The structure is separated by apparently empty channels, but they are filled with extracellular polysaccharide (COSTERTON et al., 1995; SOCRANSKY, HAFFAJEE, 2008). The presence of water channels permits the passage of nutrients and waste products within the biofilms. In this way, these channels act as a primitive ‘‘circulatory’’ system (SOCRANSKY, HAFFAJEE, 2002).

It is important to mention that there are specific relationships between the bacteria in dental biofilms and the association between them is not random (SOCRANSKY et al., 1998). These relationships are represented in facilitating the following: handling and uptake of nutrients, one bacterial species providing nutrients for other bacteria (cross-feeding), utilizing and eliminating the potentially harmful metabolic components, and establishing a convenient living environment. Moreover, the association between different bacterial species and their specific environmental conditions leads to unique growth patterns with structurally complex and mature biofilm development. This pattern of biofilm formation provides several advantages to the colonizing microorganisms. An important advantage is protecting the bacterial species inside the biofilm from environmental factors. These factors include host defence procedures and potentially toxic agents in the environment, such as antiseptics or antibiotics (SOCRANSKY, HAFFAJEE, 2002).

The majority of authors who subsequently analysed *in situ* biofilm emphasized large inter-individual differences in biofilm thickness (AUSCHILL et al., 2004; DIGE et al., 2009; MARTÍNEZ-HERNÁNDEZ et al., 2020) and vitality distribution (AUSCHILL et al., 2001; ZAURA-ARITE et al., 2001; MARTÍNEZ-HERNÁNDEZ et al., 2020). Even variations in biofilm compositions were found in samples from different oral locations; for example, anterior versus posterior sites (differing in nutrient availability), smooth surfaces versus fissures, or sites near to saliva excretion points (AUSCHILL et al., 2004). But in general, increasing biofilm age and thickness leads to higher complexity in the dental biofilm structure (ZAURA-ARITE et al., 2001; DIGE et al., 2007; MARTÍNEZ-HERNÁNDEZ et al., 2020).

Mean microbial vitality values between 60% and 77% had been detected in several *in situ* studies over 2 and 3 day periods (ARWEILER et al., 2004; AUSCHILL et al., 2005; VON OHLE et al., 2010). Furthermore, after imaging mature *in situ* formed biofilms by the confocal microscopy, it was found that the living microorganisms were mostly located on the upper layers of the dental biofilm covering dense layers of dead microorganisms (NETUSCHIL et al., 1998).

2.1.2.3 Dental biofilm complications

If left unremoved, the formation of dental biofilm can lead to severe oral complications and infections (KILIAN et al., 2016; LARSEN, FIEHN, 2017), such as caries and periodontal diseases (COLOMBO, TANNER, 2019).

For instance, the primary cause of caries is the microbial carbohydrate catabolism and the release of organic acids that induce demineralization of the tooth surfaces (TAKAHASHI, NYVAD, 2008).

Periodontal disease is also initiated and sustained by factors (substances) produced by the bacteria inside the dental biofilm. Some of these substances are soluble enzymes or harmful bacterial metabolic waste products. These products can either directly injure and digest the host's cells and tissues or initiate the host's immune and inflammatory reactions that cause damage to the periodontal tissues (KINANE et al., 2008).

2.1.3 Dental biofilm management

Biofilm management is an essential goal in preventive dentistry to eliminate bacterial biofilm. Currently, home-use oral care instruments are the most widely used method for dental biofilm elimination. The typical instruments used for dental biofilm control are the toothbrush (manual or electric), interdental brushes, and floss.

However, anti-biofilm agents are often recommended in some situations with the unavailability and incompetence of mechanical approaches (ADDY, MORAN, 1997), such as for orthodontic patients, disabled elders, and post-periodontal surgery patients, *etc.* (ELEY, 1999). In such situations, the chemically active agents are increasingly used as a valuable tool for biofilm management when manual biofilm control is compromised or not used in an appropriate way to get adequate oral hygiene (TAKENAKA et al., 2019).

In general, antiseptics have more extended activity than antibiotics because antibiotics tend to have certain targets inside the bacterial cells whereas antiseptics are targeting several points on and in microbial cells. Moreover, antiseptics can be used with relatively high concentrations directly on the oral cavity. Therefore, antiseptics are unlikely to induce microorganism's resistance (MCDONNELL, RUSSELL, 1999).

It has been stated that antiseptics can reduce biofilm formation in several ways. Antiseptic agents can prevent the constitution and growth processes of bacterial biofilm, resolve existing biofilms, or they destroy the microorganisms inside the biofilm (ADDY, MORAN, 2008). For an antimicrobial agent to have the optimum effect, it should own both a bactericidal effect followed by a bacteriostatic activity. The bactericidal effect should happen directly after the application in the oral cavity when the antimicrobial agent is at high concentration. This bactericidal effect would be expected to be lost very soon after expectoration and prospectively replaced by bacteriostatic activity. This bacteriostatic action could remain for a considerable time period in the oral cavity depending on the characteristics of the chemical agent (ADDY, MORAN, 2008).

One of the important features of a chemical agent to be an effective antiseptic *in vivo* is the substantivity, which is defined as the ability of an antimicrobial product to retain its effectiveness in the oral cavity for an extended period after application (NETUSCHIL et al., 2003). The efficient antimicrobial agent should dramatically reduce the bacterial levels inside the oral cavity to achieve a level of bacterial biofilm that will not induce oral diseases or complications.

The anti-biofilm mechanisms behind an efficient antiseptic activity are summarized as follows (BAEHNI, TAKEUCHI, 2003):

1) Reduce the bacterial adhesion to the dental surfaces to prevent the biofilm formation.

2) Reduce the bacterial viability which prevents further growth of the bacterial colonies and interferes with the co-aggregation process.

3) Removal or disruption of an existent biofilm.

There are numerous of active agents such as chlorhexidine, amine fluoride/stannous fluoride, triclosan, and octenidine, which can inhibit biofilm formation, maturation, and microbial proliferation (ADDY, MORAN, 2008). But it is important to know that not all of the chemical agents are in the same degree in regards to the antibacterial activity and the substantivity after application (MANDEL, 1988; TAKENAKA et al., 2019).

Due to the great effectiveness, the antiseptics chosen to be discussed, investigated, and above all compared in this present work were chlorhexidine and octenidine.

2.1.4 Chlorhexidine

2.1.4.1 Overview

Although many products have been used as antiseptics, chlorhexidine (CHX: $C_{22}H_{30}Cl_2N_{10}$) is regarded as the most effective antimicrobial agent for chemical control of biofilm formation and for prevention of caries for more than 50 years (VAN STRYDONCK et al., 2012). It is one of the most widely used, safest, and thoroughly investigated antiseptic (JONES, 1997). In the 1940s, CHX was developed by Imperial Chemical Industries in England and marketed in 1954 as an antiseptic for skin wounds (DAVIES et al., 1954).

CHX is a bisbiguanide symmetrical molecule, consisting of four chlorophenyl rings and two biguanide groups connected by a central hexamethylene bridge. It can be found in the market in three formulations, digluconate, acetate, or hydrochloride salts (ADDY, MORAN, 2008).

The anti-biofilm activity of CHX in the oral cavity was investigated for the first time in 1970. This study showed that using 0.2% CHX-gluconate rinse for 60 s twice per day in the absence of typical teeth cleaning prevents biofilm regrowth and the formation of gingivitis (LÖE, SCHIOTT, 1970).

At physiological pH, CHX is a strong base and a dicationic molecule, with a positive charge on all sides of a hexamethylene bridge. Because of that CHX has the ability to adsorb at negatively charged surfaces within the oral cavity. It binds to the bacterial cell membrane, where it exerts its bactericidal and bacteriostatic effects (JONES, 1997). CHX exhibits a wide spectrum of antibacterial activity that targets a wide range of Gram-positive and Gram-negative microorganisms, dermatophytes, and some lipophilic viruses, including HBV and HIV. It is active against some fungi and yeasts, including *Candida* (WADE, ADDY, 1989; DENTON, 2001).

2.1.4.2 Chlorhexidine mode of action

At low concentrations, CHX has a bacteriostatic effect due to the immediate attraction to the negatively charged targets in the microbial cell membrane. Thereby modifying the integrity of the cell surface, this encourages the liberation of low molecular weight substances such as phosphorus and potassium, which can lead to partial bacterial damage. Such partial damage prevents bacterial proliferation which in turn reduces the bacterial amount available for microbial aggregation and the subsequent formation of oral biofilms. CHX-high concentrations induce a bactericidal effect, whereby they damage and rupture cytoplasmic membranes, and cause precipitation of cell cytoplasm. This precipitation is caused by an interaction between CHX and phosphate entities within the cytoplasm, such as adenosine triphosphate and nucleic acids. In this case, CHX dramatically reduces the number of oral bacteria, and it destroys all the microorganisms either attaching or already attached to the tooth surface (DENTON, 2001; MARTÍNEZ-HERNÁNDEZ et al., 2020).

CHX has not only the antibacterial effect but also antiplaque activity by using different mechanisms of action such as breaking the glycoproteins connections between the bacteria and displacing calcium molecules which are considered the glue that binds biofilm particles together (RÖLLA, MELSEN, 1975).

2.1.4.3 Chlorhexidine substantivity

CHX binds most strongly to anionic groups (sulphates, phosphates, and carboxyl groups). This capability permits CHX to interact with the oral anionic glycoproteins and phosphoproteins, and adsorb to the oral mucosa and the tooth pellicle (MANDEL, 1988). The adsorption to oral surfaces after the application is followed by progressive desorption over time. This means that CHX can move from teeth, mucosa, saliva, *etc*. to the bacterial cells. This ultimately induces bacteriostatic activity that leads to an important reduction in the bacterial load in the oral cavity over time (GREENSTEIN et al., 1986). This persistent bacteriostatic activity of CHX lasts in excess for at least 24 h (high substantivity) (BONESVOLL et al., 1974a; TOMÁS et al., 2010; REDA et al., 2020).

Several methods have been used to investigate the long retention of CHX in the oral cavity after rinsing. At first, direct ultraviolet spectroscopy approach was used for intraoral CHX determination (RÖLLA et al., 1970; JENSEN et al., 1971). The method was, however, unspecific for CHX quantification because of the distribution by several salivary components. These components absorb within the same wavelength region as CHX (FIORENTINO et al., 2010). Therefore, techniques based on radiolabelling with carbon-14 or fluorometric method were subsequently used. Due to the higher sensitivity, ^{14}C-labelled CHX could be detected in saliva 24 h after CHX application (BONESVOLL et al., 1974a). However, this method is ethically inapplicable to humans. Fluorometric methods were limited to determine CHX in aqueous solutions and centrifuged saliva (DE VRIES et al., 1991), but did not work in whole saliva samples and failed to reliably determine very low or high CHX concentrations (TSUCHIYA et al., 1999). Different approaches of high-performance liquid chromatography (HPLC)

have been developed for CHX determination (LAM et al., 1993; MEDLICOTT et al., 1994; PESONEM et al., 1995; TSUCHIYA et al., 1999). Solid-phase microextraction (SPME) was used also to monitor free and total concentrations of CHX in pharmacokinetic investigations (MUSTEATA, PAWLISZYN, 2005). Although HPLC and SPME based methods are sensitive and specific for CHX determination, they generally need extremely long analysis times for multiple extraction and cleaning steps (FIORENTINO et al., 2010). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was also used to determine CHX retention in microtome slices of a microbial contaminated wound model which was rinsed with CHX solution (HAMERLY et al., 2015).

In MALDI-TOF analysis, the sample is first co-crystallized with a large molar excess of a matrix component. Then, laser radiation of this sample–matrix composition leads to the vaporization of the matrix which takes the analyte with it. The ionization of the analyte occurs through proton transfer from the matrix or cationization. All of the ions are given the same amount of energy and are accelerated to a detector. The ions reach the detector at different times because they have different masses. The smaller ions reach the detector first because of their smaller mass, which allows for greater velocity, while the bigger ions take longer due to their bigger mass and smaller velocity (LEWIS et al., 2000). The impacts of the ions on the detector are turned into electrical signals and are stored. A MS-peak is made up of a set of data points recorded from the detector. The highest of the peak represents the intensity of the ions at the detector (which had reached the detector), which in turn presents a quantity of the cretin substance in the sample of interest (examined sample) (KEMPKA et al., 2005). In this way, quantification of a substance can be done, depending on the calibration curve from the control condition with defined amounts of the substance (HAMERLY et al., 2015). Measurements can also be done from a conventional internal standard method, in which a known amount of an isotopically labelled analogue of an element is mixed with the investigated sample (STOCKLIN et al., 1997; STOCKLIN et al., 2000).

The substantivity feature allows CHX to work in a way of a slow-release system. This feature leads to continuous, rather than an intermittent, antimicrobial activity that can prevent microbial reproduction (MANDEL, 1988). This property of substantivity gives CHX its advantage over many other agents and makes it the "gold standard" in oral antiseptics (JONES, 1997).

2.1.4.4 Chlorhexidine pharmaceutical formulations

The gold standard CHX has been delivered in different formulations and vehicles, such as mouth rinses, sprays, toothpastes, gels, varnishes, and slow-release vehicles (SHAH et al., 2019). Solutions with alcohol or alcohol-free 0.2% CHX have been largely recommended in Europe as a mouth rinse in 10 ml volumes (20 mg dose) (KENDALL, 2002). Later, in the USA, a 0.12% mouth rinse was manufactured. This product was recommended in 15 ml solution volumes (18 mg dose) to have almost the same dose derived from 10 ml of 0.2% solutions (GAGE, PICKETT, 2002). The efficacy of the two concentrations was evaluated in a systematic review (BERCHIER et al., 2010). Regarding the biofilm inhibition, the review reported a slight but significant difference in favour of the 0.2% CHX solution. However, in the

clinical aspect, both CHX concentrations are able to reduce gingivitis to the same extent. Alcohol-free CHX rinses have become also available in the market. Such formulations have been shown to have equal effects against biofilm formation, gingivitis, and have a better taste when compared to alcohol-containing CHX rinses (VAN STRYDONCK et al., 2005; PAPAIOANNOU et al., 2016).

Spray approach was used for applying a small dose of CHX (approximately 3 mg) directly to tooth surfaces. The CHX spray showed valuable biofilm inhibition properties (KALAGA et al., 1989a). This approach seems to be especially useful for the physically and mentally handicapped groups, due to its accessibility by individuals and their carers (FRANCIS et al., 1987 a, b; KALAGA et al., 1989 b; VIANA et al., 2014).

CHX varnish has also beneficial effects on patients with chronic gingivitis. It reduces biofilm accumulation, bleeding levels, and also gingival scores (PUIG-SILLA et al., 2008; SACHDEVA et al., 2018).

CHX chips, as slow-release vehicles, have been applied as an adjunct to scaling and root planning. A degradable gelatine chip (PerioChip) containing 2.5 mg CHX has been commercially produced and evaluated in several studies with moderate periodontitis. There was significantly greater pocket depth reduction in the treated sites with the chips, in comparison with the sites receiving only mechanical treatment (SOSKOLNE et al., 1997; JEFFCOAT et al., 1998; RAJ et al. 2017; CHACKARTCHI et al., 2019).

In the last few years, the idea to incorporate CHX in a dental paste formulation emerged since most patients use a dental paste daily. With such a combination, the patient will have several advantages which are the following: mechanical cleaning with fluoride delivery, anti-biofilm effect, anti-gingivitis effect, and reduced CHX side effects with no added discomfort for patients (FIORILLO, 2019). Nevertheless, CHX was hardly formulated into toothpaste because of the interaction and inactivation between CHX particles as a dicationic antiseptic and toothpaste components, such as anionic detergent and calcium ions (ADDY et al., 1989; BARKVOLL et al., 1989). This explains the conclusions of two systematic reviews (SLOT et al., 2014; SUPRANOTO et al., 2015), where it was stated that using toothpaste with CHX is efficacious in the management of dental biofilm and gingivitis to some extent, but not to the same degree as a CHX rinsing. Furthermore, it was stated that the concentration of CHX inside the toothpaste should be more than 0.6% to have an anti-biofilm effect. Worth to mention that using CHX toothpaste induced significantly less tooth discoloration than after rinsing with CHX solutions (SUPRANOTO et al., 2015).

2.1.4.5 Optimizing the use of chlorhexidine

CHX molecule has a tendency to react with anionic surfactants (ADDY et al., 1989). To avoid any reduction of CHX activity, this tendency should be considered during the formulation of different CHX mouthwashes, spray, toothpastes, *etc.* (ADDY, 1986). Similarly, the interaction between CHX and toothpaste materials, from tooth brushing, can reduce the delivery of CHX inside the oral cavity in an

active form. Consequently, CHX should not be used nearly before or directly after using daily toothpaste. It was shown in a systematic review (KOLAHI, SOOLARI, 2006) that the best interval between using toothpaste and CHX application should be more than 30 min, advisedly for 2 h.

2.1.4.6 Clinical uses of Chlorhexidine

According to the high antimicrobial properties of CHX, a number of clinical uses have been recommended:

1) As an adjunct to oral hygiene and professional prophylaxis: CHX increases the improvement of gingival health through biofilm control, particularly following professional prophylaxis (ADDY, MORAN, 2008). Indeed, as the first research demonstrated, patients could maintain almost zero levels of biofilm with a CHX mouth rinse, without the use of any additional mechanical oral hygiene (LÖE, SCHOITT, 1970). Additionally, several clinical studies showed that CHX rinsing reduces plaque formation and gingivitis (FAVERI et al., 2006; VAN STRYDONCK et al., 2012; AHMAD, 2020). Furthermore, CHX mouth rinses may be of value in maintaining oral hygiene after scaling and root planning; this is because adequate tooth brushing may be compromised by post-treatment soreness or sensitivity (BEISWANGER et al., 1992).

2) Prophylactic pre-operative CHX rinsing and irrigation: Such pre-operative rinsing clearly reduces the microbial contamination of the surgical region, operator, and crew (WORRAL et al., 1987). Additionally, CHX rinsing reduces the occurrence of bacteraemia in treated patients (MACFARLANE et al., 1984).

3) Secondary prevention after oral surgical procedures and periodontal therapy: CHX reduces bacterial adhesion and prevents biofilm formation. As a result, it can be used post-operatively at a time and in areas where mechanical cleaning may be difficult, due to discomfort. CHX can be used until the patient can re-establish normal oral hygiene (SANZ et al., 1989; OLSSON et al., 2012). Furthermore, it was shown that CHX is able to reduce bacteraemia after tooth extraction (ARTEAGOITIA et al., 2018).

4) As a part of the oral hygiene procedures in the mentally and physically handicapped: CHX was successfully used to improve both gingival health and oral hygiene level in institutionalized physically and mentally handicapped groups (STORHAUG, 1977). Notably, the CHX spray was found to be useful and acceptable to patients and care workers (FRANCIS et al., 1987a, b; KALAGA et al., 1989b).

5) Medically compromised individuals subjected to oral infections: The application of CHX, in combination with anti-candida drugs, have been found to be very effective in preventing oral and systemic infections in the immune-compromised patients receiving chemotherapy and/or radiotherapy procedures (FERRETTI et al., 1987). Furthermore, it was shown that CHX is able to improve the oral hygiene level of patients with respiratory ventilation device and hospitalized in intensive care units (MIRZAKHANI et al., 2017).

6) High-risk caries patients: It was reported that CHX rinse or gel application has a good benefit in caries patients due to the significant reduction of *Streptococcus mutans* counts in the oral cavity (LINDQUIST et al., 1989; COELHO et al., 2017). Additionally, a combination of CHX and sodium fluoride in one rinsing solution seems to be useful to such patients (DOLLES, GJERMO, 1980).

7) Recurrent oral ulceration: It was seen in several studies that CHX, as a rinse or gel, reduces the incidence, duration, and severity of aphthous ulceration. This is due to the reduction in secondary contamination of ulcers when reducing the oral bacteria counts after application (ADDY et al., 1976; HUNTER, ADDY, 1987).

8) Removable and fixed orthodontic appliance wearers: CHX has great benefits for biofilm control, especially when the oral hygiene is compromised at the early stages of orthodontic therapy (STIRRUPS et al., 1981). It was shown also in a systematic review that using CHX varnish is efficient in caries reduction in the course of fixed orthodontic therapy (OKADA et al., 2016).

2.1.4.7 Toxicity, safety, and side effects of chlorhexidine

As a result of the CHX cationic nature, it cannot be absorbed through the skin or mucosal membrane, including the gastrointestinal tract, which eliminates the systemic toxicity from topical application or ingestion (ADDY, MORAN, 2008). Unfortunately, in spite of the positive antibacterial features, several clinical applications, and low systemic toxicity, there are some reversible local side effects from CHX usage. Side effects include the following: staining of teeth, fillings, and the tongue, increased formation of supragingival calculus, reduced taste sensation, and seldom mucosal membrane irritation. These side effects always prevent the prolonged use of CHX mouth rinse (FLOTRA et al., 1972; KOUR, KAUR, 2019). Furthermore, allergic reactions have been reported after the application of CHX in few clinical cases (EGNER et al., 2017). Because of these negative aspects concerning CHX application, it was always the trend to search for an alternative antiseptic agent with the following: similar antimicrobial spectrum, fewer adverse effects, and less toxicity and allergic reaction. One option is octenidine dihydrochloride. It has a similar antimicrobial spectrum, assumed to have fewer side effects and less toxicity. The reduced side effect by this agent is theoretically due to octenidine's similar activity to CHX, but at a lower concentration (HUBNER et al., 2010).

2.1.5 Octenidine

2.1.5.1 Overview

Octenidine dihydrochloride (OCT: $C_{36}H_{64}Cl_2N_4$), a bispyridine antimicrobial compound, was discovered in the 1980s at the Sterling-Winthrop Research Centre. It was developed as a possible local antibacterial product with broad-range activity, covering Gram-positive and Gram-negative bacteria, fungi, and several viral species (SLEE, O'CONNOR, 1983; BAILEY et al., 1984; SEDLOCK, BAILEY, 1985). Chemically, OCT has two cationic active centres, which are separated by a long aliphatic

hydrocarbon chain. Therefore, it adsorbs to negatively charged surfaces, such as bacterial cell membranes and eukaryotic cell walls (ASSADIAN, 2016).

It was shown in the *in vitro* studies that OCT has an evident antibacterial property (SLEE, O'CONNOR, 1983). Using suspension tests, it was found that the minimum bactericidal concentrations (MBCs) of OCT are ranging from 1 μg/ml to 32 μg/ml, depending on the bacterial species. There was a rapid antibacterial activity even after only one-minute application of OCT against the following: *Staphylococcus epidermidis, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans,* even in the existence of blood or wound exudate (PITTEN et al., 2003).

The early animal studies on monkeys showed that 1% OCT was able to reduce plaque scores significantly better than 1% CHX, after 7 days of application (EMILSON et al., 1981). Also, in another study, OCT dentifrice was also able to prevent dental biofilm formation and, concomitantly, inhibit the development of gingivitis in the monkey model over the 21-days experimental period (SLEE et al., 1985). Patters and co-workers used an experimental human gingivitis model to evaluate OCT efficacy in two studies. It was proved in the first one that 0.1% OCT rinsing was almost able to completely inhibit plaque formation. OCT with the concentration 0.05% was less effective than 0.1%, but still significantly better than the placebo. Additionally, gingival fluid in the OCT groups was clearly less than the control group. However, some tooth staining was observed also in all OCT groups (PATTERS et al., 1983). In the second study, there were two test groups; one test group with two-times rinsing per day and the second test group with three-times rinsing per day, for 21 days. Both OCT groups had almost 90% less plaque and 68% less gingivitis than the control group. There was no significant difference between the two OCT groups. Slight external tooth staining was noticed in 62% of the volunteers after rinsing with OCT, but it was removed by a single tooth cleaning in 90% of the conditions (PATTERS et al., 1986).

2.1.5.2 Octenidine mode of action

OCT exerts this particular antimicrobial effect due to its cationic charge. It binds readily to the negatively charged microbial cell wall. On attachment, OCT interacts with the polysaccharides in the cell membrane and alters the enzymatic systems there. Furthermore, it alters bacterial membrane permeability, which contributes to its lyses and leakage of the cytoplasm components (KRAMER, ASSADIAN, 2013). As a result, the microcellular metabolism is also disturbed, which disrupts and destroys cell functions, leading to apoptosis, programmed cell death (HARKE, 1989; GHANNOUM et al., 1990). Until now, bacterial resistance to OCT has not been seen *in vitro*. According to this fact and to the high concentrations used clinically, microbial resistance is not expected also *in vivo* (MCDONNELL, RUSSELL, 1999).

2.1.5.3 Octenidine substantivity

Since OCT binds readily to the negatively charged areas, including fibroblasts, human epithelial cells, and primary keratinocytes and is not absorbed through the mucosal membrane, at least part of the used

agent stays on the place of application and cannot be removed easily (*e.g*. by washing); thus, resulting in a depot effect that exerts a persistent antimicrobial effect (MÜLLER, KRAMER, 2007).

This substantive bacteriostatic effect is a very important aspect of being an efficient antiseptic product because it is not only important to have direct antibacterial activity, but also to possess a continuous therapeutic effect for a period of time after the application. It was stated in previous studies that OCT solution has a more persistent antibacterial and anti-adhesive efficacy in compression with CHX and essential oil (DECKER et al., 2003; WELK et al., 2016).

2.1.5.4 Clinical uses of octenidine

Due to the particular antibacterial activity of OCT and its persistent effect in the oral cavity, a number of clinical uses have been described:

1) As a part of oral hygiene procedures after periodontal therapy: It was shown clinically that 0.1% OCT application significantly reduced plaque scores and gingivitis in comparison with the placebo (BEISWANGER et al., 1990).

2) High-risk caries patients: It was reported that OCT solution is effective against the cariogenic microorganisms (*Streptococcus mutans*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus,* and *Actinomyces viscosus)* even at low concentrations (CELIK et al., 2016).

3) For endodontic irrigation: OCT can be used as endodontic irrigate due to its antibacterial efficacy and lower cytotoxicity. OCT was more active than 5.25% NaOCl solution as an antimicrobial endodontic irrigate. OCT with a concentration of 0.5% eliminated all the tested microorganisms (*Staphylococcus aureus, Enterococcus faecalis,* and *Candida albicans)* in only 15 seconds, where it took NaOCl 20 min to do the same (TIRALI et al., 2009). Additionally, 0.1% OCT was more effective as a root canal irrigant than 2% CHX (CHERIAN et al., 2016). Similarly, OCT was more active against *Enterococcus Faecalis in vitro* in comparison with 1% CHX and 3% NaOCl (MAKKAR et al., 2015). Also, such a result was found in a recent study evaluating the effect of different irrigants against *Staphylococcus epidermidis* (CHUM et al., 2019). OCT showed also an antibacterial activity against *Enterococcus faecalis* in the root canal and dentine after 1 min incubation. More effective dentine disinfection was seen after incubation in OCT for 10 min and 7 days (TANDJUNG et al., 2007).

4) Treatment of chronic ulcers**:** It was shown that treating chronic ulcers in 43 patients with OCT reduced signs of inflammation and clearly improved granulation without negative effects on the wound recovery (VANSCHEIDT et al., 2005).

2.1.5.5 Toxicology, safety, and side effects of octenidine

Systemic side effects are not expected because OCT is virtually not absorbed via the mucosal membrane (HUBNER et al., 2010). Furthermore, OCT has a good affinity towards cardiolipin, a prominent lipid in the microbial cell wall. In this way, OCT causes selective harmful implications to bacterial cell membrane without negatively affecting human epithelium or mucosal cells (BRILL et al., 2006).

In vitro experiment that investigated the biocompatibility and related cytotoxicity of OCT has found that this substance is more toxic (*i.e.* effective) to both the test microorganisms (*Escherichia coli* and *Staphylococcus aureus*) than to murine fibroblasts with biocompatibility index (the ratio of fibroblast cytotoxicity to *E. coli* or *S. aureus* toxicity) greater than one. This index was less than one by CHX, povidone-iodine solution and ointment, silver compounds, and triclosan (MÜLLER, KRAMER, 2008). Similarly, a recent study has found that OCT has a lower cell-toxic effect against fibroblasts and epithelial cells when compared with CHX (SCHMIDT et al., 2016).

However, the long usage of OCT mouth rinse produces a level of dental stain which needs longer time for dental cleaning (BEISWANGER et al., 1990). It is important to mention that more dramatic complications have been reported in several situations, such as after irrigation of penetrating hand wounds. Such irrigation can lead to chronic inflammation and aseptic tissue necrosis that persists for weeks or even months (LACHAPELLE, 2014). The same side effects happened after using a combination based on OCT and phenoxyethanol for peritoneal and bladder irrigation, as well as tympanic membrane washing (HULSEMANN, HABENICHT, 2009). Similarly, OCT should not be applied for joint irrigation, even at low concentrations such as 0.005% because it could induce a toxic alteration to the cartilage (MÜLLER, KRAMER, 2005).

To avoid such dramatic complications, OCT is contraindicated to be used in deep penetrating wounds. Moreover, OCT must not be injected or applied with pressure to tissue cavities such as peritoneal, bladder, and joint cartilage if free outflow and drain-off are not ensured (HUBNER et al., 2010).

2.1.6 Comparison between chlorhexidine and octenidine

Interestingly, concerning the antibacterial effect, OCT is 3 to 4 times more effective than CHX depending on the bacterial spices (KRAMER, ASSADIAN, 2013). For example, Celik and co-workers found that OCT was more effective than CHX at lower concentrations against the cariogenic microorganisms (*Streptococcus mutans*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus,* and *Actinomyces viscosus)* (CELIK et al., 2016). It was found also *in vitro* that OCT stays quite effective even when highly diluted. Whereas, CHX lost its antibacterial activity when diluted to less than 10% of its first concentration (ROHRER et al., 2010). In another *in vitro* study, all concentrations of OCT (0.025%, 0.05%, and 0.1%) were effective against *Candida albicans* and *Enterococcus faecalis.* Additionally, the inhibition zones observed by CHX with both concentrations 2% and 1% were significantly less than the inhibition zones induced with NaOCl and OCT (TIRALI et al., 2013). Similarly, 0.1% OCT was more efficient than 0.2% CHX in reducing the microbial counts (*Enterococcus faecalis* and *Staphylococcus aureus*) *in vitro* (MALHOTRA et al., 2016).

As shown in the previously published studies and with regard to minimum inhibitory concentration (MIC) and MBC of OCT and CHX on a wide range of pathogens (*Staphylococcus aureus, Enterococcus faecalis, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Haemophilus*

influenza, Clostridium perfringens, and *Candida albicans*), OCT seems to be a more effective antimicrobial agent compared with CHX (KOBURGER et al., 2010; LANGNER et al., 2019). Nevertheless, there is a lack of information concerning the anti-biofilm and biofilm disruption effects of each antiseptic agent, CHX and OCT, and especially the comparison between them under real *in situ* conditions. Thus, further research was necessary to fill the gaps of information concerning these two oral antiseptics.

2.2 Aim of this work

Direct proof is missing, until now, about chlorhexidine (CHX) retention on mucosal surfaces and dental pellicle after oral CHX application. In this context, MALDI-TOF provides rational mass resolving power, high mass precision, high sensitivity, and can be performed in high throughput quantifications. These features make this method a promising technique for the determination of CHX retention in samples from different locations in the oral cavity and not only in saliva like done before in other studies (FIORENTINO et al., 2010). Such retention data should provide more information about the behaviour and the pharmacokinetics of CHX, especially after the application of different medical formulations.

Additionally, previous studies have established that CHX and octenidine (OCT) are effective in preventing and controlling biofilm formation and consequently inhibiting and reducing the development of oral diseases (JONES, 1997; VAN STRYDONCK et al., 2012; ASSADIAN, 2016; WELK et al., 2016). However, up to now, the CHX activity has been only investigated on dental biofilms growing on glass specimens or plastic films (AUSCHILL et al., 2005; BRECX, THEILADE, 1984) which not necessarily reflects what is happening on biofilms that develop directly on real enamel surfaces inside the oral cavity. Likewise, the anti-biofilm and the biofilm disruption effects of OCT have not been studied yet especially in comparison with the gold standard CHX and under most natural conditions *in situ*.

This work - based on *in situ* experiments - aimed to investigate the following issues in detail:

1) The retention of CHX in different oral sites (buccal dental pellicle, interdental area, posterior buccal mucosa, anterior labial mucosa, and saliva) over a 24-h period.
2) The retention of CHX in the oral cavity after using different CHX pharmaceuticals formulations (mouth rinse, oral spray, and toothpaste).
3) The effects of CHX and OCT mouth rinse on dental biofilm formation, as well as on biofilm disruption.

The results should provide more details about the action mechanisms behind the evident efficacy of the oral antiseptics CHX and OCT.

3 Materials and Methods

3.1 Chlorhexidine retention studies

These *in situ* studies were aimed to determine the retention of CHX in different oral locations. This determination was done in the first retention study after one-time rinsing with 10 ml of 0.2% CHX for 30 s. Furthermore, the CHX quantification was performed in the second retention study after an oral application of different CHX pharmaceutical formulations and regimens.

3.1.1 Studies population

Five periodontally and systemically healthy volunteers from laboratory staff (3 males, 2 females), aged 24-42 years, were recruited for this work. The selected subjects were currently non-smokers. They provided informed consent acknowledging their willingness to participate in the experiments. Exclusion criteria included the following: pregnancy, nursing women, antibiotic therapy within the previous three months, and any systemic disease (*e.g.*, diabetes, human immunodeficiency virus/acquired immunodeficiency syndrome, or autoimmune diseases). An experienced dentist carried out a visual oral examination to assure that the subjects do not have any active dental caries, periodontal disease, gingivitis, or any other oral disease that potentially affecting the salivary fluid composition. The subjects got detailed information on how to deal with the individual splints containing the enamel surfaces.

The study protocols and informed consent were done under the guidelines of the Declaration of Helsinki. The local research ethics committee of the Medical Association of Saarland, Germany, approved oral specimen collection protocols (proposal 231/03, 2012).

3.1.2 Studies design

The first retention experiment was conducted over a 24-h period and started in the morning with the preceding individual. Oral hygienic measures were performed 60 min before beginning the experiment. No further hygienic procedures were done until the end of the 24-h period of the experiment. The volunteers refrained from eating and drinking 60 min before each individual sampling point. Trials were replicated on three weeks starting at 10:00 a.m.

One of the subjects volunteered to refrain from eating and drinking for 11 h. Samples were collected from this individual similarly to the other subjects, to discover the effects of beverage and food ingestion on the substantivity of CHX in the oral cavity.

Samples were taken from five different oral locations: buccal dental pellicle, interdental area, anterior labial mucosa opposite to the labial surface of the incisors, posterior buccal mucosa opposite to the buccal surface of the molars, and saliva. These samples were obtained from each volunteer at five-time points (1 h, 3 h, 6 h, 11 h, and 24 h) after mouth rinsing with 0.2% CHX solution in 7% ethanol for 30 s (Saarland University Pharmacy, Homburg, Germany) at the start of the experiment.

The samples were collected with a disposable micro-brush with non-absorbent and non-lining fibres (Clogherane, Dungarvan, Waterford, Ireland). Samples were taken by scratching the specific surface or immersing the brush tip into the aqueous phase of centrifuged saliva. The saliva samples were centrifuged at 2000 g for 2 min. After sample removal from the oral cavity locations or saliva, brush tips were rigorously mixed with 100 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution {10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Steinheim, Germany), 70% acetonitrile and 0.07% trifluoroacetic acid (VWR International GmbH, Darmstadt, Germany)} in individual 2 ml Eppendorf tubes, respectively. From each tube, five drops of 0.5 µl each were pipetted onto a polished MALDI target plate (AB Sciex, Darmstadt, Germany) by using the dried droplet method. The spots were then allowed to dry.

The second retention experiment was performed over a period of 12 h to evaluate the CHX retention after the application of different CHX formulations. It started in the morning at 7:00 a.m. with the selected individual. Extensive oral hygienic procedures were done with a minimum of 60 min before beginning each trail. No further oral hygienic procedures were conducted until the end of the 12-h period of the experiment. It was referred to the volunteers to take the main food meal 5 h after the start of the experiment and they were allowed to consume different beverages during the time of the experiment.

The CHX formulations and regimens were as follows: 10 ml of 0.2% CHX digluconate in 7% ethanol mouth rinse (Saarland University Pharmacy, Homburg, Germany) for 30 and 60 s, respectively; 10 ml of 0.12% CHX digluconate in 7% ethanol mouth rinse (Saarland University Pharmacy, Homburg, Germany) for 30 and 60 s, respectively; 12 squirts (one squirt equal ~137 µl) from 0.2% CHX digluconate Spray (GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, Germany) were applied directly on the teeth (6 squirts at each jaw), followed by rinsing with the total squirts for 1 min before spitting out the rest of the solution (the CHX spray total dose was ~3.288 mg); 1 g of CURASEPT ADS® 712 GEL-toothpaste without sodium lauryl sulphate (Curaden Swiss GmbH, Stutensee, Germany), and 1 g of GUM® Paroex® gel-toothpaste with sodium lauryl sulphate (Sunstar GmbH, Schönau, Germany) were used with no specific instructions concerning tooth brushing technique, except that the volunteers should brush the teeth for 2 min with 1 g of toothpaste containing 1.2 mg CHX digluconate.

After the application of different CHX digluconate formulations, 2 µl samples from whole saliva and buccal mucosal pellicle, as well as dental pellicle sample, were taken. The buccal mucosa sample was taken from a collected mucosal pellicle after scraping the mucosal area with a plastic spatula (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The pellicle sample was formed on a round enamel specimen with a standard size of 11.35 mm². The enamel surface was polished (4000 grid) and fixed on an individual palatal splint (Fig. 1).

The samples of each subject were taken at six time points, (2 h, 4 h, 6 h, 8 h, 10 h, and 12 h), after application of different CHX formulations. After removal from the oral cavity, samples were individually embedded in 100 µl CHCA-matrix solutions. Dilution steps with matrix solution were necessary to be under the limit of quantification for CHX in the MALDI-TOF technique. From each diluted sample, 5 drops with 0.5 µl each were pipetted via dried-droplet method onto a polished MALDI target plate and allowed to dry.

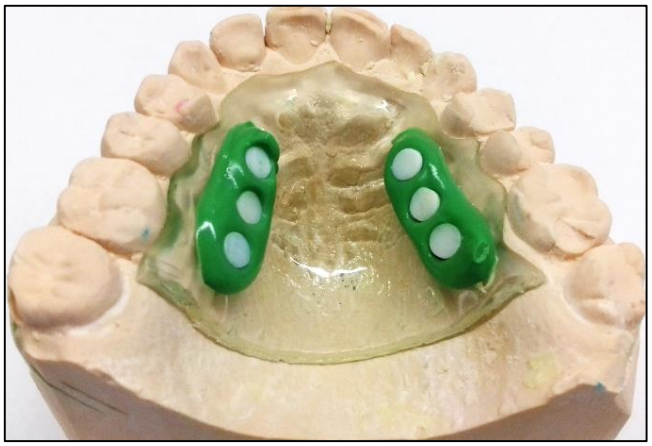

Fig. 1: Palatal acrylic splint with mounted specimens to form the dental pellicle samples for the retention experiment. Permission has been obtained to reuse an image published in the journal Clinical Oral Investigations (REDA et al., 2021b).

3.1.3 Calibration curve of chlorhexidine

A working solution of 0.1 mg/ml free base in 7% ethanol was prepared by filling up 88.81 µl from the stock solution of 0.2% CHX digluconate in 7% ethanol to 1000 µl with the matrix solution. This was directly diluted with the matrix solution in 2-fold to 1.9 x 10^{-7} mg/ml. Five replicate spots with 0.5 µl each were taken from each aliquot and spotted onto the MALDI target plate.

3.1.4 MALDI-TOF mass spectrometry

After laser radiation on the sample–matrix mixture, the CHX molecules become ionized and accelerated to a detector. The impacts of the CHX ions on the detector are turned into electrical signals and subsequently, a MS-peak is made. The highest of the peak represents the intensity of the ions at the detector which in turn represents the quantity of CHX in the sample of interest.

In this study, the MALDI-TOF approach was done using Sciex 4800 TOF/TOF mass spectrometer (Concord, ON, Canada) in positive ionization reflector mode over a range of m/z 370-600. The system used a pulsed 200 Hz solid-state Nd: YAG-laser at a wavelength of 355 nm. The laser energy was set to 2300 units, source 1 voltage to 20 kV, grid voltage to 16 kV, and reflector detector voltage at 2.19 kV. CHX standard, (protonated monoisotopic molecule $(M_i+H^+)^+$ at *m/z* 505.21 and its monoisotopic decay product $(M_i-NH_3+H^+)^+$ at *m/z* 488.21; the monoisotopic CHCA matrix dimer $(2M_i+H)^+$ at *m/z* 379.09 was utilized for internal mass calibration with a delay time of 600 Nano second. One single mass

spectrum was averaged from 20 individual spectra per spot using 25 accepted laser shots each. Mass tolerance was set to ± 0.1 u with a maximum outlier of 50 ppm. Accepted calibration settings were used to measure sample spectra with a minimum signal to noise of 20 and mass resolution (FWHM) > 8000. The measured monoisotopic peaks were extracted into Microsoft Excel worksheets. Their absolute intensities (a_i) were normalized by calculating the ratio of a_i and the sum of absolute intensities for all compounds in the investigated *m/z* range according to $I_{rel, ai} = a_i / \Sigma (a)$. This resulted in relative intensities for the monoisotopic *m/z* 505.21 signal ($=I_{rel\ 505, ai}$) of CHX. For one sample, five replicate measurements were performed using different sample spots. Their averaged relative intensities were used for calculating CHX concentrations via the calibration curve.

3.1.5 Statistical analysis

All CHX retention values were presented as mean ± standard deviation. The data were evaluated with GraphPad Prism 6, using Repeated Measures Two-Way ANOVA; Analysis of Variance as two factors were changing during the experiments. In the first retention study, the two factors were the sampling time and oral location. In the second retention study, the two factors were the sampling time and the CHX formulation. Multiple comparisons were performed with the Tukey test to find out if there are significant differences in CHX retention between the different oral sites. The same test was also conducted to see if there are any significant retention differences between every two CHX formulations. For all comparisons, $p < 0.05$ was considered statistically significant.

3.2 Anti-biofilm study

The next *in situ* experiment was conducted to investigate the effects of CHX and OCT mouth rinses on the dental biofilm formation, as well as the biofilm disruption activity of these agents.

3.2.1 Study population

Five periodontally and systemically healthy volunteers, aged 22-36 years, were recruited (1 male, 4 female). The selected subjects were current non-smokers. They provided informed consent acknowledging their willingness to participate in this experiment. The exclusion criteria in the anti-biofilm experiment were the same criteria as the previous retention experiments. The subjects got detailed information on how to deal with the individual splints containing the enamel surfaces.

The study protocols and informed consent were done under the guidelines of the Declaration of Helsinki. The local research ethics committee of the Medical Association of Saarland, Germany, approved oral specimen collection protocols (238/03, 2016).

3.2.2 Study design

This *in situ* study examined the anti- biofilm effects of CHX in two concentrations 0.1% and 0.2% as well as 0.1% OCT on dental biofilms formed on enamel specimens using two independent evaluation protocols (Fig. 2).

For the evaluation of CHX and OCT effects on biofilm formation (Fig. 2 A), the volunteers wore the splint with eight enamel specimens for 48 h. The enamel surfaces were located in the left and right buccal areas and in the molar and premolar positions (4 specimens on each upper quadrant). Biofilm formation and bacterial vitality were evaluated at 24 and 48 h. For the biofilm disruption activity analysis (Fig. 2 B), a mature biofilm was allowed to be formed on 12 enamel slabs (6 specimens on each upper quadrant) during 48 h of intraoral exposure. Then, the volunteers started mouth rinses after the 48-h biofilm formation period. The biofilm ultrastructure and bacterial vitality were evaluated at 48 h before and directly after rinsing, and repeated at 72 h of intraoral exposure. The mouth rinsing protocol was with a 10 ml solution for 30 s every 12 h.

We asked the subjects to stop all mechanical oral hygiene procedures during the 48- or 72-h experimental time. Similarly, the application of any additional agent for chemical biofilm management was not allowed. Splints with fixed enamel surfaces were not subjected to any cleaning procedures.

For the two protocols, after the intraoral exposure periods, the enamel surfaces were shortly rinsed with distilled water and processed directly for microscopic evaluation. Half of the surfaces were evaluated with transmission electron microscopy (TEM) analysis, the other half were evaluated with fluorescence microscopy (FM) analysis. After each test week, there was a 10-day washout period to eliminate the antibacterial effects of each antiseptic in the oral cavity and to restore the normal microbial

ecosystem. During these 10 days, the subjects cleaned their teeth with normal toothpaste that includes no antimicrobial elements, and they were also permitted to use inter-dental hygiene.

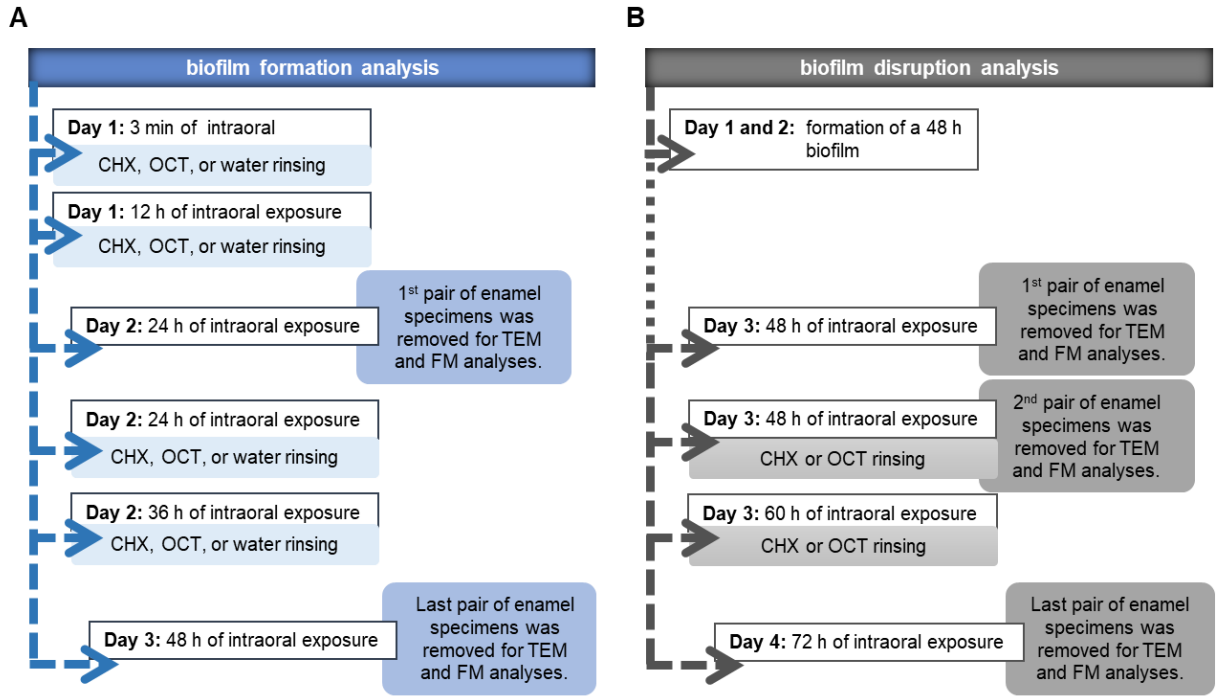

Fig. 2: Experimental set-up of the biofilm formation analysis (A) and biofilm disruption analysis (B). Both analyses were performed by all 5 subjects. Rinsing steps were carried out for 30 s at the specific time points using 10 ml of the rinsing solution, 0.2% chlorhexidine (CHX), 0.1% chlorhexidine (CHX), 0.1% octenidine (OCT), or water as a control. (TEM) transmission electron microscopy; (FM) fluorescence microscopy after vital fluorescence staining. Permission has been obtained to reuse an image published in the journal Clinical Oral Investigations (MARTÍNEZ-HERNÁNDEZ et al., 2020).

3.2.3 Specimens and splint preparation

Enamel specimens were prepared from the labial surfaces of bovine incisor teeth. Crowns of the freshly extracted cattle teeth were separated from the root at the cementum-enamel junction using a diamond cutting disc (Schott Diamantwerkzeuge GmbH, Stadtoldendorf, Germany) along with water cooling. The cutting disc was fixed to a saw machine (Conrad Apparatebau Clausthal GmbH, Clausthal-Zellerfeld, Germany). From each crown, round enamel specimens with a surface area size of 11.35 mm² were prepared for the second retention experiment. Further square specimens with a size of 4 x 4 mm² for FM analysis and 3 x 3 mm² for TEM evaluation were cut from the middle part of labial surfaces in order to standardize the enamel specimens.

The enamel surfaces were polished under continuing water cooling by a polishing machine (Buehler, Düsseldorf, Germany), using silicon carbide grinding paper (125 to 4000 grit, FEPA-P, waterproof silicon carbide paper, Buehler, Düsseldorf, Germany). Then, specimens were stored in 0.1% thymol solution (Saarland University Pharmacy, Homburg, Germany) at 4 °C.

The polished enamel specimens should be cleaned before using them in the oral cavity. According to a previously used protocol (HANNIG et al., 2005), the enamel surfaces were first washed using a 3% NaOCl (Hedinger, Stuttgart, Germany) solution for 3 min to eliminate any rest from the polishing step. Next, they were cleaned five times in distilled water (B. Braun Melsungen, Melsungen, Germany). Then, a second cleansing step was conducted for a 5-min ultrasonication (Sonorex, Bandelin, Germany). It was followed by disinfection in 70% isopropanol (Hedinger, Stuttgart, Germany) for 15 min, and then the cleaning was finished with another five washes in distilled water. In the end, and before intraoral exposure, rehydration of enamel surfaces was done by putting them in distilled water for at least 6 h.

Alginate impressions (Blueprint cremix®, Dentsply DeTrey, Konstanz, Germany) were made from the upper and lower jaws of the subjects to produce an elastic mould. Using this mould, a hard plaster model was made. Transparent custom made acrylic splints (Thermoforming foils®, Erkodent, Pfalzgrafenweiler, Germany) were made on the plaster model as a carrier of the enamel specimens. These splints were designed to cover the palatal area for the retention experiment (Fig. 1) and to cover the molar and premolar teeth on the left and right upper jaw in the anti-biofilm studies (Fig. 3).

In order to better stabilize the mounted specimens, the splints were provided with small perforations. The enamel specimens were fixed to the individual acrylic splint using silicon impression material (president light body®, Colténe, Altstaetten, Switzerland).

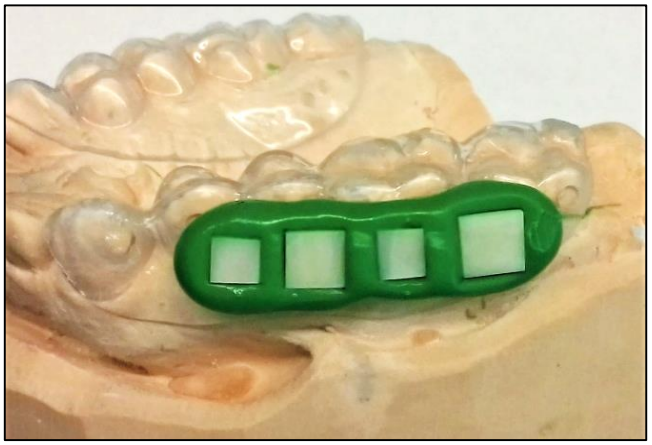

Fig. 3: Buccal acrylic splint with mounted specimens to form the biofilm samples for the anti-biofilm experiment.

3.2.4 Vital fluorescence microscopy evaluation - LIVE/DEAD staining

3.2.4.1 LIVE/DEAD staining definition

Fluorescence Microscopy analysis after LIVE/DEAD staining (LDS) is performed to distinguish and quantify living and dead microorganisms in the bacterial biofilm. The used staining system in this study (LIVE/DEAD® *Bac*Light™ Bacterial Viability kit L7012, Invitrogen by Thermo Fisher Scientific, Carlsbad, USA) consists of two stains: SYTO 9 the green-fluorescent nucleic acid dye and propidium

iodide (PI: C27H34I2N4) the red-fluorescent nucleic acid dye. These two stains vary in their spectral features and in their ability to inter the microbial cells. When the bacterial cell is not damaged and the cell membrane is intact, only the SYTO 9 will be able to enter the bacteria and be presented inside the cell, thus causing the bacterial fluorescent to be green. Whereas in the case of damaged bacteria with a compromised membrane, both stains will be able to enter the bacteria and be presented inside the cell. In this case, PI dye, which has a stronger affinity toward nucleic acids, will displace the SYTO 9 and the cells will fluoresce in red. As a final consequence of such staining, cells with an intact membrane will be stained green, whereas cells that are considered to be dead or damaged will be stained red. In the end, the percentage of bacterial adherence (coverage) and the vitality percentage can be determined.

3.2.4.2 Staining and preparing the specimens

After intraoral exposure, the biofilm-covered specimens were carefully detached from the splints, gently washed with saline and then processed for vital fluorescence staining according to the following protocol. Processing of the samples was carried out at room temperature in a 6-well-plate. At the start, the samples were covered with 10 µl of the staining solution. This solution consisted of 1 ml 0.9% NaCl (B. Braun Melsungen AG, Melsungen, Germany), 1 µl SYTO 9, and 1µl PI. After that, the samples were kept in darkness for 15 min, and then they were washed three times with saline solution to remove residues of the staining solution. Then, every specimen was fixed on a glass slide (Microscopic slides, VWR International GmbH, Darmstadt, Germany), 5 µl of mounting oil was applied on it, and then it was covered with a round cover glass (VWR International GmbH, Darmstadt, Germany) until further processing.

3.2.4.3 Fluorescence microscopic examination and images evaluation

The mounted samples were observed by a fluorescence microscope (Axio Imager 2 Microscope, Zeiss MicroImaging GmbH, Göttingen, Germany) in combination with the image processing software AxioVision 4.8 (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Four representative randomized images per specimen were taken at a magnification of 1000-fold. The biofilm colonization (coverage) and biofilm vitality were evaluated by using a digital program called ImageJ (ImageJ2, National Institutes of Health, LOCI, University of Wisconsin, USA), which is an open-source micrographs analysis program for multidimensional image data evaluation with a focus on scientific imaging.

To evaluate the bacterial colonization, the total area of the FM image was first measured and then only the selected biofilm-covered area was measured. The colonization percentage was determined by calculating the proportion of the selected biofilm area to the total image area.

For the vitality percentage, both green and red channels from the microscopic image were saved in the grey scale and then the integrated density of both channels was measured with ImageJ. After calculating the total density value of both channels, the biofilm vitality percentage (the percentage of

the green channel density) can be easily calculated. It is essential to eliminate the background effect of the images by measuring only the selected area of the living bacteria in the green channel and the damaged bacteria in the red channel. This step is imperative in avoiding any misleading results due to the background integrated density.

3.2.5 Transmission electron microscopy evaluation

3.2.5.1 Samples preparation

After intraoral exposure, the biofilm-covered enamel surfaces considered for TEM analysis were rinsed gently with sterile water and then fixed with a fixing solution for one hour at 4 ºC. The fixing solution consists of 1% glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany) and 1% paraformaldehyde (Science Services, München, Germany) in 0.1 M cacodylate buffer. Then, the samples were washed five times with 0.1 M cacodylate buffer for 10 min each and then stored in the last buffer solution at 4 ºC until further processing.

For imaging of the organic biofilm structure, specimens were post-fixed with 2% osmium tetroxide in cacodylate buffer for 1 h. Then, the samples were rinsed five times with distilled water and dehydrated in increasing concentrations of ethanol. The dehydration was done at room temperature. The specimens went through series of 50% (2x 10 min), 70% (2x 20 min), 90% (2x 30 min) and 100% (2x 30 min) ethanol. Afterward, they were further immersed in 100% acetone twice for 30 min each and kept overnight in an acetone/Araldite mixture (Agarscientific, Stansted, United Kingdom), including a 3% accelerator (mixture A) at room temperature. On the next day, mixture A was poured out and a second mixture, mixture B, was prepared (Araldite mixture with 2% accelerator). Specimens were stored again overnight in mixture B at room temperature in the air chamber. Then, a new mixture B was utilized to fill half of the embedding forms. Notes with identification numbers were inserted at the bottom part, and the embedding forms were filled until the top with mixture B, once more. Next, specimens were incubated for polymerization for 48 h at 65 °C. After polymerization, samples were decalcified in hydrogen chloride (HCl) and re-embedded in Araldite. Finally, all the samples were cut in ultra-thin sections (about 50-80 nm) in an ultramicrotome using a diamond knife (Leica EM UC7, Germany) and mounted on Pioloform-coated copper grids. Then, the samples were contrasted with aqueous solutions of uranyl acetate and lead citrate at room temperature. After an intensive rinse with distilled water, biofilm specimens are then ready to be evaluated with TEM.

3.2.5.2 TEM analysis

Characteristic images of the biofilm ultrastructure, under the influence of CHX and OCT rinsing or water rinsing (control), were performed at magnifications ranging from 2500- to 80000-fold by a TEM TECNAI 12 Biotwin (FEI, Eindhoven, Netherlands). For the biofilm thickness assessment, two representative images per specimen were evaluated at the magnifications of 11000- and 13000-fold.

Four measurements of the biofilm thickness (μm) at each micrograph were made by using ImageJ software and the average value from these measurements was taken for statistical evaluation

3.2.6 Statistical analysis

Statistical analysis for the comparison between the experimental groups in the anti-biofilm experiment was performed with GraphPad Prism 6 software package. The analysis was conducted by Repeated Measures One-Way ANOVA Analysis of Variance with Geisser-Greenhouse correction to control the violations of sphericity. One-Way ANOVA Test was followed by multiple comparisons made by the Tukey test to compare each test group with the other ones.

It is noteworthy to mention that the Shapiro-Wilk Normality Test was used to investigate if the data follow the Gaussian distribution. Additionally, the tests for equal variances were conducted using the Brown-Forsythe test. Statistical significance was set at $p < 0.05$.

4 Results

The following results were obtained through MALDI-TOF mass spectrometry, vital fluorescence microscopy, and TEM evaluations. The MALDI-TOF technique provided reliable detection limits for the determination of CHX after mouth rinsing as well as after the application of different pharmaceutical products.

The evaluation of bacterial biofilm colonization and bacterial vitality using fluorescence microscopy approach allowed the investigation of the effect of CHX and OCT mouth rinses on the oral biofilm under *in situ* conditions. Furthermore, TEM provided the possibility to study biofilm ultrastructure under the effect of the aforementioned oral antiseptics. In the current experiment, not only the effects of CHX and OCT on the biofilm formation were evaluated, but also the biofilm disruption activity of CHX and OCT on the mature biofilm was investigated.

4.1 Retention studies results

4.1.1 Chlorhexidine calibration curve

A reproducible calibration curve was created from a diluted sequence of CHX. This calibration curve in combination with the MALDI-TOF approach were used as a quantitative tool for the determination of CHX values in experimental samples. The CHX protonated monoisotopic molecule $(M_i+H^+)^+$ was detected at m/z 505.21 g/mol (Fig. 4).

The calibration curve was linear from 1.5 x 10^{-3} µg/ml to 0.39 µg/ml of CHX free base with a regression correlation coefficient of R^2 = 0.9972 (Table 1, Fig. 5). These values were the limits of quantification of CHX. Beyond 0.39 µg/ml, detection saturation was observed. Below 1.5 x 10^{-3} µg/ml, the predefined minimum S/N ratio of 20 prevented quantification of smaller signals (Fig. 6). After the determination of CHX concentration in the matrix sample mixtures, the dilution factor was applied for calculating the CHX concentrations in the undiluted oral samples.

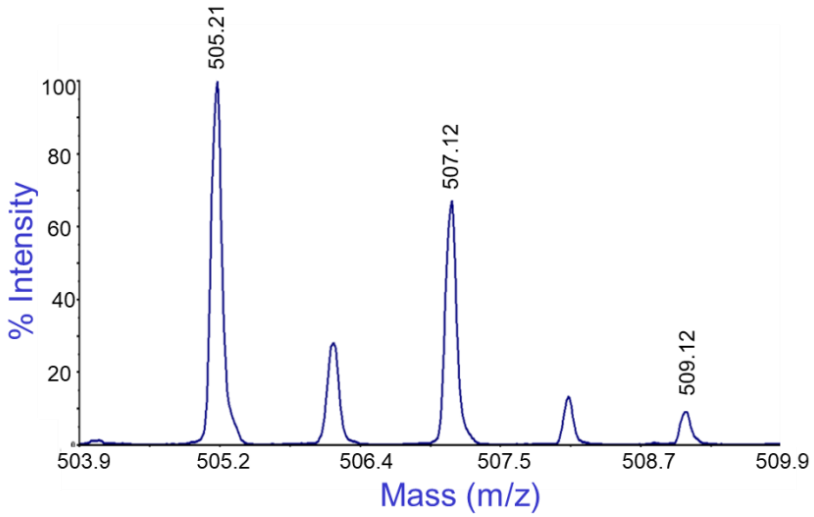

Fig. 4: Typical mass spectrum of chlorhexidine according to MALDI-TOF technique.

Table 1: The chlorhexidine (CHX) values (µg/ml) and their relative intensity (Rel. Int) where the calibration curve was linear.

CHX µg/ml	0.3905	0.1953	0.0976	0.0488	0.0244	0.0122	0.0061	0.0031	0.0015	0.0008
Rel. Int 505	0.1297	0.0702	0.0379	0.0176	0.0089	0.0028	0.0027	0.0015	0.0010	0.0000

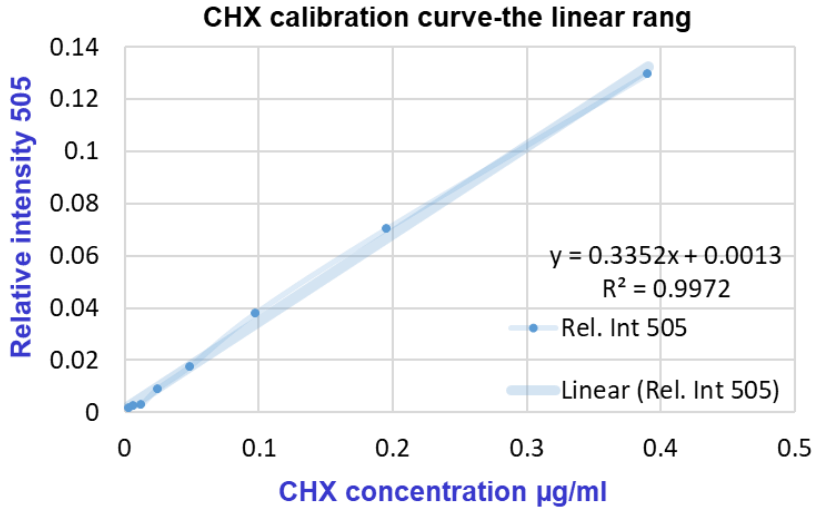

Fig. 5: Calibration curve of chlorhexidine (CHX) concentrations versus their relative intensity values. The curve ranges from 1.5 x 10^{-3} µg/ml to 0.39 µg/ml. The linear equation and the quadratic correlation coefficient are given for this range. Permission has been obtained to reuse a diagram published in the journal Clinical Oral Investigations (REDA et al., 2021b).

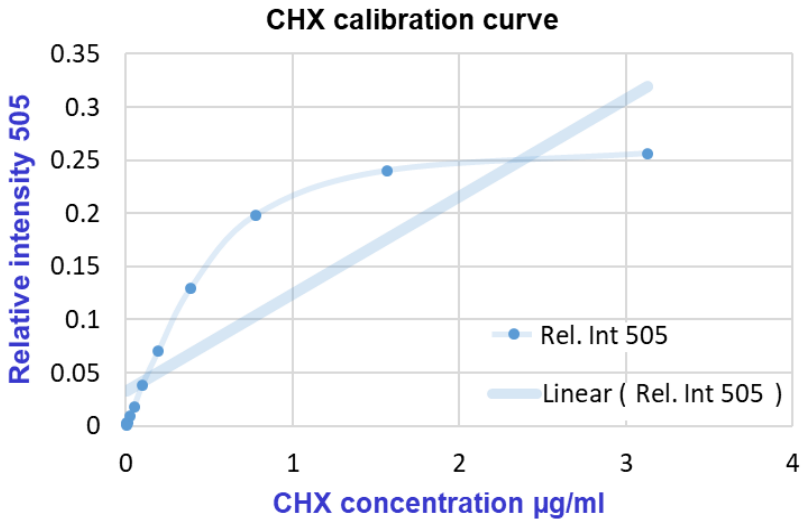

Fig. 6: The complete calibration curve of chlorhexidine (CHX) concentrations versus their relative intensity values Beyond 0.39 µg/ml, detection saturation was observed. Below 1.5 x 10^{-3} µg/ml, the predefined minimum S/N ratio of 20 prevented quantification of smaller signals.

4.1.2 Chlorhexidine retention in different oral locations after mouth rinsing

Possible discrepancies in CHX sampling efficacy was avoided due to the use of standardized micro-brushes with non-absorbent fibres in their tips for sampling from different oral sites. The micro-brush tips did not enrich CHX after the saturation of their tip absorption capacities with salivary film covering the teeth (buccal or interdental area) or mucosa. This made sampling independent from the wiped area in oral sites and enabled comparison of CHX retention in saliva to CHX retention in different oral sites.

The concentrations of oral CHX in different sites decreased with time after mouth rinsing (Fig. 7). The highest reduction in CHX-retention has happened in the first 6 h and plateaued at low concentrations in the oral cavity. It was even detected 24 h after application (Table 2).

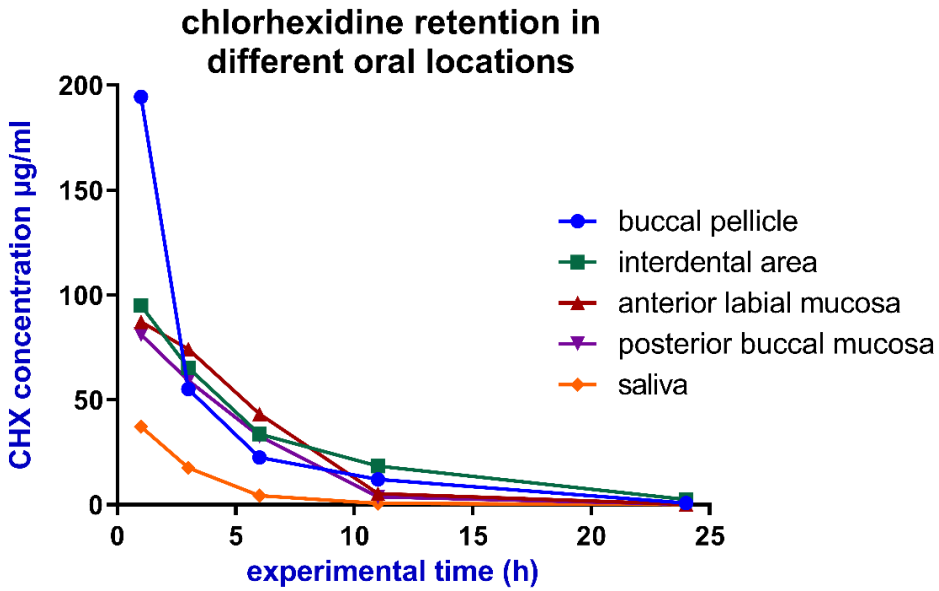

Fig. 7: Mean chlorhexidine (CHX) concentrations (µg/ml) in five different oral locations (dental pellicle, interdental area, anterior labial mucosa, posterior buccal mucosa, saliva) of five volunteers under normal diet during 24 h after mouth rinsing with 10 ml chlorhexidine solution (0.2%) for 30 s. Permission has been obtained to reuse a diagram published in the journal Archives of Oral Biology (REDA et al., 2020).

For a normal diet, CHX retention one-hour after rinsing was higher in the dental pellicle than all other oral locations, with a mean concentration of 194.5 µg/ml (Fig. 8 A). The highest substantivity of CHX was detected in the interdental area with a mean concentration of 2.4 µg/ml, 24 h after CHX rinsing. Relatively high CHX retention was observed at the mucosal surfaces (Fig. 8 C and D). No significant difference in CHX retention between anterior labial mucosa and posterior buccal mucosa was seen ($p = 0.89$). Although, no significant differences in CHX retention between the four oral locations: dental pellicle, interdental area, buccal and labial mucosa were detected ($p > 0.05$), the CHX retention in saliva was significantly lower compared to the retention in the aforementioned four oral sites ($p < 0.05$) (Table 2, Fig. 8 E).

Table 2: Chlorhexidine retention in five different oral locations (buccal dental pellicle, interdental area, anterior labial mucosa, posterior buccal mucosa, and saliva) of five volunteers during 24 h after mouth rinsing with 10 ml chlorhexidine digluconate solution (0.2%) for 30 s. Permission has been obtained to reuse a table published in the journal Archives of Oral Biology (REDA et al., 2020).

Chlorhexidine in the oral cavity (µg/ml)†, normal diet.					
time after rinsing (h)	buccal dental pellicle	interdental area	anterior labial mucosa	posterior buccal mucosa	saliva*
1	194.5 ± 59.1	94.9 ± 39.1	87.2 ± 59.8	81.1 ± 37.12	37.3 ± 15.2
3	55.1 ± 37.6	65.2 ± 28.6	74.1 ± 30.2	59.2 ± 27.25	17.6 ± 9.8
6	22.5 ± 25.5	33.7 ± 23.3	43.2 ± 25.7	32.6 ± 39.78	4.3 ± 3.1
11	12.1 ± 16.2	18.4 ± 17.3	5.3 ± 3.9	3.7 ± 3.31	0.6 ± 0.4
24	0.7 ± 0.4	2.4 ± 2.3	0.2 ± 0.1	0.2 ± 0.1	0.06 ± 0.03

† Mean concentrations (µg/ml) ± Standard Deviation.

* Chlorhexidine retention in saliva was significantly lower than the other oral locations. Statistically significant association ($p < 0.05$).

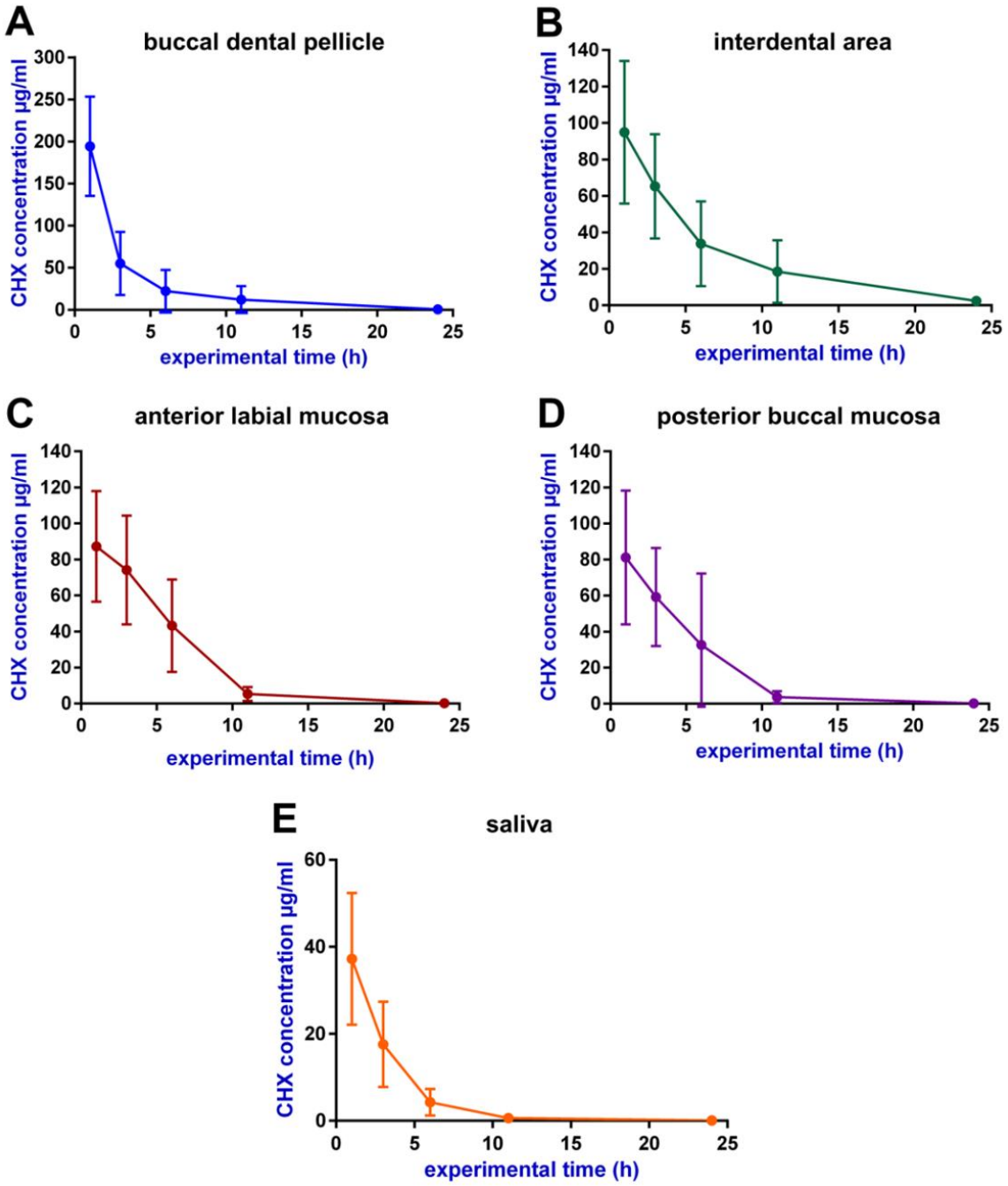

Fig. 8: Mean chlorhexidine (CHX) concentrations (µg/ml) ± standard deviation in five different oral locations (A: buccal dental pellicle, B: interdental area, C: anterior labial mucosa, D: posterior buccal mucosa, E: saliva) of five volunteers under normal diet during 24 h after mouth rinsing with 10 ml chlorhexidine digluconate solution (0.2%) for 30 s. Permission has been obtained to reuse diagrams published in the journal Archives of Oral Biology (REDA et al., 2020).

The fasting volunteer displayed unorthodox results. Retention of CHX in all oral sites was higher in comparison to the normal diet, and CHX stayed at high concentrations during the fasting period of 11 h in almost all oral sites. In the dental pellicle and mucosa, the CHX concentrations were 315.1 µg/ml and 84.2 µg/ml, respectively, 1 h after mouth rinsing, and they remained virtually the same (302.2 and 79.8 µg/ml) for 11 h (Table 3, Fig. 9).

Again, the CHX retention in saliva was significantly lower compared to the retention in the other four oral sites ($p < 0.05$) (Table 3, Fig. 9).

Table 3: Chlorhexidine concentrations (µg/ml) in five different oral locations (dental pellicle, interdental area, anterior labial mucosa, posterior buccal mucosa, and saliva) of the abstinent volunteer during 11 h after mouth rinsing with 10 ml of chlorhexidine solution (0.2%) for 30 s.

Chlorhexidine in the oral cavity (µg/ml)†, fasting.					
time after rinsing (h)	dental pellicle	interdental area	anterior labial mucosa	posteior buccal mucosa	saliva*
1	315.13	93.07	84.23	100.72	28.87
3	217.66	143.04	89.93	100.37	15.67
6	327.01	75.39	84.00	73.2	22.85
11	302.23	41.17	79.81	64.99	2.32

† Mean concentrations (µg/ml).

* Chlorhexidine retention in saliva was significantly less than the other locations. Statistically significant association ($p < 0.05$).

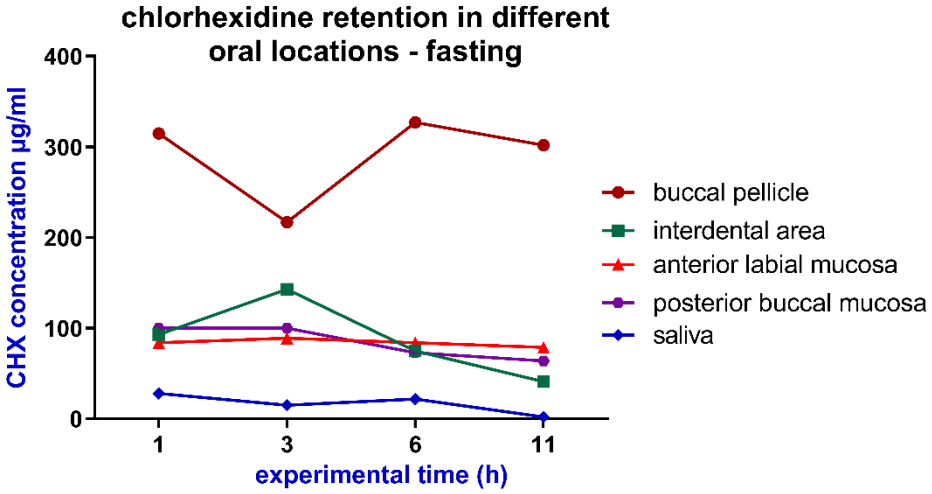

Fig. 9: Mean chlorhexidine (CHX) concentrations (µg/ml) in five different oral locations (buccal dental pellicle, interdental area, anterior labial mucosa, posterior buccal mucosa, saliva) of the abstinent volunteer during 11 h after mouth rinsing with 10 ml chlorhexidine solution (0.2%) for 30 s.

4.1.3 Chlorhexidine retention after application of different pharmaceutical regimens

The concentrations of oral CHX at different sites decreased with time after the application of different CHX protocols (Figs. 10-12).

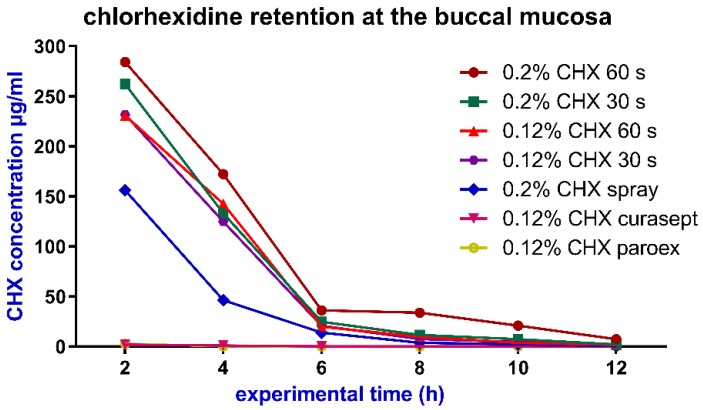

Fig. 10: The mean concentrations (µg/ml) of chlorhexidine (CHX) at the buccal mucosa over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a diagram published in the journal Clinical Oral Investigations (REDA et al., 2021b).

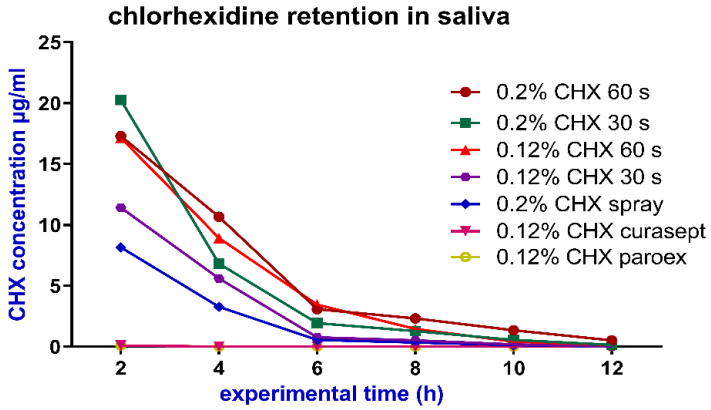

Fig. 11: The mean concentrations (µg/ml) of chlorhexidine (CHX) in saliva over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a diagram published in the journal Clinical Oral Investigations (REDA et al., 2021b).

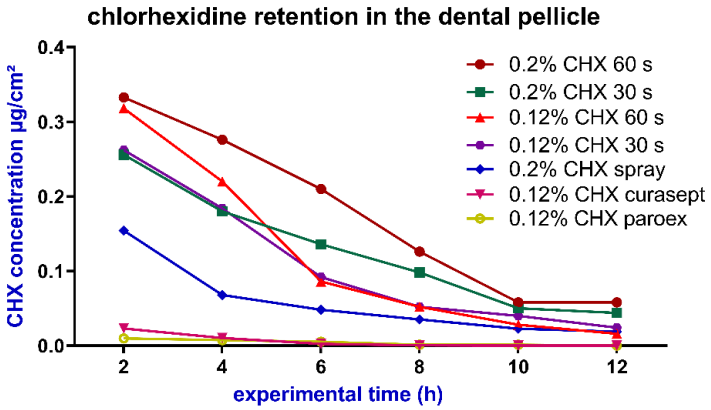

Fig. 12: The mean retention (µg/cm²) of chlorhexidine (CHX) in the dental pellicle over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a diagram published in the journal Clinical Oral Investigations (REDA et al., 2021b).

The highest decline in CHX-concentration in the oral cavity was during the first 6 h after application, and then it sustained low concentrations in the oral cavity with a level of micrograms per millilitre for at least 12 h (Tables 4 and 5).

Table 4: Chlorhexidine (CHX) retention of five volunteers at the buccal mucosa over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a table published in the journal Clinical Oral Investigations (REDA et al., 2021b).

Chlorhexidine at the buccal mucosa (µg/ml)†							
time (h)	0.2% CHX 60 s rinse	0.2% CHX 30 s rinse	0.12% CHX 60 s rinse	0.12% CHX 30 s rinse	0.2% CHX spray	0.12% CHX curasept tooth paste	0.12% CHX paroex tooth paste
2	284.1 ± 65.1	262.3 ± 78	232.9 ± 9.2	231.7 ± 25.7	156.1 ± 39.5	1.8 ± 1.9	2.7 ± 2.3
4	172.3 ± 51.4	133.1 ± 42.3	127.1 ± 64.5	124.7 ± 63.6	46.3 ± 22.2	0.9 ± 0.8	0.3 ± 0.2
6	36.2 ± 23.3	24.7 ± 15.9	19.9 ± 13.1	20.7 ± 13.1	13.8 ± 20.3	-	-
8	33.9 ± 18.8	11.6 ± 9.1	9.7 ± 5.1	7.5 ± 6.3	3.7 ± 2.4	-	-
10	20.9 ± 16.3	7.2 ± 7.3	3.6 ± 3.3	4.6 ± 2.8	2.2 ± 1.9	-	-
12	7.3 ± 5.2	2.1 ± 1.8	1.6 ± 3.3	1.3 ± 1.8	0.4 ± 0.4	-	-

† Mean concentrations (µg/ml) ± Standard Deviation.

- = chlorhexidine less than the limit of quantification

Table 5: Chlorhexidine (CHX) retention of five volunteers in saliva over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a table published in the journal Clinical Oral Investigations (REDA et al., 2021b).

Chlorhexidine in saliva (µg/ml)†							
time (h)	0.2% CHX 60 s rinse	0.2% CHX 30 s rinse	0.12% CHX 60 s rinse	0.12% CHX 30 s rinse	0.2% CHX spray	0.12% CHX curasept tooth paste	0.12% CHX paroex tooth paste
2	17.3 ± 10.7	25.7 ± 17.7	17.2 ± 6.8	11.4 ± 5.5	7.5 ± 5.6	0.1 ± 0.1	0.1 ± 0.1
4	10.7 ± 6.8	6.8 ± 4.7	8.9 ± 8.5	5.6 ± 3.9	3.3 ± 3.9	-	-
6	4.6 ± 4.2	1.9 ± 1.6	3.4 ± 3.9	0.6 ± 0.3	0.6 ± 0.5	-	-
8	2.3 ± 2.8	1.3 ± 1.2	1.4 ± 1.5	0.47 ± 0.3	0.4 ± 0.3	-	-
10	1.4 ± 1.2	0.5 ± 0.6	0.4 ± 0.2	0.07 ± 0.03	0.1 ± 0.2	-	-
12	0.5 ± 0.6	0.2 ± 0.1	0.2 ± 0.2	0.02 ± 0.03	-	-	-

† Mean concentrations (µg/ml) ± Standard Deviation.

- = chlorhexidine less than the limit of quantification

After the application of different formulations, there was significantly higher retention of CHX at the buccal mucosa compared with the retention in saliva (Tables 4 and 5; Fig. 13)

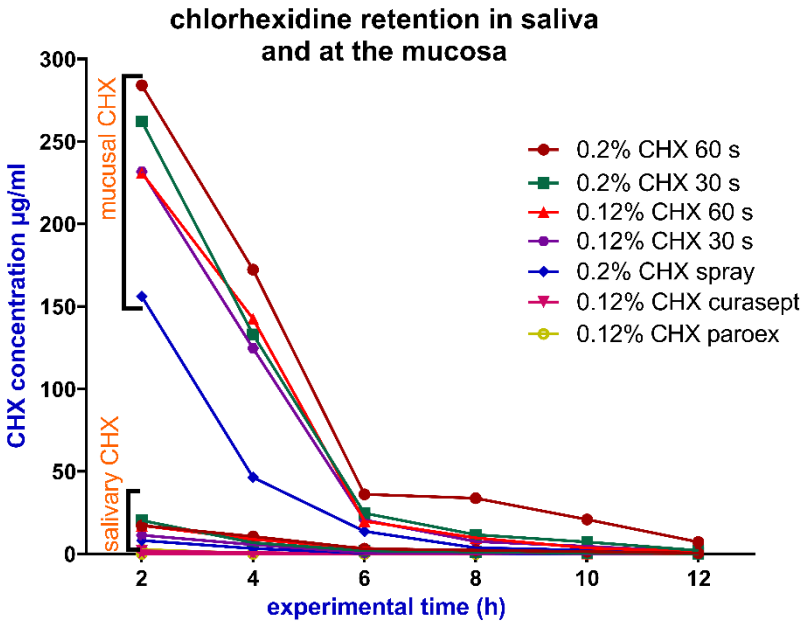

Fig. 13: The mean concentrations (µg/ml) of chlorhexidine (CHX) at the buccal mucosa in comparison with saliva over 12 h after application of different chlorhexidine formulations.

After mouth rinsing, there were relatively higher retentions of CHX in the mucosal pellicle, saliva, and dental pellicle when the rinse was performed either at a higher concentration or for a longer rinsing time. For example, 12 h after mouth rinsing there was higher substantivity in the mucosal pellicle when 0.2% of CHX was used for 60 s (7.3 ± 5.2 µg/ml) comparing with the concentration when 0.12% CHX was used for 60 s (1.6 ± 3.3 µg/ml) (Table 4). Furthermore, the difference was statistically significant in some cases such as between 0.2% for 60 s and the 0.12 % for 60 s ($p = 0.015$), as well as between 0.2% for 60 s and 0.12% for 30 s ($p = 0.005$).

Similarly, both the application of higher concentration and longer rinsing time cause a more CHX retention in saliva and the dental pellicle (Tables 5 and 6). However, the difference between the rinsing protocols in these locations was not statistically significant ($p = 0.49$ in saliva and $p = 0.16$ in the pellicle).

Interestingly, considerable CHX retention was detected after spray application. This retention lasted in the oral cavity for at least 12 h. Furthermore, there were no significant differences in CHX retention between the spray application and the rinsing regimens in saliva and dental pellicle except with 0.2% CHX rinsing for 60 s ($p = 0.03$, $p = 0.005$; respectively). However, in the buccal mucosa pellicle, the differences between the spray application and the four rinsing regimens were statistically significant.

In the present study, the retention of CHX changed as the dosage of the CHX in different regimens changes. In other words, when the dosage of the drug raised from 1.2 mg in the toothpaste to 3.28 mg in the spray, to 12 mg in the 0.12% mouth rinse, and 20 mg in the 0.2% mouth rinse, the mean CHX retention in the mucosal pellicle increased from 2.7 µg/ml after tooth brushing, to 156.1 µg/ml after spray application, to 232.9 µg/ml after 0.12% mouth rinse, and to 284.1 µg/ml after 0.2% CHX rinsing at the 2-h time point (Table 4).

Similarly, this condition of increased retention in accordance with higher dosage at application was also seen in saliva and the pellicle (Tables 5 and 6). For example, as seen in Table 6, there was significantly lower adsorption of CHX in the dental pellicle when the toothpastes were used (0.01 - 0.02 µg/cm²) compared with the retention obtained when the different rinsing protocols were applied (0.25 - 0.33 µg/cm²) at 2-h time point.

Table 6: Chlorhexidine (CHX) retention of five volunteers in the dental pellicle over 12 h after application of different chlorhexidine formulations. Permission has been obtained to reuse a table published in the journal Clinical Oral Investigations (REDA et al., 2021b).

	Chlorhexidine in the dental pellicle (µg/cm²)†						
time (h)	0.2% CHX 60 s rinse	0.2% CHX 30 s rinse	0.12% CHX 60 s rinse	0.12% CHX 30 s rinse	0.2% CHX spray	0.12% CHX curasept tooth paste	0.12%CHX paroex tooth paste
2	0.33 ± 0.15	0.25 ± 0.09	0.32 ± 0.25	0.26 ± 0.15	0.15 ± 0.11	0.02 ± 0.02	0.01 ± 0.01
4	0.28 ± 0.09	0.18 ± 0.08	0.22 ±0.17	0.18 ± 0.11	0.07 ± 0.04	0.01 ± 0.01	0.01 ± 0.01
6	0.21 ± 0.15	0.13 ± 0.06	0.09 ± 0.03	0.09 ± 0.08	0.05 ± 0.04	-	-
8	0.12 ± 0.06	0.10 ± 0.04	0.05 ± 0.04	0.05 ± 0.03	0.04 ± 0.04	-	-
10	0.06 ± 0.02	0.05 ± 0.04	0.03 ± 0.02	0.04 ± 0.03	0.02 ± 0.02	-	-
12	0.06 ± 0.01	0.04 ± 0.03	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	-	-

† Mean retention (µg/cm²) ± Standard Deviation.

- = chlorhexidine less than the limit of quantification.

4.2 Anti-biofilm study results

4.2.1 Chlorhexidine and octenidine effects on biofilm formation

To analyse the effect of CHX and OCT rinsing on *in situ* biofilm formation on real enamel surfaces, five volunteers wearing splints with attached bovine enamel specimens applied the mouth rinses with 0.1% CHX, 0.2% CHX, 0.1% OCT, or water as a control every 12 h during a 48-h time period. At two time points, 24 h and 48 h, the enamel surfaces were taken and the resulting biofilms were evaluated by FM after staining with fluorescent dyes, which allows the bacterial vitality evaluation. Furthermore, the thickness and morphology of the dental biofilm were assessed by TEM (Fig. 2 A).

4.2.1.1 FM-evaluation

The effect of the CHX and OCT rinsing on the oral bacterial colonization was evaluated successfully using fluorescence microscopy at 24- and 48-h time intervals. Representative images of the bacterial colonization at 24 and 48 h of intraoral exposure are presented in figures 14 and 15, respectively. A thick biofilm containing high proportions of living microorganisms was seen on the control surfaces (rinsed with water) after 24 h of intraoral exposure (Fig. 14 A and B). In addition to the bacteria with coccoid morphology which are the most common microorganism in the oral biofilm, fibrils and rod-shaped bacteria were also visible. In contrast, scattered microbial colonies or isolated microorganism were found on CHX- and OCT-rinsed specimens (Fig. 14 C-H). Rinsing with 0.2% CHX reduced the microbial colonization and induced a significant reduction in bacterial vitality. CHX rinsing with a concentration of 0.1% was also able to reduce the bacterial vitality and reduce the bacterial adhesion. Oral rinsing with 0.1% OCT was even more effective and able to almost completely eliminate bacterial colonization in comparison with the water control (Fig. 14 G and H, Fig. 16 A and B).

In this context, the reduction in the mean values of oral bacteria colonization was from 20% in the control group to 1.5%, 4.5%, and 0.4% in the 0.2% CHX, 0.1% CHX, or 0.1% OCT groups, respectively. Similarly, the reduction in bacterial vitality decreased from 70% in the control to 4%, 12.3%, and 1.25% in the 0.2% CHX, 0.1% CHX, and 0.1% OCT, respectively.

After 48 h of intraoral exposure (Fig. 15), mature and multi-layered biofilm containing a high portion of living microorganism was seen on the control samples (Fig. 15 A and B). In contrast, a significant reduction in biofilm coverage with few isolated dead bacteria was observed on the 0.2% CHX treated surfaces (Fig. 15 C and D). Also, 0.1% CHX rinse was very effective to reduce bacterial adhesion and only a few bacterial colonies were seen on the enamel surfaces (Fig. 15 E and F). Additionally, bacterial vitality after rinsing with both CHX concentrations was significantly reduced (Fig. 16 D). Similarly, after rinsing with 0.1% OCT, there was a significant reduction in biofilm coverage and bacterial vitality when compared with water rinsing (Fig. 16 C and D). This reduction was similar to the 0.2% CHX effect. There was almost no bacterial adhesion on the OCT treated surfaces (Fig. 15 G and H).

The reduction in the biofilm coverage was from 63% in the control to 1.15%, 11.3%, and 0.5% in the 0.2%, 0.1% CHX, and 0.1% OCT groups, respectively. Furthermore, the reduction in bacterial vitality was from 71.5% in the control biofilms to 4.5%, 11.5%, and 1.9% in the 0.2% CHX, 0.1% CHX, and 0.1 %OCT groups, respectively.

After comparing the different treatment regimens concerning the colonization and vitality, the results are summarized as follows: At 24-h time point, there was no significant difference between both concentrations of CHX and OCT regarding the biofilm coverage, whereas there was a significant difference between 0.1% CHX and 0.1% OCT ($p = 0.0036$) in regards of the biofilm vitality (Fig.16 A and B). Whereas, at the 48-h evaluation time point, 0.2% CHX and 0.1% OCT were more effective than 0.1% CHX in reducing the bacterial colonization and biofilm coverage ($p = 0.021$, $p = 0.016$; respectively). Whereas, there was no significant difference in biofilm vitality reduction between the rinses at this point (Fig. 16 C and D).

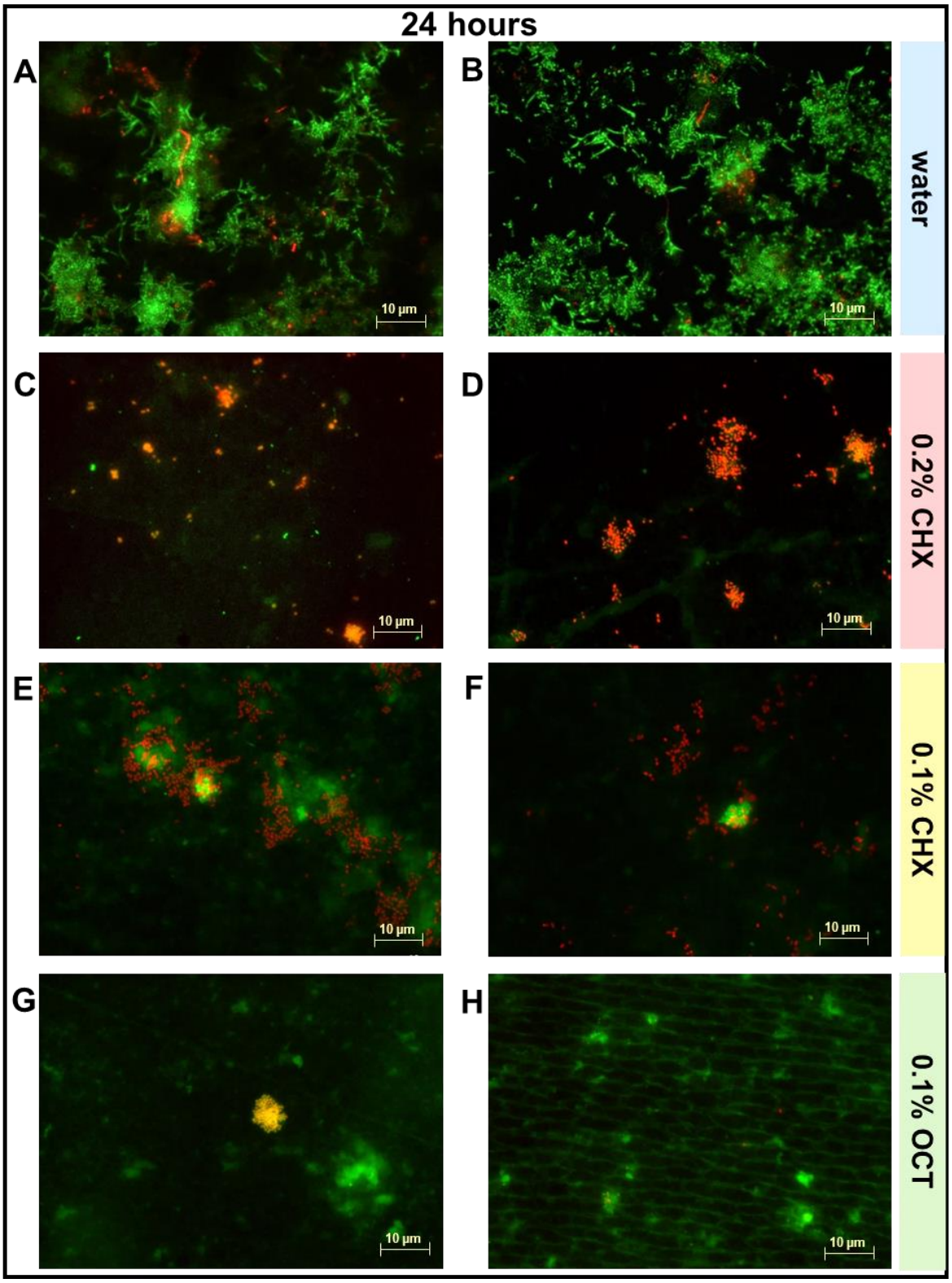

Fig. 14: Effect of chlorhexidine (CHX) and octenidine (OCT) on biofilm formation. The figure shows representative fluorescence images after LIVE/DEAD staining (living bacteria are stained green and dead bacteria are stained red) of the 24-h biofilm formed on enamel surfaces after two times rinsing with water (A and B) covered with vital biofilm, as well as enamel surfaces after two times rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) covered with scattered dead bacteria. Permission has been obtained to reuse images published in the Journal of Dental Research (REDA et al., 2021a).

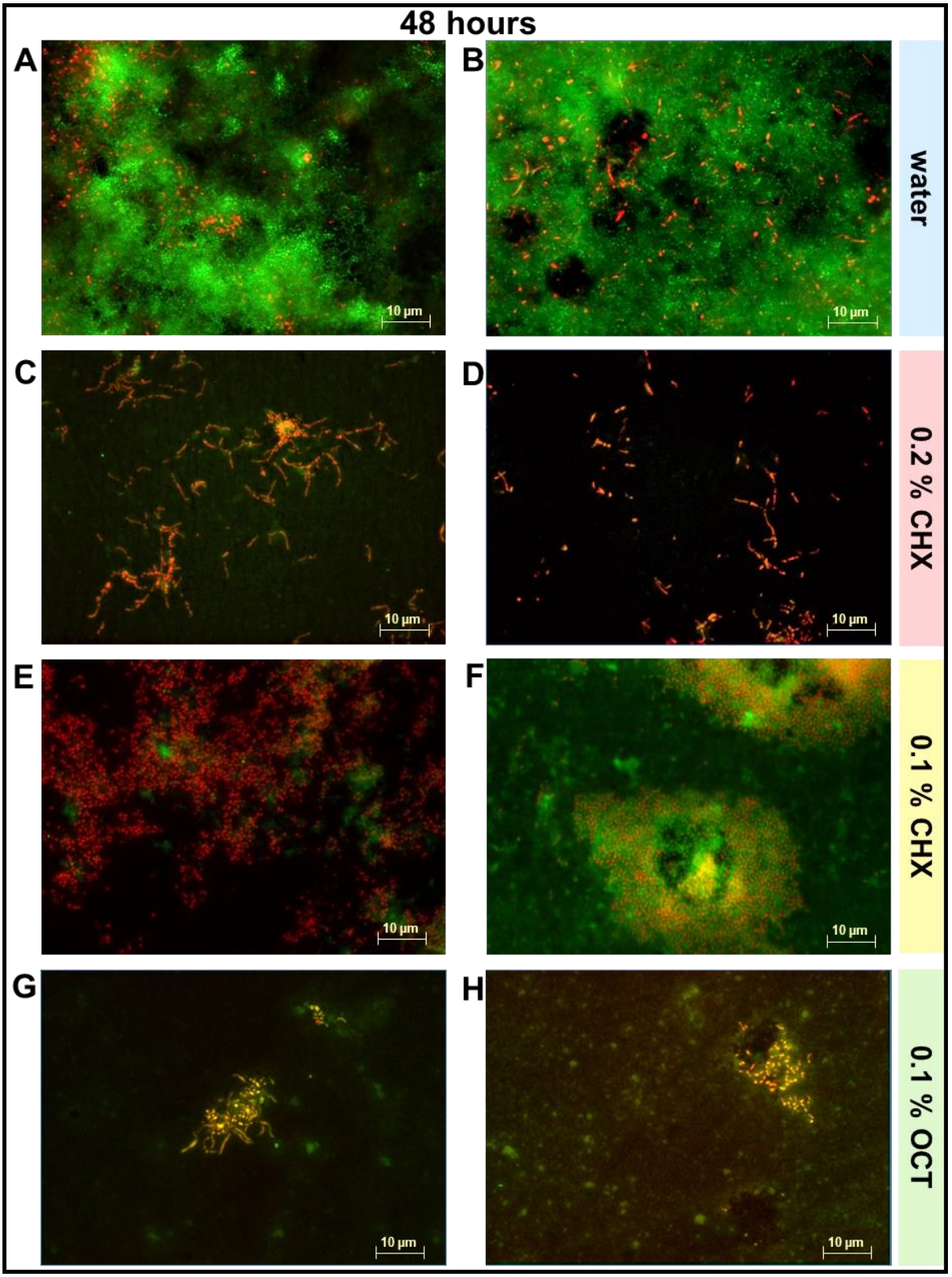

Fig. 15: Effect of chlorhexidine (CHX) and octenidine (OCT) on biofilm formation. The figure shows representative fluorescence images after LIVE/DEAD staining (living bacteria are stained green and dead bacteria are stained red) of the 48-h biofilm formed on enamel surfaces after 4 times rinsing with water (A and B) covered with vital mature biofilm, as well as enamel surfaces after 4 times rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) covered with scattered dead bacteria. Permission has been obtained to reuse images published in the Journal of Dental Research (REDA et al., 2021a).

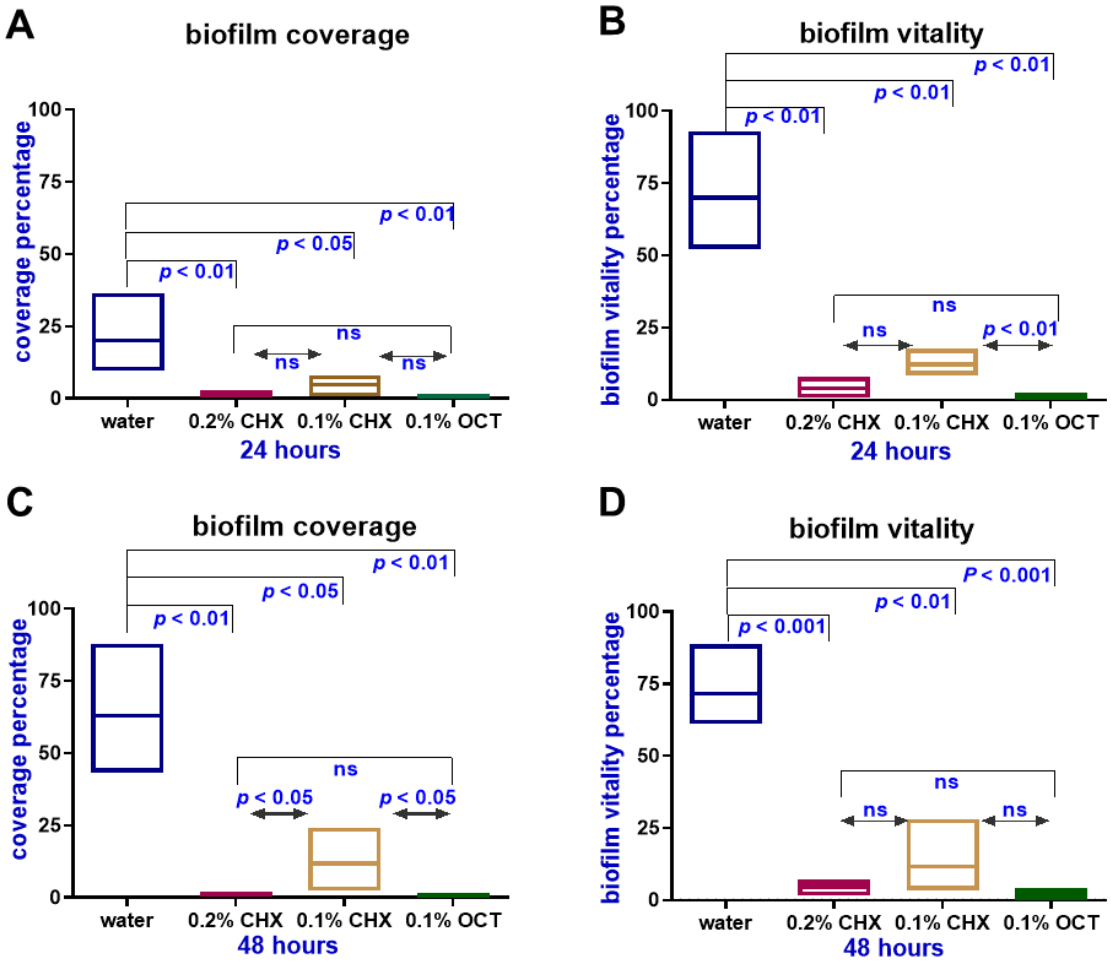

Fig. 16: Quantification of biofilm coverage and bacterial vitality at 24 h (A and B) and 48 h (C and D). In comparison with water rinsing, chlorhexidine (CHX) and octenidine (OCT) rinsing solutions caused a significant reduction in biofilm coverage and biofilm vitality at both time-points. Analysed were 320 independent images (160 images at each time point); min to max values are shown as floating bars and the middle line refers to the mean. Statistically significant association: ($p < 0.05$). ns: not significant.

4.2.1.2 TEM-evaluation

The TEM images show clear differences in the thickness and ultrastructural appearance of the biofilm found on the control specimens (rinsed with water) compared to the biofilm found on the test specimens (rinsed with CHX and OCT) at 24 and 48 h of intraoral exposure (Figs. 17-19).

All enamel surfaces after 24 h of intraoral exposure were covered by a globular structured pellicle layer with adherent microorganism. The pellicle of the control specimens was covered either partly or entirely with a multi-layer of bacteria. The majority of the bacteria observed were cocci in addition to some short rod-shaped bacteria (Fig. 17 A and B). They appeared generally intact and well preserved, but in some cases, lysed microorganisms were also observed. In contrast, on the CHX rinsed specimens, the pellicle surface was, in most places, not colonized by microorganisms and when bacteria were attached to the surface, it was only a monolayer of microorganisms. Most of the detected microorganisms revealed prevalently a coagulated cytoplasm and compromised cell membrane

(Fig. 17 C-F). Also, some lysed bacteria (cell ghosts) were found (Fig. 17 C). In agreement with the FM images, no bacterial colonization was seen on the test surfaces rinsed with 0.1% OCT. The specimens were covered only by a dental pellicle layer (Fig. 17 G and H).

The thickness of the pellicle layer and microbial deposits varied between the control and test specimens (Fig. 20 A). On the specimens rinsed with water, a biofilm with a thickness of 3.617 ± 1.735 µm was observed covering the surfaces. Whereas the biofilm thickness significantly decreased after rinsing with the test solutions at 24 h of intraoral exposure evaluation. This thickness was (1.15 ± 0.18 µm) by using 0.2% CHX, (0.50 ± 0.26 µm) by using 0.1% CHX, and (0.73 ± 0.11) in case of 0.1% OCT. There was a statistically significant difference between the control and treated surfaces concerning biofilm thickness, whereas there was no significant difference between the rinsing protocols (Fig. 20 A).

After 48 h of intraoral exposure (Figs. 18 and 19), it was observed that a pellicle layer covered all specimens. On the control surfaces, (Fig. 18 A-D) a multilayer biofilm was observed coating the surface of the specimens. The majority of the microorganisms appeared generally intact and well preserved. Furthermore, some of them were trying to multiply (Fig. 18 D). Whereas on the CHX treated specimen, (Fig. 18 E–H, Fig. 19 A and B) a pellicle layer was observed covering these surfaces with some microorganisms. These bacteria were seen prevalently damaged with a coagulated cytoplasm. Furthermore, some lysed bacteria were found (cell ghosts) (Fig. 18 G). Again, in the 48 h evaluation point, no bacterial adhesion was detected on the treated surfaces with 0.1% OCT (Fig. 19 C and D).

The biofilm thickness on the test surfaces was significantly reduced ($p < 0.0001$) in comparison with the thickness on control specimens (Fig. 20 B). The biofilm thickness was 1.49 ± 0.44 µm on 0.2% CHX treated surfaces, 1.37 ± 0.29 on 0.1% CHX treated surfaces and 0.54 ± 0.09 on 0.1% OCT treated surfaces *vs.* 12.32 ± 6.58 µm in control surfaces.

According to the TEM analysis, there were significant differences between the biofilm found on the control specimens and the biofilm on the treated specimens with CHX or OCT regarding the thickness and ultrastructural appearance. These differences were found at both evaluation time points, after 24 and 48 h *in situ*. Whereas, there were no significant differences between the oral rinses regarding the biofilm thickness at both time points (Fig. 20). The TEM results are in good agreement with the FM findings observed in the present study and underline the evident anti-biofilm potential of CHX and OCT solutions under most natural conditions *in situ*.

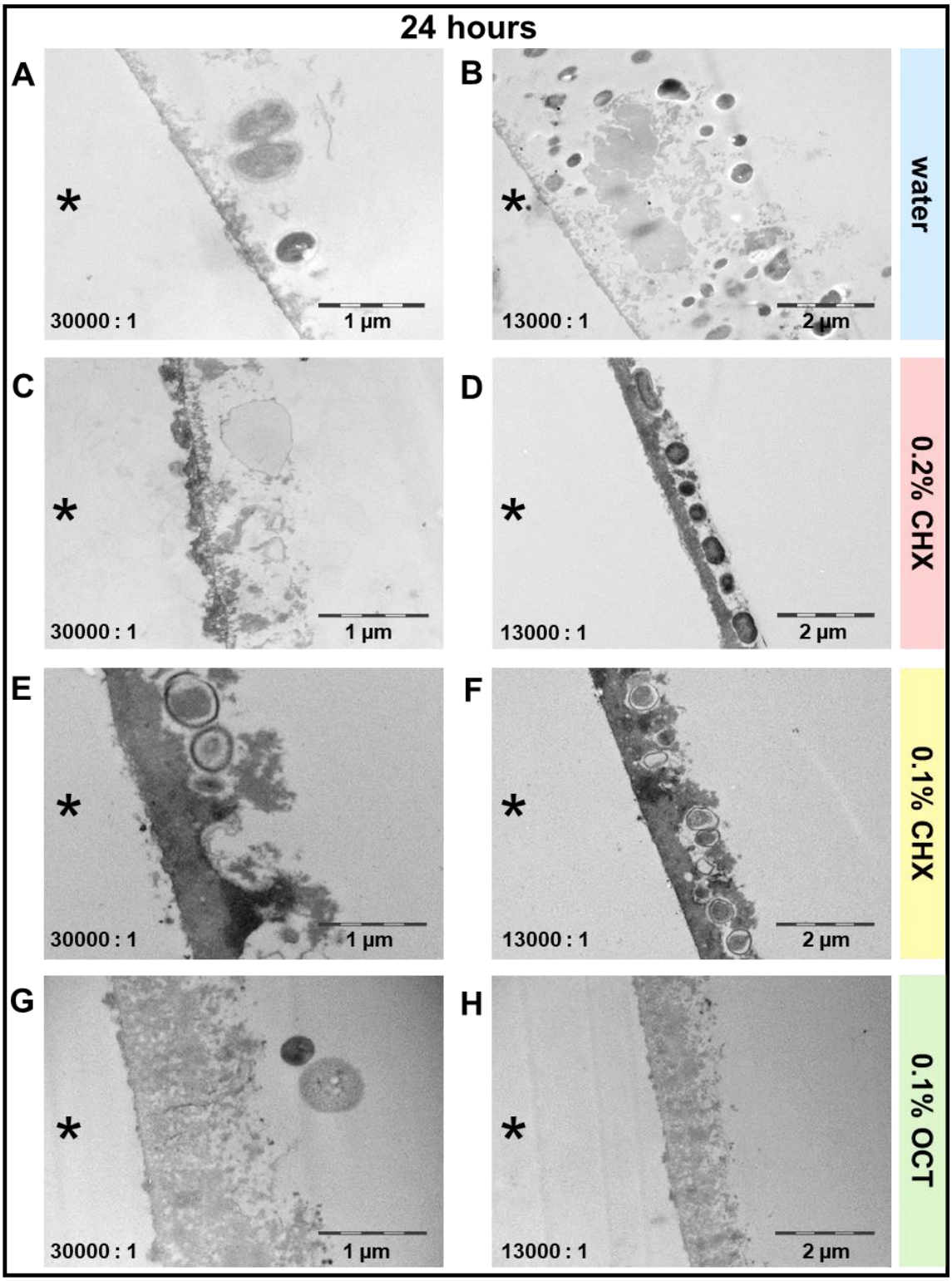

Fig. 17: Effect of chlorhexidine (CHX) and octenidine (OCT) on 24-h biofilm formation. The figure shows representative TEM micrographs of the 24-h biofilm formed on enamel surfaces after 2-times rinsing with water (A and B) covered with multi-layered vital biofilm, as well as TEM micrographs of enamel surfaces after 2-times rinsing with 0.2% CHX (C and D) and 0.1% CHX (E and F) covered with mono-layered biofilm with damaged bacteria. The figure shows also that there was no bacterial coverage on the surfaces after rinsing with 0.1% OCT (G and H). * Previous enamel surface. Permission has been obtained to reuse images published in the Journal of Dental Research (REDA et al., 2021a).

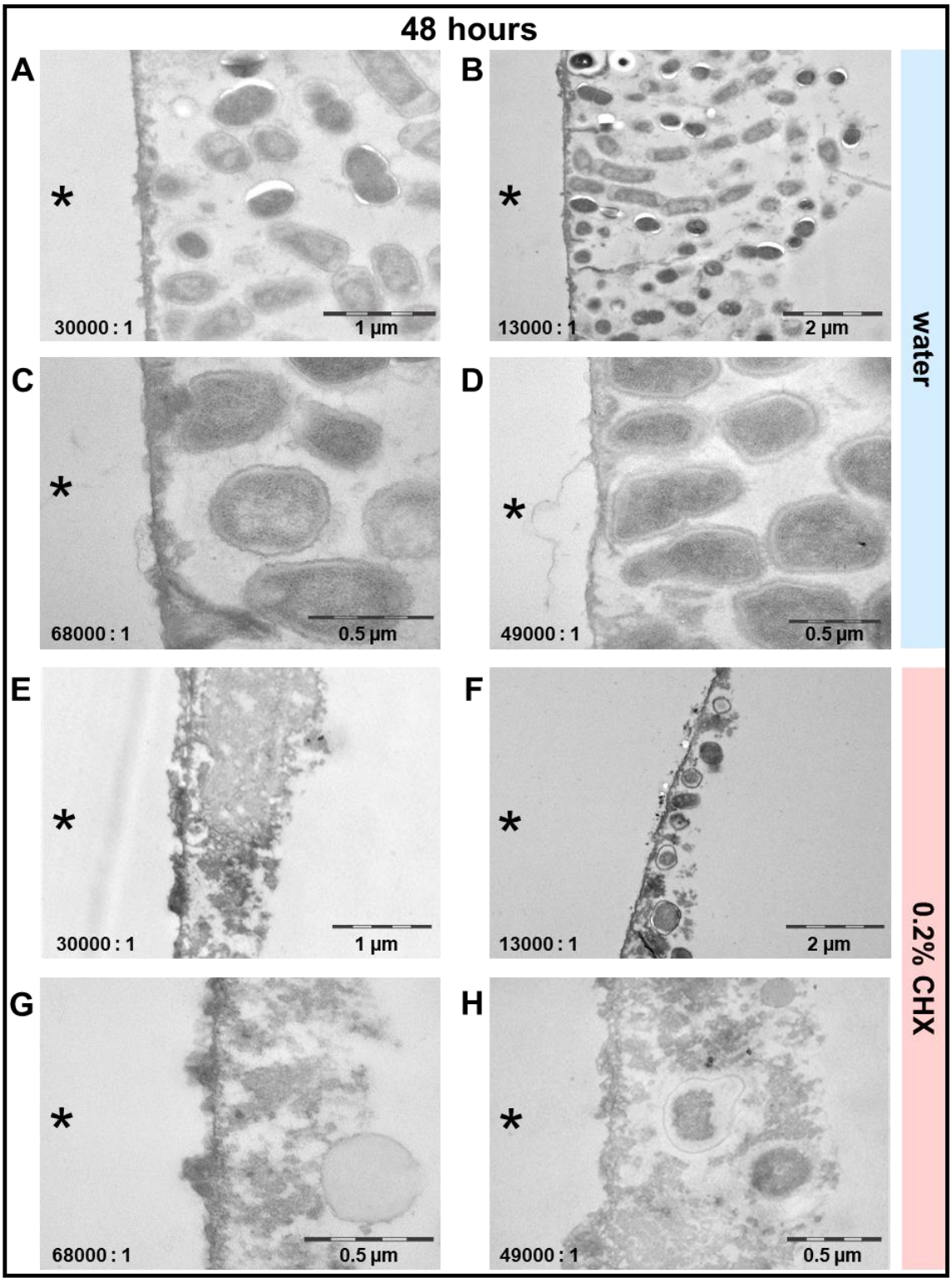

Fig. 18: Effect of 0.2% chlorhexidine (CHX) on 48-h biofilm formation. The figure shows representative TEM micrographs of the 48-h biofilm formed on enamel surfaces after 4-times rinsing with water (A-D) covered with multi-layered vital biofilm, as well as TEM micrographs of enamel surfaces after 4-times rinsing with 0.2% CHX (E-H) covered with mono-layered biofilm with damaged bacteria (cell ghosts). * Previous enamel surface. Permission has been obtained to reuse images published in the journal Clinical Oral Investigations and Journal of Dental Research (MARTÍNEZ-HERNÁNDEZ et al., 2020; REDA et al., 2021a).

.

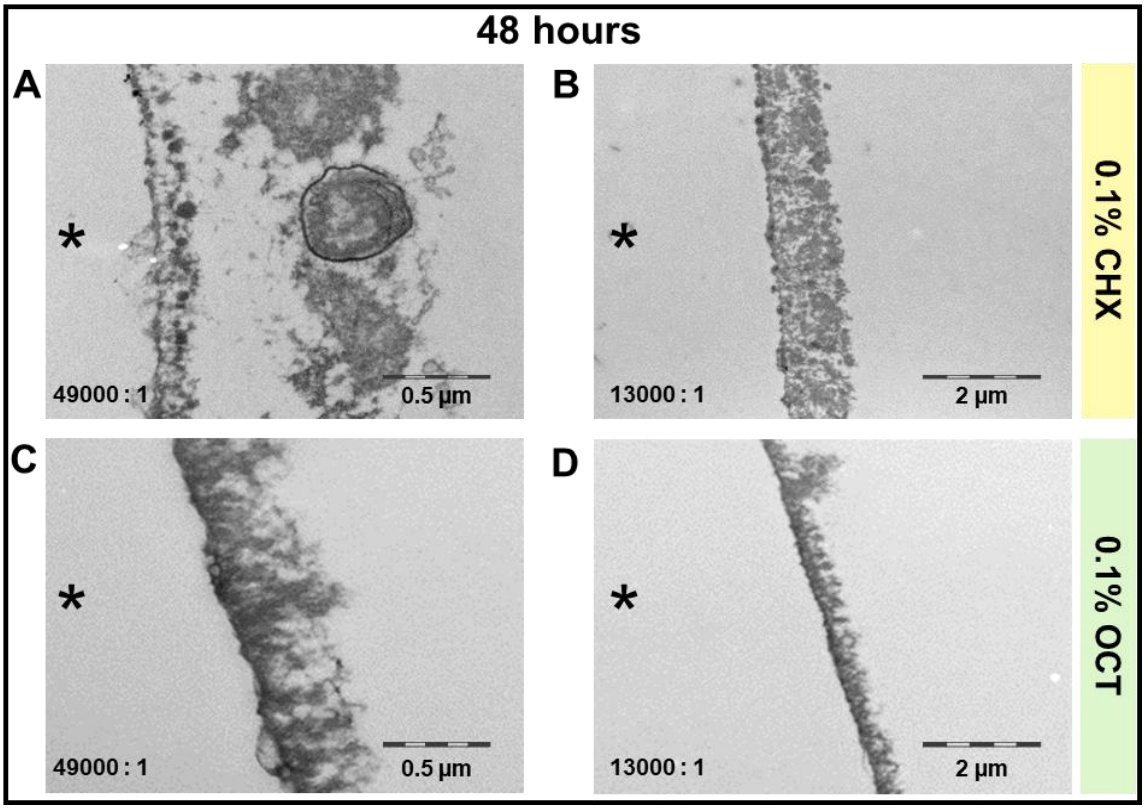

Fig. 19: Effect of 0.1% chlorhexidine (CHX) and 0.1% octenidine (OCT) on 48-h biofilm formation. Representative TEM micrographs of the 48-h biofilm formed on enamel surfaces after 4-times rinsing with 0.1% CHX (A and B), or 0.1% OCT (C and D) without any bacterial coverage. * Previous enamel surface. Permission has been obtained to reuse images published in the Journal of Dental Research (REDA et al., 2021a).

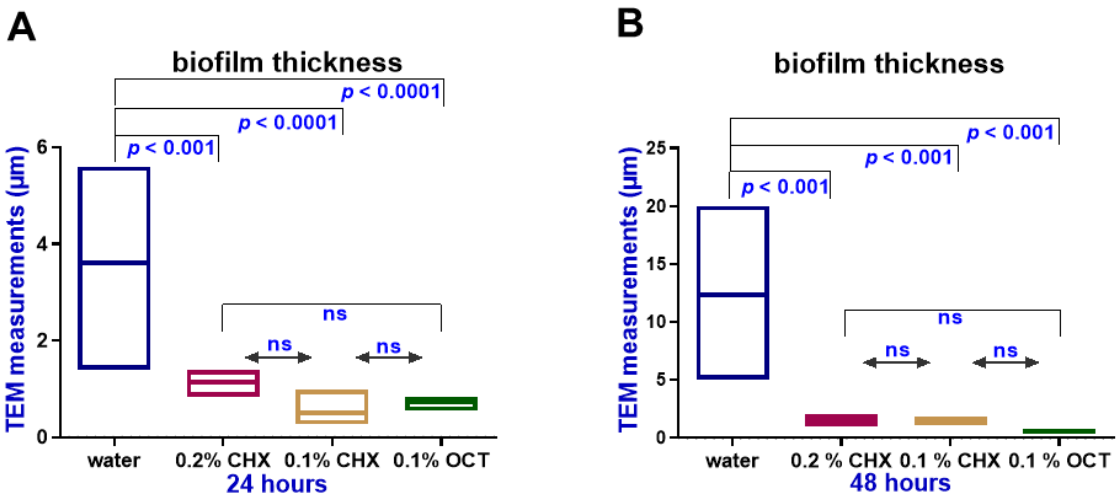

Fig. 20: Quantification of biofilm thickness at 24 h (A) and 48 h (B). In comparison with water rinsing, chlorhexidine (CHX) and octenidine (OCT) rinsing solutions caused a significant reduction in the biofilm thickness at both time points. Mean values from analyses of 80 micrographs (40 at each time point) are shown. Min to max values are shown as floating bars and the middle line refers to the mean. Statistically significant association: ($p < 0.05$). ns: not significant.

4.2.2 Chlorhexidine and octenidine disruption effect on mature biofilm

The disruption potential of CHX and OCT on preformed mature biofilm was evaluated by two times application of mouth rinse on a 48-h biofilm over a period of 24 h and a total of 72 h intraoral exposure. Again, the evaluation was performed by using FM and TEM approaches.

4.2.2.1 FM-evaluation

The disruption effect of CHX and OCT rinses on the existent mature biofilm assessed by FM can be seen in figures 21 and 22. Before the rinsing (at 48 h of intraoral exposure), matured, dense, and multi-layered biofilms were seen covering the entire specimen of the control surfaces (Fig. 21 A and B). Microorganisms were identified as having a coccoid, and rod-shaped form. In contrast, a significant shift to lower vitality was observed in the 48-h biofilm after the first CHX or OCT application (Fig. 21 C-H). Rinsing with 0.2% CHX and 0.1% OCT solutions were able to disrupt the multi-layered mature biofilm. Furthermore, a statistically significant reduction in the biofilm coverage was detected and some parts of the enamel specimens were clean without any bacterial accumulation especially after the second rinsing (Fig. 22 C-D, Fig. 22 G-H). However, the 0.1% CHX was effective in biofilm disruption, but less than the 0.2% CHX or 0.1% OCT rinses. It is clear in the FM evaluation (Fig. 21 E and F, Fig. 22 E and F) that even though the specimens treated with 0.1% CHX are fully covered with bacteria, but the maturity and the complexity of the biofilm were less dominant.

After the statistical analysis and comparing the different treatment regimens, it is important to note that the higher concentration of CHX (0.2%) was significantly more active in reducing the bacterial vitality and biofilm coverage than 0.1% CHX. Moreover, according to the fluorescence images, 0.2% CHX had the most disruptive activity against the 48-h mature biofilm especially after the second rinsing (Fig. 22 C and D). However, there was no significant difference between 0.2% CHX and 0.1% OCT in both the vitality and coverage parameters (Fig. 23). Thus, rinses with 0.2% CHX and 0.1% OCT have great biofilm disruptive potential.

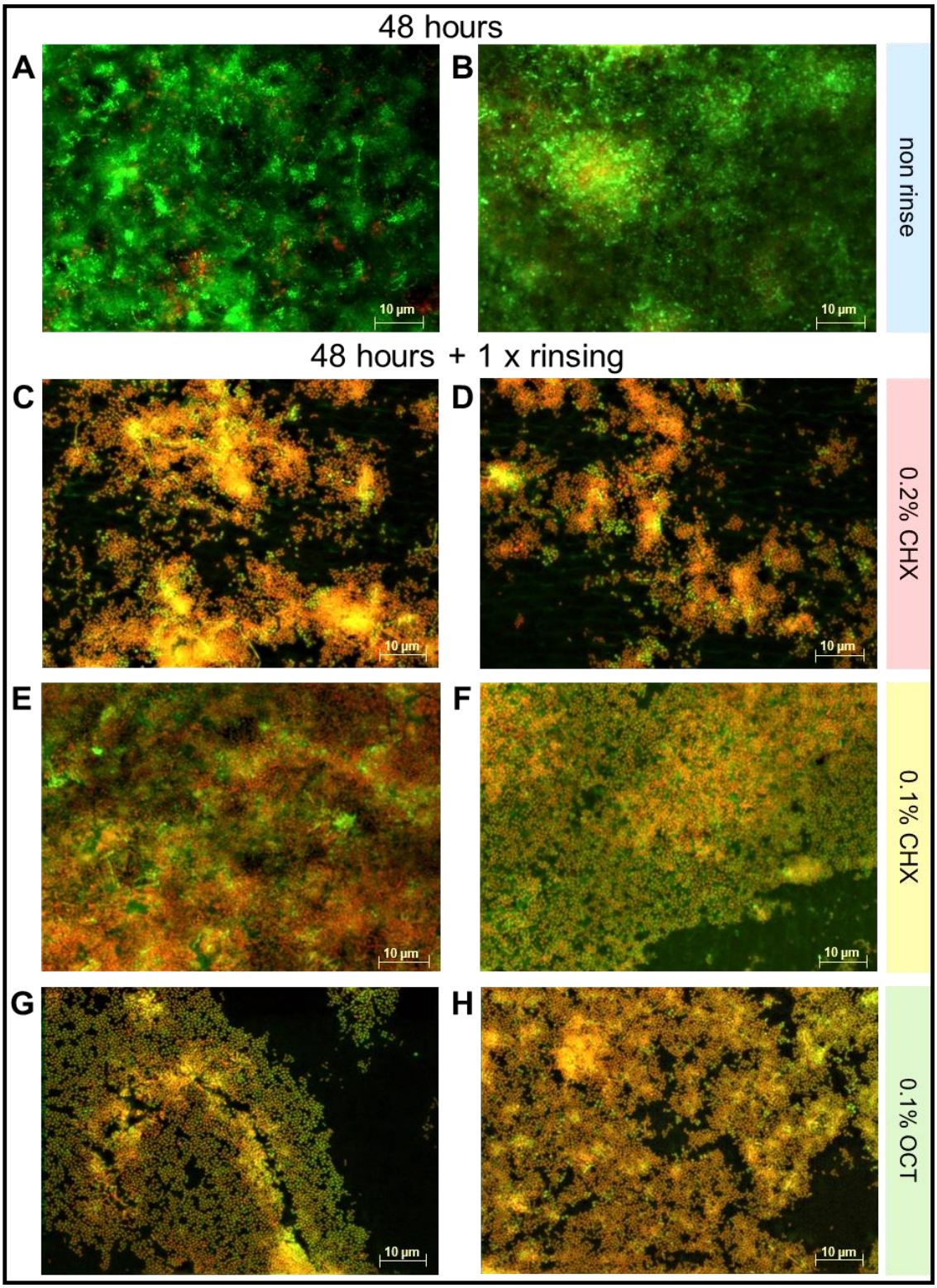

Fig. 21: Effect of chlorhexidine (CHX) and octendine (OCT) on biofilm disruption. The figure shows representative fluorescence images after LIVE/DEAD staining (living bacteria are stained green and dead bacteria are stained red) of the 48 h-biofilm formed on enamel surfaces before rinsing (A and B) with vital mature biofilm as well as directly after one-time rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) with a monolayer of non-vital bacteria. Permission has been obtained to reuse images published in the journal Clinical Oral Investigations and Journal of Dental Research (MARTÍNEZ-HERNÁNDEZ et al., 2020; REDA et al., 2021a).

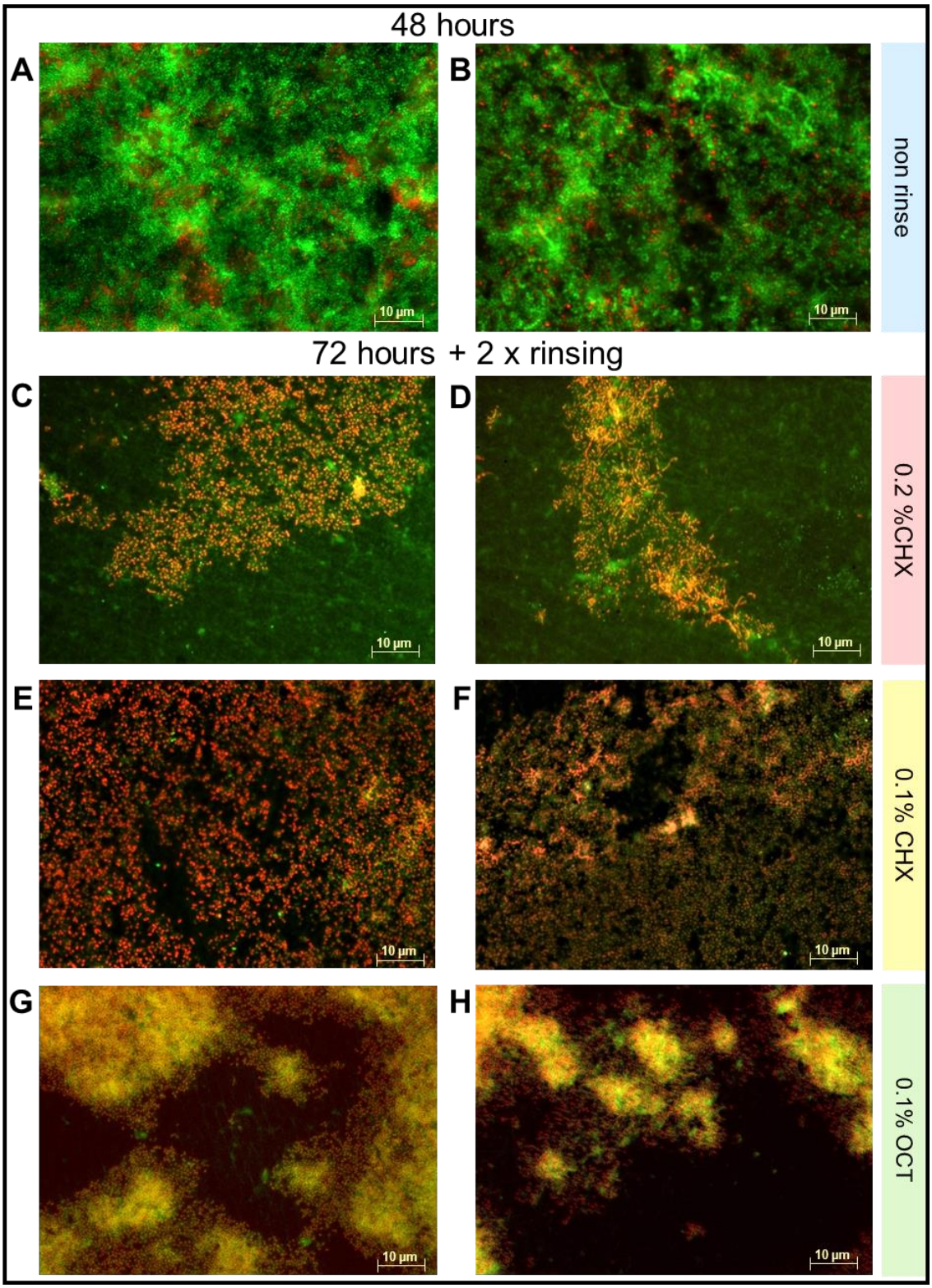

Fig. 22: Effect of chlorhexidine (CHX) and octendine (OCT) on biofilm disruption. The figure shows representative fluorescence images after LIVE/DEAD staining (living bacteria are stained green and dead bacteria are stained red) of the 48 h-biofilm formed on enamel surfaces before rinsing (A and B) with vital mature biofilm as well as of the 72-h biofilm after two-times rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) with a monolayer of non-vital bacteria. Permission has been obtained to reuse images published in the Journal of Dental Research (REDA et al., 2021a).

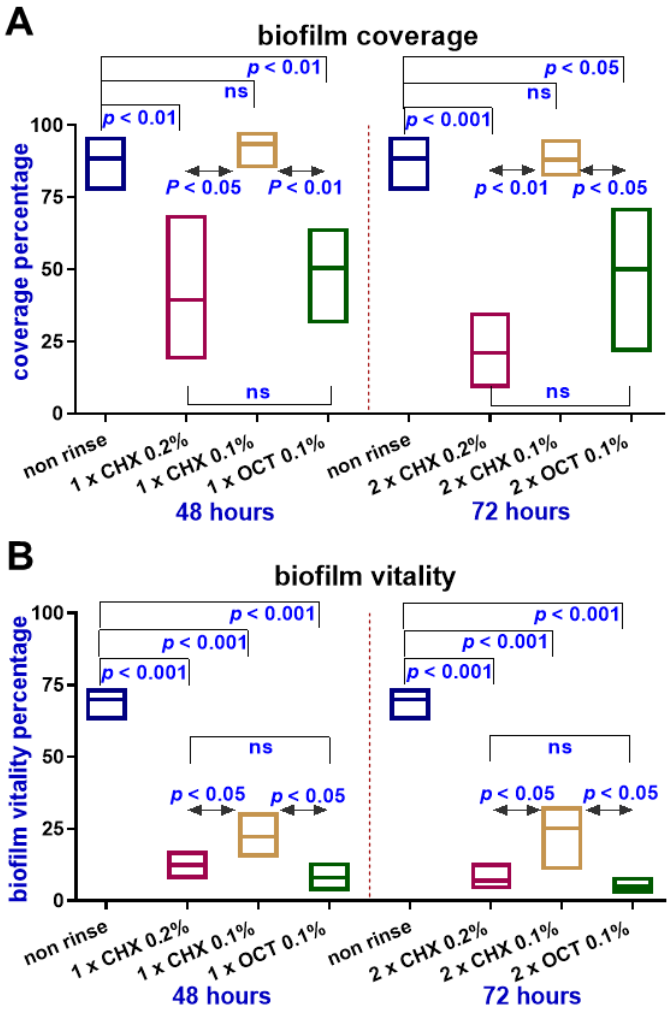

Fig. 23: Quantification of biofilm coverage (A) and bacterial vitality (B) at 48 h and 72 h. In comparison with the control before rinsing, 0.2% chlorhexidine (CHX) and 0.1% octenidine (OCT) rinsing solutions caused a significant reduction in the biofilm coverage and biofilm vitality. Rinsing with 0.1% CHX caused a significant reduction in the biofilm vitality only. There are significant differences in vitality and coverage parameters between 0.2% CHX and 0.1% CHX as well as between 0.1% OCT and 0.1% CHX. Analysed were 280 independent images; min to max values are shown as floating bars and the middle line refers to the mean. Statistically significant association: ($p < 0.05$). ns: not significant.

4.2.2.2 TEM-evaluation

The disruption effect of CHX and OCT rinses on a complex biofilm was also evaluated by TEM analysis. The results are presented in figures 24 and 25. After 48 h of intraoral exposure (Fig. 24 A and B, Fig. 25 A and B), a pellicle layer was observed on the control surfaces consistently covering the enamel surfaces. Additionally, a multilayer biofilm was seen coating the surface of the control specimens. The microorganisms were found prevalently well preserved and trying to proliferate.

After a single CHX or OCT rinsing (after 48 h of intraoral exposure), alterations of the biofilm were observed. The number of bacterial sheets covering the treated specimens was reduced and the bacteria detected within the treated biofilms were damaged. After a second CHX or OCT rinse and a

total of 72 h intraoral exposure, further disintegration of the biofilm was found. The percentage of lysed bacteria with a coagulated cytoplasm increased (Fig. 25 C-H).

It is worth noting that the thickness of the biofilm significantly changed between the evaluation at 48 h without mechanical control of the biofilm (16.7 ± 3.2 µm) and the biofilm thickness after the first rinsing with 0.2% CHX (4.1 ± 1.7 µm), 0.1% CHX rinsing (3.2 ± 0.7 µm), or 0.1% OCT rinsing (2.1 ± 0.8 µm) ($p < 0.001$). Additionally, a significant reduction in the biofilm thickness was observed between the control specimens, and the thickness of the biofilm observed at 72 h of intraoral exposure after two times rinsing with 0.2% CHX (4.3 ± 1.4 µm), 0.1% CHX (2.9 ± 1.6 µm), or 0.1% OCT (2.3 ± 0.7 µm) (Fig. 26).

Again, the biofilm dissolving activity of the CHX and OCT rinses was confirmed by comparing TEM images and the numbers of the bacterial layer before oral rinsing and after the application of CHX or OCT. On the control surfaces (before the oral rinsing) there was a mature biofilm with multi-layers of different oral bacteria (Fig. 24 A and B, Fig. 25 A and B). Whereas after the application of either CHX or OCT there was only a mono-layer of microorganisms covering the enamel specimens. Moreover, the enamel surfaces were even partly devoid of bacteria after the second CHX or OCT rinsing (Fig. 24 C-H, Fig. 25 C-H). Most of the detected bacteria on the treated surfaces revealed a coagulated cytoplasm and damaged cell membrane. Lastly, the statistical analyses of the data taken from the TEM evaluation confirmed the biofilm disruptive ability of CHX and OCT rinsing and that there were no significant differences between the aforementioned agents (Fig. 26).

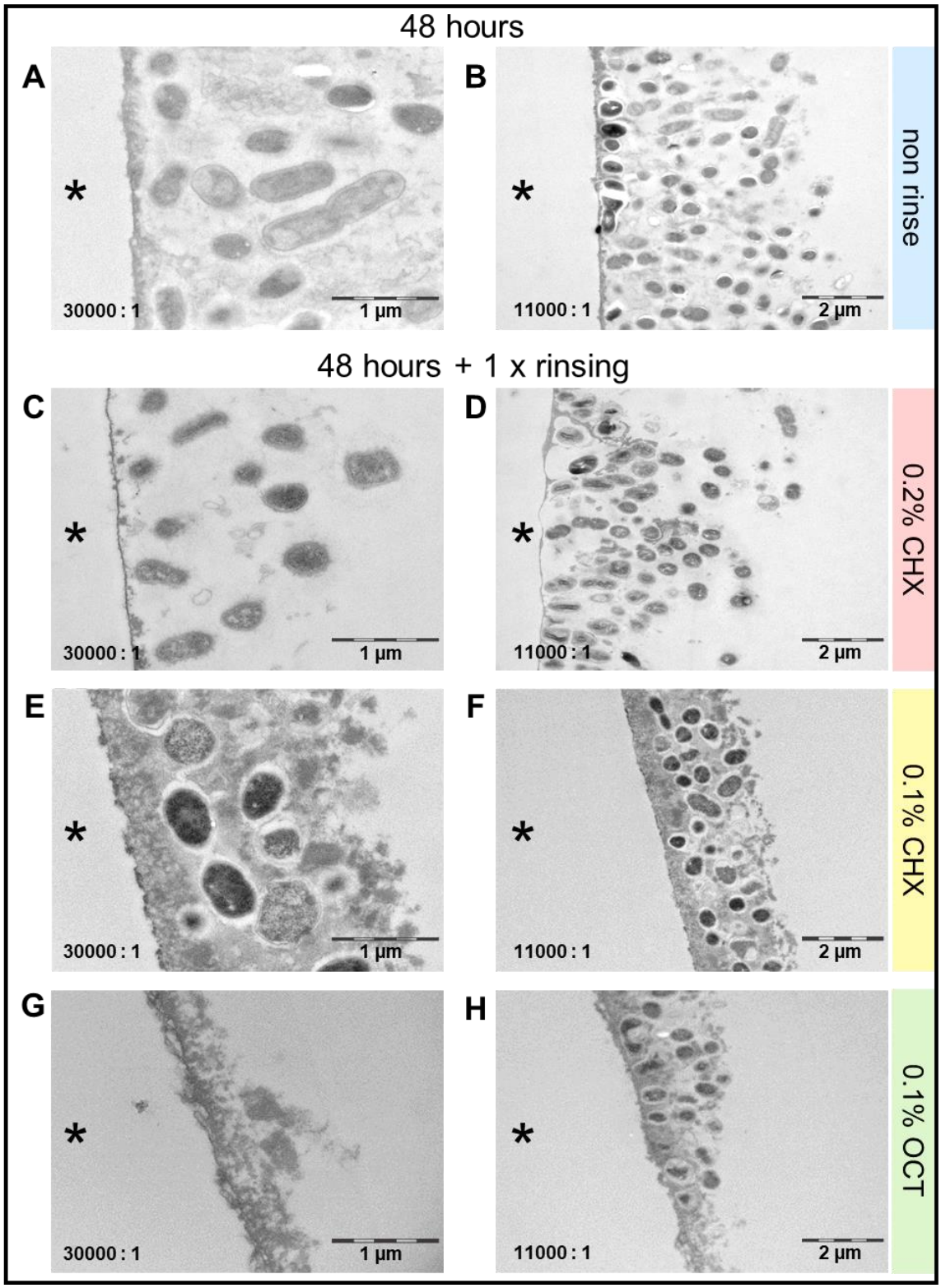

Fig. 24: Effect of chlorhexidine (CHX) and octendine (OCT) on biofilm disruption. The figure shows representative TEM micrographs of the 48-h biofilm formed on enamel surfaces before rinsing (A and B) with multilayer biofilm as well as directly after one-time rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) with monolayer biofilm. * Previous enamel surface. Permission has been obtained to reuse images published in the journal Clinical Oral Investigations and Journal of Dental Research (MARTÍNEZ-HERNÁNDEZ et al., 2020; REDA et al., 2021a).

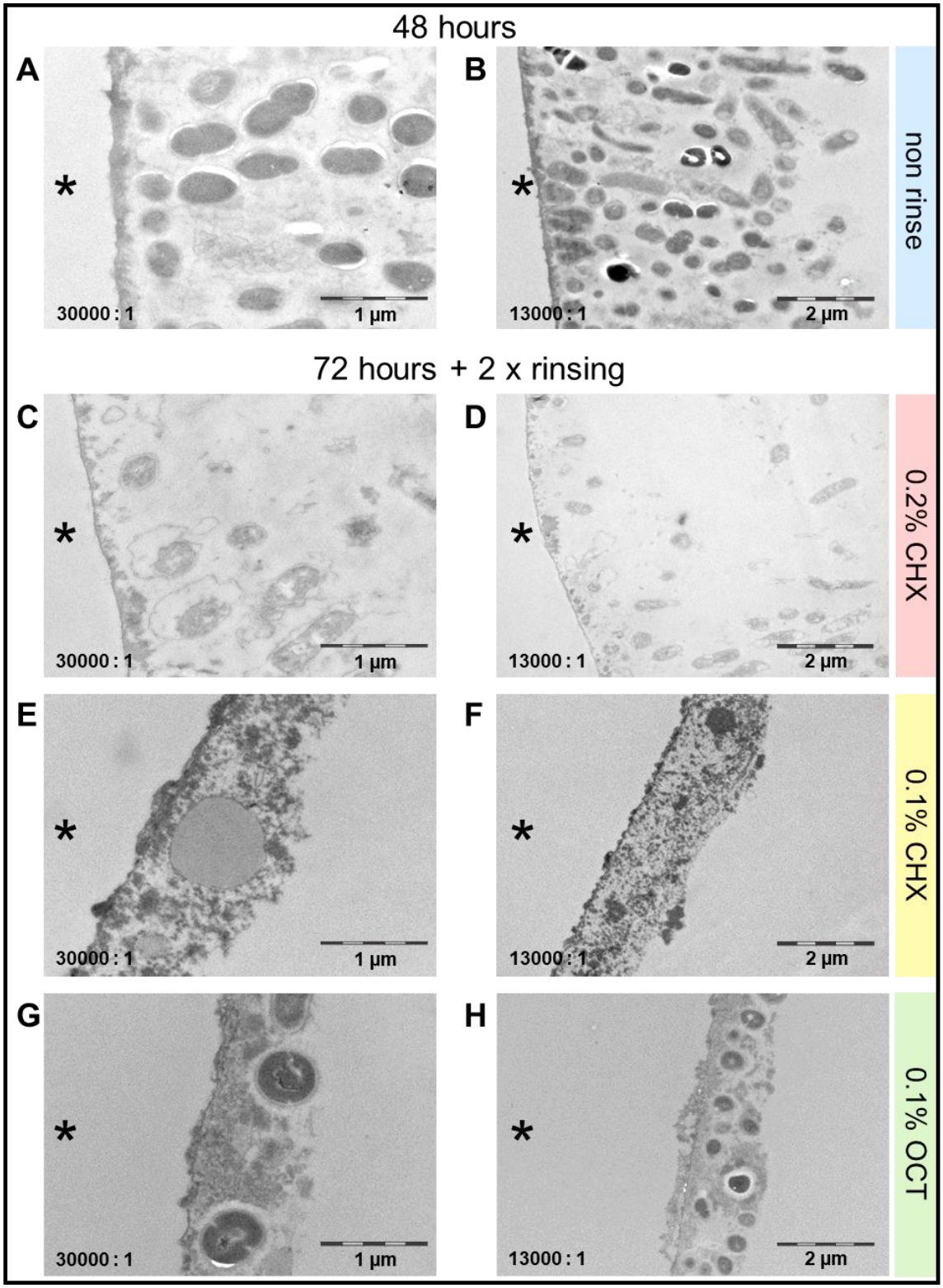

Fig. 25: Effect of chlorhexidine (CHX) and octendine (OCT) on biofilm disruption. The figure shows representative TEM micrographs of the 48-h biofilm formed on enamel surfaces before rinsing (A and B) with vital multilayer biofilm as well as the 72-h biofilm after two-time rinsing with 0.2% CHX (C and D), 0.1% CHX (E and F), or 0.1% OCT (G and H) with monolayer non-vital biofilm. * Previous enamel surface. Permission has been obtained to reuse images published in the journal Clinical Oral Investigations and Journal of Dental Research (MARTÍNEZ-HERNÁNDEZ et al., 2020; REDA et al., 2021a).

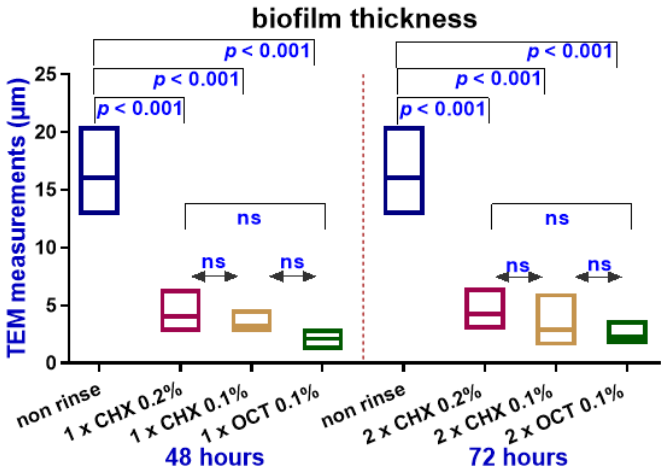

Fig. 26: Quantification of biofilm thickness at 48 h and 72 h. In comparison with the control before rinsing, chlorhexidine (CHX) and octenidine (OCT) rinsing solutions caused a significant reduction in the biofilm thickness. There were no significant differences between 0.2% CHX, 0.1% CHX, or 0.1% OCT. Analysed were 70 independent images; min to max values are shown as floating bars and the middle line refers to the mean. Statistically significant association: ($p < 0.05$). ns: not significant.

5 Discussion

Antiseptic products are worldwide used as an adjunctive therapy to improve preventive and treatment measures through reducing the bacterial load in the oral cavity, particularly by patients with oral diseases such as caries and periodontitis. The antiseptic agent CHX is the most representative of the chemo-prophylactic substances. Therefore, in order to monitor the CHX concentrations after oral application of different CHX formulations and to evaluate how long CHX remains in different oral sites, oral pharmacokinetic experiments as a part of this research were carried out.

Worth to mention that CHX has some side effects such as tooth discoloration and bitter taste, which prevent the prolonged use of this agent. One possible alternative is OCT, a highly active antiseptic agent against gram-positive and gram-negative microorganisms. However, the CHX- and OCT-induced modifications in the dental biofilm had not been well evaluated and compared especially on natural enamel specimens and by using both FM and TEM approaches together. Therefore, part of the current work was designed to investigate the effects of CHX and OCT rinses on biofilm formation and biofilm disruption on native enamel under *in situ* conditions. This experiment evaluated bacterial vitality and, for the first time, the ultrastructure of dental biofilms under the influence of CHX and OCT solutions.

5.1 Discussion of materials and methods

5.1.1 Experimental design

5.1.1.1 In situ biofilm model

The efficacy of different chemical agents is studied usually on bacterial biofilms which are generated either by using *in vitro* models or *in situ* models. The *in vitro* model is based on laboratory techniques to produce a biofilm from one or several oral bacteria (GUGGENHEIM et al., 2001). The *in vitro* studies are considered as the first step to investigate the clinical efficacy of oral antiseptics (SHANI et al., 2000; SHAPIRO et al., 2002). However, the findings from *in vitro* experiments should be interpreted with caution for many reasons. Foremost, there is a big difference between *in vitro* and *in vivo* biofilm formation, mainly because of the existence of saliva in the oral cavity which plays a key role in the physiological bio-adhesion. As a result, the adhesion process *in vivo* is totally different from *in vitro* conditions (HANNIG, HANNIG, 2009).

In addition to the salivary role, both the structural organization of the microbial community in the oral biofilm and the modified pattern of gene expression therein cause reduced sensitivity of microbial cells to antiseptics (STEWART, CONTERTON, 2001). The complexity of bacterial biofilm may prevent the penetration of the antiseptics. Furthermore, the chemical agent can kill and inhibit the superficial layer of the microbial biofilm leaving the bacterial cell in the depths of the biofilm undamaged. Additionally, the presence of a sub-population of ‘‘persister’’ microorganisms, which are

specialized survivor cells, leads to an increased resentence of some biofilms to the antibacterial agents (KEREN et al., 2004). Charged polymers also that form part of the biofilm matrix and the neutralizing enzymes which are attached to this matrix can bind to oppositely charged inhibitors and deactivate the antibacterial power (ALLISON, 2003). Lastly, bacterial cells in the biofilm usually become more resistant because of mutations affecting the drug targets on the bacteria or because of the production of modifying residues and enzymes, which can react with the inhibitors (STEWART, CONTERTON, 2001).

Due to the aforementioned reasons, the oral biofilm is considered as a novel phenotype which can lead to a lower sensitivity of the bacteria to antimicrobial agents. That means the MIC for a bacterial cell grows on a surface can range from 2- to 1000-fold greater than the same cells grown planktonically (STEWART, CONTERTON, 2001; JOHNSON et al., 2002). For example, the MIC of CHX and amine fluoride was 300 times and 75 times greater, respectively, when *Streptococcus sobrinus* was grown as a biofilm compared with the MIC for planktonic cells (SHANI et al., 2000).

Therefore, considering the mentioned drawbacks of *in vitro* models, there was a desperate need to use an *in situ* model where the biofilm grows in a way similar to the growth pattern inside the oral cavity as well as under the same conditions and thereafter evaluating the oral antiseptics efficacy on these *in situ*-formed biofilms (HANNIG, 1999; HANNIG et al., 2013a). In order to accurately replicate oral cavity conditions, an *in situ* approach was adopted in the present study.

5.1.1.2 The removable mini-splint

To allow the dental pellicle and oral biofilm to form under *in situ* conditions on enamel specimens in this study, a specific approach was needed to fix these surfaces inside the oral cavity. One option was to use a removable acrylic splint which is considered a reliable tool where several enamel surfaces can be fixed using the silicone impression material (light body) and then easily removed after the end of the experimental time without disturbing the adherent biofilm (PRADA-LÓPEZ et al., 2016). This approach can be considered as a valid way for *in situ* biofilm studies because as stated in a previous study there is almost no difference between the normal biofilm formed on the natural tooth and the biofilm formed on enamel specimens fixed on acrylic splints (MACPHERSON et al., 1991).

While this splint model is well accepted by the volunteers because it can be taken out of the mouth for a short time to perform the oral hygiene measurements or during meals time, there are also few negative aspects concerning this model. The negative aspects of the splint model are the absence of food effect on the biofilm development and also these splints can't be used for long-term experiments (more than a few days) because wearing them for extended periods of time will be difficult and exhausting for the volunteers.

Another option was to use dental bonding agents for fixing the specimens inside the oral cavity for long time under the effects of food and beverages. Unfortunately, to achieve that, an etching step should

be applied to the tooth surface before bonding the specimens which can cause decalcification and minor damage to the tooth surface and as a result, this approach was deemed unethical.

5.1.1.3 Experimental conditions

All the subjects followed specific experimental criteria to avoid factors that can mislead the obtained results because of individual habits of the subjects and to reduce the inter- and intra-individual variations in such kind of *in situ* studies. These criteria include the following points:

1) Last tooth brushing step should be done at least 30 min before the start of the experiment to avoid the interaction between residues of the toothpaste particles and the tested agents (KOLAHI, SOOLARI, 2006).

2) Oral hygiene procedures were done during the experimental time without any additional chemical agents or toothpastes, again to avoid the interaction between these products and the tested agent and to avoid the additional misleading antibacterial effects which can be delivered from the chemical agents or toothpastes.

3) Each experimental trial was started in the morning and the last food meal was taken at least one hour before each tested product to avoid the interaction between the food particles and the tested agents at the application time point.

4) All the subjects had almost matched lunchtime points to have a similar effect at the same time on the substantivity of the test products by all the subjects.

5) *In situ* sample collections by standardized micro-brushes in the first retention experiment resulted in comparable data groups from saliva as well as from the other oral locations because all CHX concentrations were correlated to the wetted tip volume of the micro-brushes. This point of reference was independent of different and non-standardisable superficial abrasion area sizes within the oral cavity. The calculated CHX concentrations cannot be given as amounts of CHX per defined tissue area, because the present data are independent of the wiped area size. To gain that specific information, surgical sections of defined sizes would be necessary to the CHX retention evaluation in this part, which is ethically not allowed on humans. In contrast, the used sampling approach is minimally invasive, not even inducing any mucosal irritation.

6) In the second retention study and for a more standardized experimental sampling, 2 µl samples were taken from saliva and buccal mucosa pellicle in addition to the dental pellicle sample formed on a standardized enamel surface (11.35 mm^2).

7) The experimental design of the work was a crossover design where repeated measurements were conducted. In this design, the subjects cross over from one treatment to another during the course of the experiment. Whereas in the parallel design the volunteers are randomized to a specific treatment and remain on that treatment throughout the duration of the experiment. The crossover design could yield

more efficient comparisons between treatments than a parallel design by reducing the variation between experimental runs, which could occur if every run started with a new volunteer. The utilization of the crossover design seems more reasonable because each subject serves as his/her own matched control. However, the major disadvantage in the crossover design is that carryover effects which is defined as the effects of previous treatment on the current treatment. In other words, if a patient receives therapy A during the first experimental trial and therapy B during the second trial, then the obtained values from the second trial could be a result of the direct effect of therapy B applied during the second run, and/or the carryover or residual effect of treatment A. Significant carryover effects can bias the interpretation of data analysis and lead to statistical bias. To eliminate the impact of these carryover effects, incorporation of long washout periods in the experimental design of the cross over studies should be considered.

8) In the present work, an important washout period of 10 days was applied. A washout period is defined as the time between two treatments. That means, instead of stopping and then immediately starting the new treatment, there will be a period of time to eliminate the activity of the first treatment and to restore the original conditions before a new experimental trial. In this way, the results of the next treatment will be not violated by the carryover effects from previous treatments. The rationale for using the washout period is to nullify any negative bias effects that a crossover experiment may have. The main aim of using a washout period in the retention studies was to eliminate any rest of CHX in the oral cavity from a treatment regimen which can affect the retention values of the next treatment regimen. Whereas the aim in the anti-biofilm study was to restore the normal bacterial ecosystem before testing a new active agent.

9) The volunteers should not use any medication before or during the experimental time to avoid any additional antibacterial effect on the studied biofilm beside the effect of the tested antiseptics.

10) In the present work, the biofilms were formed on the enamel specimens for 24 h, 48 h, and even 72 h to evaluate the effects of the test products on a real mature biofilm.

5.1.2 MALDI-TOF evaluation

Previously, there were many analytical methods for CHX determination starting from direct ultraviolet spectroscopy which was a simple method and easy to carry out, but not specific for CHX detection (JENSEN, CHRISTENSEN, 1971). The determination was disturbed by endogenous components from the salivary matrix (PESONEM et al., 1995). Therefore a method based on the use of radiolabel ^{14}C (BONESVOLL et al., 1974a) and a fluorometric method (DE VRIES et al., 1991) have been conducted. The first one was able to detect CHX in saliva 24 h after administration due to the higher sensitivity of the radioisotope techniques and also able to use the whole saliva sample. In contrast, the reported fluorometric method was unable to analyse the whole saliva samples and their linearity limitation (6.7 - 20 µg/ml) fails to determine lower and higher concentrations of CHX in saliva samples. Different

approaches of high-performance liquid chromatographic (HPLC) has been used for CHX detection (LAM et al., 1993; MEDLICOTT et al., 1994; PESONEM et al., 1995; TSUCHIYA et al., 1999), and also solid-phase micro extraction (SPME) was used for pharmacokinetic investigation (MUSTEATE, PAWLISZYN, 2005). In 2015, MALDI-TOF MS technique was used to detect CHX at therapeutic concentrations in microtome slices of a bacterially contaminated model wound treated with the CHX (HAMERLY et al., 2015).

Although HPLC and SPME are relatively selective and accurate, they require many extraction procedures and more steps for calculating the CHX concentrations. Even more, sample preparation can be laborious and time-consuming (FIORENTINO et al., 2010). Thus, while both techniques are useful, both of them impose some restrictions when compared to the sensitivity, speed, and accuracy offered by MALDI-MS. Furthermore, MALDI-TOF MS was used in the present study for analysing different kinds of samples from different oral locations besides the saliva. This mass spectrometry method allows high sample throughput, even if 5 replicate measurements were performed for one sample, because the laser shots were completed within 2 s for each measurement. Additionally, none of the samples needed time-consuming sample preparation, enrichment steps, or clean-up-techniques. Moreover, the characteristic isotope pattern of the two chlorine isotopes ($^{35}Cl/^{37}Cl$ at ~3:1 ratio) easily allowed the recognition of CHX in the measured mass spectra, as human endogenous molecules do not contain chlorine covalently bound to organic components. That means, neither salivary compounds nor ingestion of food and beverages interfered with the identification of CHX isotope patterns.

The low limit of quantification in the present experiment (1.5 ng/ml) and the adequate range of linearity demonstrate the MALDI-technique as a high sensitivity approach to determine and quantify CHX in experimental samples.

Taken together, samples collecting and processing for the MS-measurement in the MALDI-TOF approach is relatively simple. There is no need for any additional procedures or cleaning steps of the samples before the quantification. Therefore, the MALDI-TOF technique appears to be a useful method for the pharmacokinetic investigation of different oral medications in the scientific and industrial domains. This method can answer questions about the availability, substantivity, and hence the frequency of application required for a certain therapy.

5.1.3 Fluorescence microscopy evaluation after LIVE/DEAD staining

Traditionally, bacterial vitality is determined by plating a diluted bacterial sample on a solid medium followed by counting the colonies that form. This is the routine procedure in microbiology laboratories worldwide. However, this technology in the oral biofilm field has several shortcomings. First and foremost, only half of the oral bacterial strains are cultivable (AAS et al., 2005). Additionally, there will be a lot of bacterial cells in the oral cavity which are still vital but are not able to form colonies on nutrient media despite them having other measurable activity and requiring special conditions to

proliferate. Therefore, the plate count technique always gives an underestimation of the actual vitality of a biofilm sample. It was even proposed that, for stressed cells, plate counts could give vitality to less than 50% of the truly vital inhabitants (JONES, 1987). Another drawback of this technique involves a significant delay to obtain the final results, usually 24 h to 5 days. Such a delay can be problematic especially when the results are needed as soon as possible.

For the aforementioned reasons and more, many attempts have been made to establish quick techniques for the determination of bacterial vitality, usually based on the exclusion, uptake, or metabolism of coloured stains. One of these methods is the fluorescence-based, two-colour vitality assays LIVE/DEAD staining (LDS), using *Bac*Light™ bacterial vitality kit. This kit consists of two dyes, propidium iodide (PI) and SYTO 9, which both stain nucleic acids. SYTO 9 is a green fluorescent and membrane-permeable molecule which stains all cells. In contrast, PI is a red intercalating stain and membrane impermeable. It is therefore excluded by "healthy" cells. When both dyes are presented within a damaged bacterial cell, the PI, with its stronger affinity toward nucleic acids, will displace the SYTO 9 and cells will fluoresce in red. Consequently, the percentage of bacterial adherence (coverage) and the vitality percentage can be determined.

The LDS approach followed by fluorescence microscopic evaluation has been used in previous experiments because of its high reliability, simplified preparation steps, and rapidity (HANNIG et al., 2013a; HANNIG et al., 2013b). This approach was considered as a useful tool for evaluating potential anti-biofilm effects of mouth rinses by determining the percentage of adherent bacteria on a surface, together with how vital they are during biofilm formation (WEIGER et al., 1995; AL-AHMAD et al., 2008). Therefore, fluorescence microscopy after LDS was used in the present study to evaluate and compare the efficacy of different mouth rinses on both aspects: the anti-biofilm effect and the biofilm disruption activity.

The counting procedure can be applied to evaluate the bacterial colonization and to calculate the vitality percentage, especially when few bacteria are attached to the surface as initial biofilm or in samples of salivary flora (HANNIG et al., 2007; TAWAKOLI et al., 2013). In contrast, by mature biofilms, it is difficult to determine and count single bacteria in the colonies and with-in dentinal tubules. Thus, a scoring system has been developed to evaluate the bacterial colonization and for the assessment of the ratio between red and green fluorescence (*i.e.*, the ratio between dead and alive bacteria) (RUPF et al., 2012; FENG et al., 2015).

In spite of the rapidity of the scoring system in regards to biofilm qualitative and quantitative evaluation, it can only give approximate values especially for the mature biofilm where the living bacteria with green fluorescence are on the superficial layer hiding the dead bacteria under them, which can give a misleading evaluation toward more viable percentage. Furthermore, there is an additional

drawback in the scoring system which is the personal bias where every individual can see the same fluorescence image in a different way.

To overcome these aforementioned negative aspects, the biofilm evaluation in this study was done using a digital program called ImageJ which is able to measure the whole FM image area in addition to the biofilm covered area and then the percentage of the bacterial colonization can be easily calculated. This program is also able to measure the grey integrated density of both FM channels, the red one (indicates to dead bacteria) and the green one (indicates to living bacteria) and then consequently the vitality percentage can be calculated.

5.1.4 Electron microscopic investigation

TEM is a common method for evaluating the ultrastructural pattern of oral biofilm and morphological appearance (BRECX, THEILADE, 1984; HANNIG, JOINER, 2006). Thus, TEM evaluation is very useful for studying the ultrastructural alterations in oral biofilm induced by different oral antiseptics. TEM is the gold standard for biofilm evaluation at the micro- and nano-scale levels. This detailed visualization of the biofilm structure, as well as bacterial morphology, will help to get further information about the action mechanisms behind the clinical efficacy of CHX and OCT. Furthermore, it makes it possible to accurately measure the biofilm thickness in the control conditions and under the influence of the antibacterial agents as well. Unfortunately, and due to huge efforts for sample preparation, TEM can be considered as a laborious and time-consuming evaluation method. An additional issue is the small evaluated ultrathin-section (50 - 80 nm) taken out of a small enamel surface, (3 mm x 3 mm), which is sometimes not reflecting the general situation of the bacterial biofilm either under the control or test conditions. However, by using good quality TEM images it is practical to get a meaningful impression of the oral biofilm at the ultrastructural level.

As described above, there are different methods to evaluate the oral biofilms, thus it would be of great value to use the advantages of several investigating techniques at the same time to have a detailed, comprehensive view about the oral biofilm both in a control condition and under the influence of different oral antiseptics. The vital fluorescence approach gives an overview of the microbial colonization and vitality pattern, whereas TEM gives detailed information at the ultrastructural level. Therefore, this combination of the two methods is able to build a ‘three-dimensional’ ultra-structure vision of the oral biofilms in addition to the vitality evaluation, which is very important to investigate the effects of the chemical antiseptics on the quality and the quantity of oral biofilm. This is of great importance for evaluating the efficiency of oral hygiene agents for biofilm control.

5.1.5 Statistical analysis

The statistic tests have great importance, especially in comparative studies. They help the researcher to find out if there are any significant differences between the experimental groups and consequently between several treatment agents. In some cases, the difference between the control group and very

effective treatment is quite obvious and no further tests are needed to prove that, whereas when different treatment regimens are investigated, statistic tests are needed to clarify if the detected differences between the treatment groups are significant or not. The decision, which statistical tests should be used is not easy or straightforward. There are many considerations and variances that should be taken into consideration before choosing the appropriate statistical test such as the distribution pattern, sample size, pairing, *etc*.

In this study and for the retention experiments, repeated measures of two-way analysis of variance ANOVA was taken as a statistical test because two factors were changing during the experiments. In the first retention experiment, the time of sampling and the oral site were changing. In the second retention study, the sampling time was changing as well as the CHX treatment formulation and regimen. Furthermore, repeated measures test by both factors (paired test) was chosen because oral samples were taken in the first retention experiment from the same subject and the subjects in the second retention experiment cross over from one therapy to another during the experiment. Multiple comparisons were conducted in the retention experiments with the Tukey test to find out if there were any significant differences in oral CHX retention between the different oral sites in the first experiment and between the different CHX formulations and regimens in the second.

The statistic test in the anti-biofilm experiments was Repeated Measures of one-way ANOVA with multiple comparisons. The reasons behind choosing a parametric test were the following:

1) According to the normality test (Shapiro-Wilk test), the statistic units in the experimental groups were sampled from a population that follows a Gaussian distribution or at least most of the data were not inconsistent with a Gaussian distribution.

2) In this work, the statistic units were 5 in each test group and for that it was better to avoid Nonparametric tests, because when the number of samples is too small (less than 7 values) the Nonparametric tests have little power to detect the differences between the groups.

Repeated measures test (paired test) was also used because treatments were given repeatedly to each subject. It is important to note that the Repeated-measures ANOVA test is quite sensitive to violations of the assumption of sphericity or circularity. Violation of sphericity means that factors from treatment A will affect the results obtained from treatment B in the crossover design. This will happen when the repeated measurements are made too close together so that random factors from previous treatment are not washed out completely or dissipate before the start of the new treatment run in the crossover experimental design. This will cause a particular value to be high (or low) in the next measurement.

To avoid violating the assumption of sphericity in the present work, an important washout period of 10 days was applied. Furthermore, the order of treatments was random and each subject followed a different treatment sequence in his experimental trial. Additionally, Geisser and Greenhouse correction

was added to the Repeated Measures-ANOVA to avoid any statistic bias that could occur from the violation of the assumption of circularity. The Geisser and Greenhouse correction was particularly applied in the anti-biofilm study because of the usage of highly effective agents such as CHX and OCT in the treatment groups. Therefore, when there is a doubt that the treatment effect is not completely washed in spite of the washout period, it would be better to add Geisser and Greenhouse correction. It reduces the values of the degrees of freedom, and thus increases the p-value. Multiple comparisons with the Tukey test were done to compare the control group with each therapy group and to compare each therapy group with the other.

Lastly, the Brown-Forsythe test was applied for the assessment of equal variances because it is less sensitive to small deviations from normality. Bartlett's test was not recommended from the statistic program because it is more sensitive even for slight deviation from Gaussian ideal and may give a small *p*-value even when the differences among standard deviations are negligible.

5.2 Discussion of results

5.2.1 Discussion of retention results

Oral pharmacokinetic studies were carried out in order to monitor the CHX retention after application and to estimate how long CHX stays at bactericidal or bacteriostatic concentrations in different oral locations. Such kind of experiments is also needed to obtain more information about some factors that affect the retention of the antiseptic in the oral cavity, such as drug concentration, application time, and delivery system.

The effective concentrations of CHX were evaluated previously in earlier studies (STANLEY et al., 1989; TSUCHIYA et al., 1994). The MIC of CHX for primary cariogenic bacteria like *Streptococcus mutans, Actinomycetes,* or *Lactobacilli* ssp. ranged from 0.39 to 6.25 µg/ml (TSUCHIYA et al., 1994) and the MICs for 52 subgingival bacterial strains ranged from 8 to 250 µg/ml (STANLEY et al., 1989). In the current work, mean CHX-retentions ranging from 0.6 to 18.4 µg/ml were determined after 11 h in the mouth. Therefore, it can be stated that the antimicrobial activity of CHX against several species of pathogenic oral microorganisms remains for a considerable time after CHX rinsing.

Up to now, the retention of CHX was mainly determined in saliva (LAM et al., 1993; MEDLICOTT et al., 1994; PESONEM et al., 1995) or in saliva-coated hydroxyapatite and buccal epithelial cells *in vitro* (TSUCHIYA et al., 1999). Additionally, it was proved in former experiments that CHX rinsing leads to changes in the dental pellicle layer such as increasing the pellicle thickness (CARPENTER et al., 2005; JOINER et al., 2006; DE SOUZA-E-SILVA et al., 2017). Moreover, brownish tooth staining after prolonged CHX usage obviously suggests that there must be a CHX associated modification of the dental pellicle. Until the present study, there was missing proof that CHX is retained and enriched onto and within the acquired dental pellicle formed *in situ*. However, we showed in the current work that CHX, in fact, adsorbs onto and within the dental pellicle under *in situ* conditions and that the dental pellicle acts as a reservoir for CHX-release towards the saliva for a considerable time after CHX application.

The usage of MALDI-TOF MS has shown that CHX retention varied at different oral locations in each subject and between subjects. The inter-individual differences between the subjects were rather large and led to a high standard deviation of the CHX retention. This could be due to the individual difference between the volunteers in the oral environment (especially oral pH value) in addition to dietary habits during the time of the experiment. For example, consuming acidic foods and drinks can lower the pH within the oral cavity which can lead to reduced CHX retention (BONESVOLL et al., 1974b). Worth noting that many previous studies had reported such an inter-individual variation after CHX mouth rinsing (BONESVOLL et al., 1974a; MEDLICOTT et al., 1994; PESONEM et al., 1995; TSUCHIYA et al., 1999).

A fast reduction of CHX retention in the first few hours after mouth rinsing was noticed in the present study. The main meal intake could be the rational reason for such a rapid decline. This might be attributed to the interaction between negatively charged food particles with positively charged CHX-molecules. This interaction could lead to the CHX desorption from oral surfaces. Nevertheless, a significant quantity of CHX still presented in the oral cavity for an extended time even after the one-time 30 s oral rinsing. Such a rapid decline in oral CHX retention after food and beverage was also observed in former experiments (MEDLICOTT et al., 1994; TSUCHIYA et al., 1999).

As can be seen from the retention experiments results (Tables 2 and 5), the concentrations of salivary CHX stayed at microgram pro millilitre scales up to 10 h after oral application. These findings are in good agreement with the results from former experiments of CHX retention in saliva (JENSEN, CHRISTENSEN, 1971; MEDLICOTT et al., 1994; PESONEM et al., 1995; TSUCHIYA et al., 1999; MUSTEATA, PAWLISZYN, 2005).

The retention of CHX at the buccal mucosa was significantly higher than the CHX-retention detected in saliva at all-time points. This elevated retention was also clear in the dental pellicle. Such retention might be attributed to the high adsorption of CHX to the dental pellicle and mucosal pellicle. These findings agree with a former *in vitro* experiment proposing that the existence of proteins in pellicles and buccal epithelial cell walls encourage the adsorption of CHX (TSUCHIYA et al., 1999).

The retention of CHX in the anterior labial mucosa was slightly, but not significantly higher than in the posterior buccal mucosa ($p = 0.89$) (Table 2). It is probably that CHX was more diluted at the posterior locations as they are nearer to the main salivary glands (parotid glands). Moreover, there might be an abrasive impact against the retained CHX on the posterior location during food chewing.

The highest persistent of CHX in the mouth was detected in the interdental area. The enriched and immobilized CHX which is kept in the interdental area appears to be protected from dilution and abrasive effects of eating and drinking.

Food intake is considered to be an effective factor for reducing the retention of CHX in the oral cavity (JONES, 1997). To investigate the food effect in current work, CHX retention during fasting was evaluated. The results showed clearly high retention of CHX, which can be directly attributed to the absence of dilution and abrasion effects of beverages and food mastication. These results agree with previous studies concerning the effects of food intake on the substantivity of CHX (MEDLICOTT et al., 1994; TSUCHIYA et al., 1999; MUSTEATA, PAWLISZYN, 2005). It is worth noting that the long-lasting retention of CHX at higher concentrations in the oral cavity indicates the high stability of CHX in the oral cavity without decomposition over time. The mechanical effects during the consumption of food and beverages are the most important factor for reducing CHX concentrations after the application. Additionally, such a high retention of CHX in the fasting time could point to the importance of using it

directly before sleeping to keep it in high concentrations in the oral cavity during the night in a similar way to fasting and to have the best antibacterial effects after rinsing.

Regarding the effect of CHX formulas and regimens on oral retention, when identical amounts of 10 ml from CHX mouth solution were used, an increase in either the concentration or the rinsing time led to a slight rise in the CHX retention in saliva, mucosal pellicle, and dental pellicle. The maximum level of CHX retention was achieved with the 0.2% CHX rinsing for 60 s. While the differences in CHX retention between the four rinsing protocols were not statistically significant neither in saliva nor in the dental pellicle, the 0.2% CHX rinsing for 60 s, however, caused significantly higher retention than 0.12% at the buccal mucosa. Such retention results explain the conclusion of a systemic review (BERCHIER et al., 2010) where they found that with regards to biofilm inhibition effect; there was a small, but significant difference in favour of the 0.2% CHX solution. It was reported also in previously published studies that there was higher retention by rising the CHX concentration from 0.1 mg/ml to 1 mg/ml (TSUCHIYA et al., 1999). Bonesvoll and co-workers also raised CHX retention by raising the concentration or increasing the rinsing time (BONESVOLL et al., 1974b). They also noticed that there was a slight deviation towards less retention at the highest concentration tested. This means that there is a binding capacity for CHX in the oral surfaces, which explains why there was only a small difference in retention between 0.2% and 0.12% in favour of the higher one. Additionally, this finding indicates that increasing the concentrations of CHX for more than 0.2% or rinsing with more than 10 ml (more than 20 mg CHX dose) will not induce remarkable higher CHX retention and more persistent bacteriostatic activity because of the binding capacity for CHX in the oral cavity.

Attempts to overcome the side effects of prolonged CHX use have resulted in the development of different delivery systems and proposing several CHX formulations, which employ little amounts of CHX and deliver the drug to specific areas in the oral cavity. The CHX spray is one of these formulations, which delivers small dose from CHX of approximately one-seventh of the dose used in a 0.2% rinsing protocol and obtains, as shown in the present study, a considerable CHX retention and good substantivity for at least 12 h. Furthermore, there were no statistically significant differences in CHX retention in saliva and dental pellicle between the rinsing protocols and spray application with an exception of 0.2% CHX for 60 s where there was more CHX retention. Such results indicate that low dose from CHX solutions delivered by the spray system could provide adjunctive benefits to oral hygiene and gingival health with minimal side effects. In accordance with that, it was stated in previous studies that CHX retention after spray application is able to provide almost the same beneficial biofilm-inhibiting effects as it provided from rinsing solution (DEVER, 1979; KALAGA et al., 1989a; STOEKEN et al., 2007).

The spray approach is particularly useful for elderly people and physically and mentally handicapped groups (FRANCIS et al., 1987a, b; KALAGA et al., 1989b; BURTNER et al., 1991; CLAVERO et al., 2003; VIANA et al., 2014). This method also allows focusing on the treatment of

specific regions such as periodontal or implants surgical sites (FRANCETTI et al., 2000; FRANCETTI et al., 2004).

It was thought that adding CHX in the dentifrice formulations will be helpful for patients with periodontal disease to get beneficial approaches, mechanical and chemical cleaning. However, components, in which pastes and gels are usually formulated, are from anionic substances such as sodium lauryl sulphate (SLS), which interferes with the action and bioavailability of CHX via forming salts of low solubility and low antibacterial activity (KOLAHI, SOOLARI, 2006). Therefore, two different kinds of CHX toothpaste were tested in the current work, one with SLS (paroex) and another without it (curasept), to evaluate the retention of CHX in the oral cavity after tooth brushing and the effect of SLS on such retention.

In this study, no statistically significant differences were observed between the toothpaste with SLS and the one without SLS in regard to the retention of CHX in the saliva, buccal mucosa, and dental pellicle.

The results showed that CHX toothpaste is retained in the oral cavity at low concentration and not to the same extent as a CHX mouth rinse or even after CHX spray application. Differences in CHX retention between CHX toothpaste and other CHX formulations in the three oral locations were highly significant. The reason behind this low retention could be due to the mechanical action of the tooth brushing that can remove the newly attached CHX particles at the dental pellicle. Furthermore, rinsing the mouth several times with water after tooth brushing will also wash away most of the CHX particles from the oral cavity surfaces. In contrast, most of the retained CHX after mouth rinsing or spray application will stay in the oral cavity for a more extended time after application because usually the patient does not rinse the mouth with water after using the mouthwash or spray. To overcome the problem of washing CHX after tooth brushing, it was suggested in previous studies that CHX gel could be delivered in trays or applied directly on teeth surfaces with the finger (PAI et al., 2004). This style of application, especially with high CHX concentration, has provided more antibacterial efficiency and caused a significant reduction of plaque scores and gingival index (SUPRANOTO et al., 2015). Such a reduction was comparable with the mouth rinse in some studies (PAI et al., 2004; SLOT et al., 2010; MAGAZ et al., 2018).

Another factor that can influence the retention of CHX after toothpaste application is the CHX concentration, *i.e.* dosage of the drug. It was concluded in a systematic review (SLOT et al., 2014) that the positive effects of CHX toothpaste were noticed only when the concentration of CHX was more than 0.6%. Therefore, when 0.12% CHX toothpaste in this study was used, the dosage was 1.2 mg which is clearly lower than a 20 mg CHX dose delivered by rinsing and this could explain why there was negligible retention after tooth brushing and almost no substantivity 2 h thereafter.

Worth noting that the results in the present experiment mirrors (in a clinical setting) the conclusion from a systematic review (SUPRANOTO et al., 2015), which showed that CHX mouth rinse is significantly more efficient on biofilm control than CHX toothpaste or gel because CHX retention after mouth rinsing is much higher than the retention after CHX toothpaste application.

Returning to the CHX MICs of oral bacteria and according to the retention study results, the antibacterial concentrations for the elimination of primary cariogenic bacteria such as *Streptococcus mutans, Actinomycetes,* and *Lactobacilli* were between 0.36 and 6.25 µg/ml (TSUCHIYA et al., 1994). These concentrations were achieved by all the CHX regimens including the toothpaste. Whereas the needed CHX concentrations for the subgingival bacteria responsible for periodontal diseases which ranged between 8 and 250 µg/ml (Stanley et al., 1989) were only achieved by the CHX rinsing solutions or at least from spray application, but not after the toothpaste application which had given us at most 2 µg/ml 2 h after tooth brushing.

It is important to mention that the CHX retention values in saliva and at the mucosal pellicle after the application of 0.2% CHX for 30 s in the second retention experiment were different than the values in the first one, especially at the first time point. This is presumably due to the differences in the study design, evaluation time points, and sampling technique between both retention studies.

Generally, in the present study, 0.2% CHX for 60 s gave the best retention values that provide persistent antibacterial activity in the oral cavity. In accordance with that, García-Caballero and co-workers found that rinsing with 0.2% CHX had the greatest persistent antibacterial impact on salivary microorganisms, underlining the effect of the concentration and form of application on the CHX substantivity (GARCÍA-CABALLERO et al., 2009).

Taking together, the results of the present study showed that CHX can be present and potentially active against some pathogenic bacteria in the oral cavity for at least 12 h after oral application. Additionally, the CHX concentration and the delivery system play an essential role in the CHX retention in the oral cavity after application.

5.2.2 Discussion of anti-biofilm results

The CHX- and OCT-induced morphological alterations in the dental biofilm have not been well investigated and compared yet, especially using the TEM technique on real enamel surfaces. Therefore, the current work was designed to examine and compare the anti-biofilm and the biofilm disruption effects induced by CHX and OCT mouth rinse every 12 h under *in situ* conditions.

5.2.2.1 Chlorhexidine and octenidine rinsing affects in situ biofilm formation on enamel

Dental biofilm is defined as a microbial community embedded in the bacterial matrix on the tooth surface (SOCRANSKY, HAFFAJEE, 2002; MARSH, 2004). In general, the development of bacterial biofilm could be seen as an increase in the microbial quantity and the formation of big aggregates, including density and the diversity of the microbiota (HANNIG, JOINER, 2006).

In the present study, the 24-h control biofilms (water rinsing) in the FM evaluations were identified as microbial communities colonizing the enamel specimens, covering about 20% of the surface. Subsequently, a remarkable increase in the number of bacteria and the complexity of the biofilm structure was noticed between 24 and 48 h of intraoral exposure. The 48-h control biofilms were covering about 63% of the enamel surfaces. The comparison of biofilm images between 24- and 48-time points leads us to conclude that the structural complexity increased with increasing biofilm age and thickness. This condition was also observed in previous studies (ZAURA-ARITE et al., 2001; DIGE et al., 2009). Microbial cell division and fibril-like constructions between bacteria in the 48-h biofilm can be considered as a sign of microbial proliferation and bacterial coaggregation that leads to further biofilm maturation and the formation of multi-layered colonies. The control enamel surfaces (water rinsing) were covered by a mature and complex bacterial biofilm with a mean bacterial vitality of around 70% at 24-and 48-h evaluation times. This finding is in good agreement with previous *in situ* studies that have shown that dental biofilm vitality over 2- and 3-day periods is between 60 and 77% (ARWEILER et al., 2004; AUSCHILL et al., 2005).

Worth mentioning is that the inter-subject variations in the biofilm vitality, coverage, structure, and thickness that was seen by FM and TEM analyses might be attributed to individual factors, such as salivary flow rate and dietary habits.

Evident anti-biofilm activity of CHX and OCT solutions was confirmed in the current work by investigating the microbial vitality, biofilm coverage, biofilm thickness, and ultrastructure. A low presence of microorganisms or even a total absence of bacteria was detected after CHX or OCT application, supporting the well-established antibacterial features of CHX (JONES, 1997) and OCT (ASSADIAN, 2016). All the aforementioned investigated parameters of treated biofilms showed significant differences in comparison to the parameters of control biofilms. This anti-biofilm activity of CHX and OCT was clear at 24- and 48-h assessment points and was confirmed by a significant reduction in the mean values of oral bacteria colonization. In parallel with the strong reduction of the bacterial

colonization by all the rinses, a comparable dramatic reduction of bacterial vitality was detected. It was significantly reduced to less than 10% after CHX or OCT application.

This clear anti-biofilm effect is mostly attributed to the bactericidal activity of CHX and OCT on the oral bacteria directly after application. They act on the bacterial cell membrane and disrupt the membrane integrity and then they promote the formation of vesicles and precipitation in the cytoplasm, which leads in the end to microbial death (BRECX, THEILADE, 1984; JONES, 1997; HUBNER et al., 2010; ASSADIAN, 2016).

In this context, it has been stated in former studies that the usage of 0.2% CHX leads to an immediate reduction of the bacterial vitality (≥90%) in the salivary microorganisms (TOMÁS et al., 2009; COUSIDO et al., 2010; GARCÍA-CABALLERO et al., 2013). OCT was also found to be extremely effective in reducing *Streptococcus mutans* levels in saliva and compared favourably with CHX (KOCAK et al., 2009).

As time passes, both antibacterial agents are present at low concentrations in the oral cavity due to the dilution effect of saliva as well as food and beverages effects. In such concentrations, both agents exhibit bacteriostatic effects (TOMÁS et al., 2008; DOGAN et al., 2008; DOGAN et al., 2009). Hence, the further bacterial adherence is hindered and the microorganism available to establish a biofilm or attach to an existing biofilm are clearly reduced (NEWCOMBE et al., 1995; TOMÁS et al., 2009; RUPF et al., 2012), which is in agreement with the findings detected in the present study. Similarly, a previous *in situ* experiment has shown an important reduction of bacterial vitality to 20% after the CHX application (AUSCHILL et al., 2005). The experimental differences in that study compared to the present work are the use of glass specimens and the reduced contact of the rinses to the specimens because of the splint design, which led to a lower antibacterial effect in comparison to the present study. It was shown also in another study that *ex vivo* CHX application remarkably reduced the microbial vitality in 3-day biofilms from 67% in control biofilms to 2% and 0.7% in CHX-treated biofilms at 1 and 10 minutes, respectively (VON OHLE et al., 2010).

In the case of OCT, a complete killing of *Streptococcus salivarius* biofilms was detected after the exposure to 0.1% OCT for at least 30 s *in vitro* (AL-SEBAIE, 2014). Furthermore, Sennhenn-Kirchner and co-workers investigated the antibacterial activity of OCT on biofilms created by aerobic oral microorganism on rough titanium specimens. The experiment showed that the application of OCT for 8 min caused a clear reduction of the biofilm vitality by 99.8% (SENNHENN-KIRCHNER et al., 2010).

In the comparison between the different treatment regimens concerning the tested biofilm parameters, at the 24-h evaluation point, there was only a significant difference between 0.1% CHX and 0.1% OCT in regards to the biofilm vitality. At 48 h, 0.2% CHX and 0.1% OCT were significantly more effective than 0.1% CHX regarding the biofilm coverage.

It is worth mentioning that the higher retention detected after using higher CHX concentration in the first part of the present work (retention study) can explain why 0.2% CHX was more effective as an anti-biofilm agent in compression with the 0.12% CHX.

A meaningful observation was made in several studies that 0.1% OCT as an antimicrobial agent was better than many other agents such as polyvinylpyrrolidone-iodine and even CHX. It was stated in a previous study that OCT is quite active in reducing salivary and cariogenic microbial amount after 15, 30, 60, and 120 min (DOGAN et al., 2008). Furthermore, the same working group found that the use of OCT for 5 days caused the most significant reduction in cariogenic bacteria with greater antimicrobial activity than 0.2% CHX and 7.5% polyvinylpyrrolidone-iodine (DOGAN et al., 2009). Additionally, it was found also that OCT has a similar and even better antibacterial activity than CHX and NaOCl against *Staphylococcus epidermidis* (CHUM et al., 2019).

It is worth mentioning that an effective anti-bacterial agent is the one that has a direct (bactericidal) and persistent (bacteriostatic) efficacy otherwise the oral flora will colonize the tooth surface and establish a new biofilm again shortly after the antiseptic application (ADDY, MAROM, 2008).

Collectively, both antimicrobial agents CHX and OCT have this anti-biofilm effect through several effective antibacterial mechanisms. The first mechanism is bactericidal in nature, whereby CHX and OCT destroy the oral microorganisms either in saliva, which are trying to attach to the tooth surface, or in the already attached bacterial biofilms. The second mechanism is the bacteriostatic activity, whereby CHX and OCT are able to damage the oral bacteria and prevent them from proliferation even for an extended time after application. Furthermore, it was proved in the retention study that CHX adsorbs at the pellicle-coated tooth surface and therefor it can prevent the attachment of oral bacteria and damage them.

Lastly, according to the FM evaluation in the present study, 0.1% OCT was similar to the 0.2% CHX and more active than 0.1% CHX at both evaluation times 24 and 48 h in regards to the anti-biofilm effects.

The TEM quantitative analyses revealed that the mean biofilm thicknesses were 4 and 12 µm after 24 and 48 h of intraoral exposure, respectively, under the control conditions and water rinsing. In contrast, the TEM images showed that CHX and OCT rinsing caused a significant reduction in both the biofilm thickness and amount of bacteria in comparison with water rinsing. Similarly, it was reported in a former experiment by using confocal microscopy that CHX can produce an 89% reduction in biofilm thickness on glass discs (AUSCHILL et al., 2005).

Additionally, through qualitative analyses, electron microscopy images showed more cellular ghosts on the CHX-treated specimens as compared to those found in the control enamel specimens. In some parts on the enamel surface, no loss of cytoplasmic ingredient could be found, because the cell walls were intact, surrounding the cytoplasm of a coagulated appearance. This is in agreement with

former experiments demonstrating that CHX caused leakage of cytoplasmic constituents and cytoplasm coagulation (HUGO, LONGWORTH, 1965; HUGO, LONGWORTH, 1966; BRECX, THEILADE, 1984). Furthermore, it was stated that CHX induced a general perturbation of lipid bilayer membranes (GILBERT, MOORE, 2005) causing the bacterial membrane to lose its structural integrity (CHAWNER, GILBERT, 1989; VITKOV et al., 2005).

Interestingly, in cases of treated specimens and unique to the control specimens, only a small increase in the biofilm thickness and the number of microbial cells was seen between 24 and 48 h of intraoral exposure, confirming a retarded oral biofilm development on the enamel induced by CHX or OCT application. This finding could suggest that with continued exposure to the antibacterial rinses twice daily used in the current work, this could be the maximal thickness of the bacterial layer that can develop. This finding indicates the importance of CHX or OCT rinses for biofilm control focused on persons incapable to reach appropriate oral hygiene levels or after oral surgical procedures where the usual hygiene measures are restricted.

It is important to mention that an increased pellicle thickness of unstructured loose material covering the CHX treated specimens was observed in the current study (Fig. 17 C, Fig. 18 G and H). It was reported in a former experiment that CHX application even at a low concentration (0.012%) increases the adsorption of proteins on the enamel specimens (DE SOUZA-E-SILVA et al., 2017). Such a finding can clarify why, in the present study, the pellicle sheet after the CHX application was thicker on the treated specimens compared to the control ones. Interestingly, it was found in the former study that many proteins with potential protective functions against caries and erosion such as proline-rich proteins, calcium-binding proteins, and statherin were among the increased number of adsorbed proteins detected after CHX rinsing (DE SOUZA-E-SILVA et al., 2017). Because of that, it might be stated that CHX treatment can lead not only to bacterial biofilm inhibition but also could increase the dental protective potential against caries lesions by the formation of a thicker proteinaceous protective coating.

Since the ultrastructural alteration caused by OCT rinsing was not evaluated before, the present study delivers the first TEM images of oral biofilms formed on natural enamel surfaces treated by OCT solution *in situ*. The mean observation is in agreement with the LDS images; OCT was able to totally inhibit the bacterial adhesion on the test surfaces. Only an acquired enamel pellicle was seen on the TEM micrographs either at 24- or 48-h time points without any bacterial colonization. Such an interesting finding can be explained by the evident antibacterial effect of OCT over time which was proved to stay very active even when highly diluted. Whereas CHX lost its antimicrobial effect when diluted to lower than 10% of its original concentration (ROHRER et al., 2010).

5.2.2.2 Chlorhexidine and octenidine rinsing disrupts mature dental biofilm

Since the killing of bacterial biofilm does not automatically induce the elimination of the microbial rests, the biofilm disrupting effect is an important concept for anti-biofilm efficacy. In this context, it is

not only important for an agent to reduce the bacterial vitality but also to be able to disrupt the mature biofilm and clean the enamel surface. In this way, there will be neither residual resistant bacteria to reform a new bacterial biofilm nor largely dead colonies attached to the tooth surface which could act as a base for additional mediated adherence of salivary living microorganisms.

The biofilm disrupting effect was evaluated in this study by using an experimental design where a mature biofilm was formed on the dental surfaces for 48 h and then the application of CHX or OCT rinsing solution was done twice daily to investigate the ability to detach previously adsorbed bacteria.

After the first rinsing, an immediate significant reduction in the microbial vitality was observed. After further rinsing during the next 24 h, there was a more remarkable reduction in both the microbial vitality and bacterial colonization on the treated specimens.

According to the LDS images, the reduction in biofilm vitality was detected after using both CHX concentrations and OCT. Whereas, the biofilm disruption effect, which is presented as a reduction in the biofilm coverage, was noticed only by 0.2% CHX and 0.1% OCT but not on the 0.1% CHX treated surfaces. That means 0.2% CHX and 0.1% OCT have more evident biofilm disruption activity than 0.1% CHX.

The significant reduction in biofilm vitality is due to the effective antibacterial properties of CHX and OCT as described before in the introduction. Additionally, 0.2% CHX and 0.1% OCT were able to disrupt the biofilm presumably by breaking the glycoproteins connections between the bacteria, as well as displacing calcium molecules, which are considered as the glue that binds biofilm particles together (RÖLLA, MELSEN, 1975). Furthermore, CHX and OCT are able to devitalize the planktonic organisms in saliva (KOCAK et al., 2009; DOGAN et al., 2009; COUSIDO et al., 2010; GARCÍA-CABALLERO et al., 2013), thus reducing the number of bacteria available for adherence and form new biofilm layers.

By electron microscopy analysis, a complex multi-layer biofilm was observed at 48 h of intraoral exposure in the absence of mechanical teeth cleaning (control surfaces). The majority of the microorganisms observed were cocci and rod-shaped bacteria. The microorganisms were found prevalently and well preserved, however, after the first rinsing with CHX as well as OCT an immediate effect was observed on the biofilm complexity. The biofilm disrupting effect was more evident at 72 h of intraoral exposure. This disrupting effect significantly reduced the biofilm thickness and the number of bacterial cells of the biofilm after two-times rinsing when compared with the biofilm formed on the control enamel surfaces at 48 h of intraoral exposure. Most of the bacterial cells on the biofilm treated with CHX and OCT lost their structural integrity as seen at higher magnifications after 72 h of intraoral exposure. Moreover, leakage of the cellular elements, precipitation, and coagulation of the cytoplasm of the treated bacteria were detected (Fig. 24 C–H, Fig. 25 C-H).

It is important to note that the disruption effect of 0.1% CHX rinsing was more obvious in the TEM analysis than FM/LDS analysis. While the 0.1% CHX failed to induce a clear reduction in the biofilm coverage on the treated surfaces, it was able to significantly reduce the biofilm thickness by almost 80% after only one rinsing (Fig. 26). The biofilm was changed from multilayer mature biofilm to monolayer biofilm. However, it was able to cover the whole enamel surface, causing no change in the biofilm coverage percentage in the FM/LDS analysis. In other words, the disruptive activity of 0.1% CHX was clearer in the TEM images and analysis indicating that after the rinse application, the maturity and the complexity of the biofilm were less dominant. Such results indicate clearly the importance of using different experimental techniques in the experimental design to have more detailed results to explain the mechanism of action of the oral antiseptics.

In the case of 0.1% OCT, it was able to reduce the biofilm thickness of the mature biofilm by almost 90% after only one rinsing. The multilayer biofilm was not only changed to a monolayer but there were also many places on the enamel surface without any bacterial colonization, which is in agreement with the LDS results. Such a result was also obtained with 0.2% CHX rinsing where there were many places without any bacterial adhesion.

Former experiments had evaluated the disruption potential of CHX against mature biofilms. However, these studies showed contradictory findings. García-Caballero and co-workers reported that rinsing the 48-h mature biofilms with 0.2% CHX solution for 30 s reduced the biofilm vitality from 78% under the basic situation to 5% after CHX rinsing (GARCÍA-CABALLERO et al., 2013). A similar finding was also seen in another work where the *ex vivo* CHX application for one and 10 min induced a clear reduction of the microbial vitality from 67% under basal conditions to 2% and 0.7% after CHX application, respectively (VON OHLE et al., 2010). On the contrary, only a small and superficial antimicrobial activity was detected after extra oral rinsing of 48-h mature biofilm with 0.2% CHX for 60 s (ZAURA-ARITE et al., 2001). Important to note that the *ex vivo* application of CHX and the thicker biofilm in the aforementioned work could have a major role in producing such a minor activity of CHX. Similarly, there was a minor antibacterial effect even after 5 min CHX treatment of mature multispecies biofilms that were formed on bovine enamel specimens *in vitro* (PRATTEN et al., 1991).

In agreement with our finding, OCT was quite effective at killing pre-formed biofilms of *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA). The average biofilm thickness on the control specimens was 15 μm as seen in the confocal microscopy images, whereas the average thickness of OCT treated biofilms was 1 μm. OCT was able to induce patchy breaks in the biofilm by causing a disruption of the bacterial colonies and a loss of the microbial cells (AMALARADJOU, VENKITANARAYANAN, 2014). Similarly, Junka and co-workers found that 1 min contact time of OCT is able to completely inactivate *in vitro* pre-formed biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (JUNKA et al., 2014).

Taking these aforementioned studies into consideration, the biofilm disruption activity was not well studied in the literature under real *in situ* conditions especially in the case of OCT, which was evaluated only *in vitro*. Because of that, our findings came to emphasize the importance of *in situ* studies and confirm for the first time the biofilm-disruptive potential of CHX and OCT under most natural conditions on enamel surfaces. Lastly, although OCT has significant antibacterial effects, further experiments are needed to investigate OCT's relative safety, biocompatibility, and absence of negative side effects.

5.3 Conclusions

5.3.1 Conclusion of retention studies

This work shows a new reliable, selective, and sensitive technique with appropriate determination limits for CHX detection without the need for time-consuming sample preparation. MALDI-TOF provides rational mass resolving power, high mass accuracy, and can be implemented in high throughput measurements, making this method a promising approach for detecting CHX or other antiseptics in samples from different oral locations and not only in saliva like previous studies.

The current work also confirms the assumption that oral surfaces (dental pellicle and oral mucosa) are the main reservoirs of CHX in the oral cavity. With time, the CHX particles are desorbed from the oral surfaces toward the saliva to produce a persistent bacteriostatic effect in the whole oral cavity for an extended time.

It is shown in this study that the retention of CHX is much higher after mouth rinsing comparing with toothpaste application. According to the retention values, CHX toothpaste with a minimum concentration of 0.12% should not be considered as an adequate and sufficient pharmacological treatment of periodontal diseases and could be applied only for high caries risk patients. When a significant reduction of oral bacteria is required, such as for oral surgery preparations or for the treatment of periodontal diseases, 0.2% CHX application for 60 s gives the best retention values, which can provide a direct and persistent antibacterial efficacy in the oral cavity. Also, according to the good retention values after the spray usage, this kind of CHX application can be recommended especially in particular cases such as physically and mentally handicapped people.

5.3.2 Conclusion of anti-biofilm study

It is concluded in the study that the formulation based on CHX in both concentrations 0.1% and 0.2% as well as 0.1% OCT agent have a significant effect on reducing biofilm formation, confirmed through the reduction of the oral bacterial colonization and bacterial vitality on the experimental specimens. Additionally, this study demonstrates an important biofilm disruption potential of the 0.2% CHX and 0.1% OCT rinsing, since they are effective to dislodge pre-existing mature biofilms, even in the total absence of manual biofilm control. The findings of the present study confirm the effectiveness of 0.2% CHX and 0.1% OCT rinsing during the intra-oral evaluation and also highlight the importance of performing *in situ* researches to investigate the efficacy of antibacterial products.

As a final conclusion, since 0.1% OCT is almost as effective as 0.2% CHX in the anti-biofilm activity, but with half of the concentration, *i.e.* theoretically lower long-term side effects, it may be considered as a potent alternative to CHX-based preparations. However, preference in clinical effectiveness between OCT and CHX will request direct comparisons of both agents in long term clinical studies.

6 References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. Journal of Clinical Microbiology 43:5721-5732
2. Addy M (1986) Chlorhexidine compared with other locally delivered antimicrobials. A short review. Journal of Clinical Periodontology 13:957-964
3. Addy M, Carpenter R, Roberts WR (1976) Management of recurrent aphthous ulceration. British Dental Journal 141:118-120.
4. Addy M, Jenkins S, Newcombe R (1989) Studies on the effect of toothpaste rinses on plaque regrowth. (I). Influence of surfactants on chlorhexidine efficacy. Journal of Clinical Periodontology 16:380-384
5. Addy M, Moran JM (1997) Clinical indications for the use of chemical adjuncts to plaque control: chlorhexidine formulations. Periodontology 2000 15:52-54
6. Addy M, Moran JM (2008) Chemical Supragingival Plaque Control. In Clinical Periodontology and Implant Dentistry, Lindhe J, Lang NP, Karring K, eds. (Oxford, Blackwell Publishing, Ltd), pp. 734-765.
7. Ahmad BZ (2020) The efficacy of chlorhexidine gel as an adjunctive treatment for patient with chronic periodontitis. Indian journal of Forensic Medicine & Toxicology 14:544-550
8. Al-Ahmad A, Wiedmann-Al-Ahmad M, Auschill TM, Follo M, Braun G, Hellwig E, Arweiler NB (2008) Effects of commonly used food preservatives on biofilm formation of *streptococcus mutans in vitro*. Archives of Oral Biology 53:765-772
9. Al-Hashimi J, Levine MJ (1989) Characterization of *in vivo* salivary-derived enamel pellicle. Archives of Oral Biology 34:289-295
10. Allison DG (2003) The biofilm matrix. Biofouling 19:139-150
11. AL-Sebaie D (2014) The assessment of antibacterial effect of 0.1% octenidine solution on *in vitro* biofilms of *Streptococcus salivarius* using bacterial LIVE/DEAD viability staining and CFU counting. Pakistan Oral & Dental Journal 34:281-284
12. Amaechi BT, Higham SM, Edgar WM, Milosevic A (1999) Thickness of acquired salivary pellicle as a determinant of the sites of dental erosion. Journal of Dental Research 78:1821-1828
13. Amalaradjou MAR, Venkitanarayanan K (2014) Antibiofilm effect of octenidine hydrochloride on *staphylococcus aureus*, MRSA and VRSA. Pathogens 3:404-416
14. Armstrong WG (1967) The composition of organic films formed on human teeth. Caries Research 1:89-103
15. Arteagoitia I, Andrés CR, Ramos E (2018) Does chlorhexidine reduce bacteraemia following tooth extraction? A systematic review and meta-analysis. PLoS One 13:e0195592
16. Arweiler NB, Hellwig E, Sculean A, Hein N, Auschill TM (2004) Individual vitality pattern of *in situ* dental biofilms at different locations in the oral cavity. Caries Research 38:442-447

17. Assadian O (2016) Octenidine dihydrochloride: chemical characteristics and antimicrobial properties. Journal of Wound Care 25:S3-S6
18. Auschill TM, Arweiler NB, Netuschil L, Brecx M, Reich E, Sculean A (2001) Spatial distribution of vital and dead microorganisms in dental biofilms. Archives of Oral Biology 46:471-476
19. Auschill TM, Hein N, Hellwig E, Follo M, Sculean A, Arweiler NB (2005) Effect of two antimicrobial agents on early *in situ* biofilm formation. Journal of Clinical Periodontology 32:147-152
20. Auschill TM, Hellwig E, Sculean A, Hein N, Arweiler NB (2004) Impact of the intraoral location on the rate of biofilm growth. clinical oral investigations 8:97-101
21. Baehni PC, Takeuchi Y (2003) Anti-plaque agents in the prevention of biofilm-associated oral diseases. Oral Diseases 1:23-29
22. Bailey DM, Degrazia CG, Hoff SJ, Schulenberg PL, O'Connor JR, Paris DA, Slee AM (1984) Bispyridinamines. a new class of topical antimicrobial agents as inhibitors of dental plaque. Journal of Medicinal Chemistry 27:1457-1464
23. Barkvoll P, Rella G, Svendsen AK. Interaction between chlorhexidine digluconate and sodium lauryl sulfate *in vivo*. Journal of Clinical Periodontology. 1989; 16: 593-595.
24. Beiswanger BB, Mallat ME, Jackson RD, Mau MS, Farah CF, Bosma ML, Bollmer BW, Hancock EB (1992) Clinical effects of a 0.12% chlorhexidine rinse as an adjunct to scaling and root planning. Journal of Clinical Dentistry 3:33-38
25. Beiswanger BB, Mallatt ME, Mau MS, Jackson RD, Hennon DK (1990) The clinical effects of a mouthrinse containing 0.1-percent octenidine. Journal of Dental Research 69:454-457
26. Berchier CE, Slot DE, Van der Weijden GA (2010) The efficacy of 0.12% chlorhexidine mouthrinse compared with 0.2% on plaque accumulation and periodontal parameters: a systematic review. Journal of Clinical Periodontology 37:829-839
27. Bonesvoll P, Lokken P, Rölla G, Paus PN (1974a) Retention of chlorhexidine in the human oral cavity after mouth rinses. Archives of Oral Biology 19:209-212
28. Bonesvoll P, Lokken P, Rölla G (1974b) Influence of concentration, time, temperature and pH on the retention of chlorhexidine in the human oral cavity after mouth rinses. Archives of Oral Biology 19:1025-1029
29. Brecx M, Theilade J (1984) Effect of chiorhexidine rinses on the morphology of early dental plaque formed on plastic films. Journal of Clinical Periodontology 11:553-564
30. Brill F, Goroncy-Bermes P, Sand W (2006) Influence of growth media on the sensitivity of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to cationic biocides. International Journal of Hygiene and Environmental Health 209:89-95
31. Burt BA (1998) Prevention policies in the light of the changed distribution of dental caries. Acta Odontologica Scandinavica 56:179-186

32. Burtner AP, Low DW, Mc Neal DR, Hassell TM, Smith RG (1991) Effects of chlorhexidine spray on plaque and gingival health in institutionalized persons with mental retardation. Special Care in Dentistry 11:97-100
33. Carlen A, Borjesson AC, Nikdel K, Olsson J (1998) Composition of pellicles formed *in vivo* on tooth surfaces in different parts of the dentition, and *in vitro* on hydroxyapatite. Caries Research 32:447-455
34. Carpenter GH, Pramanik R, Proctor GB (2005) An *in vitro* model of chlorhexidine induced tooth staining. Journal of Periodontal Research 40:225-230
35. Celik EU, Tunac AT, Ates M, Sen BH (2016) Antimicrobial activity of different disinfectants against cariogenic microorganisms. Brazilian Oral Research 30:e125
36. Chackartchi T, Hamzani Y, Shapira L, Polak D (2019) Effect of subgingival mechanical debridement and local delivery of chlorhexidine gluconate chip or minocycline hydrochloride microspheres in patients enrolled in supportive periodontal therapy: A retrospective analysis. Oral Health and Preventive Dentistry 17:167-171
37. Chawner JA, Gilbert P (1989) A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine. Journal of Applied Bacteriology 66:243-252
38. Cherian B, Gehlot PM, Manjunath MK (2016) Comparison of the antimicrobial efficacy of octenidine dihydrochloride and chlorhexidine with and without passive ultrasonic irrigation - an *in vitro* study. Journal of Clinical and Diagnostic Research 10:Zc71-Zc77
39. Chum JD, Lim DJZ, Sheriff SO, Pulikkotil SJ, Suresh A, Davamani F (2019) *In vitro* evaluation of octenidine as an antimicrobial agent against *Staphylococcus epidermidis* in disinfecting the root canal system. Restorative Dentistry & Endodontics 44: e8
40. Clavero J, Baca P, Junco P, González MP (2003) Effects of 0.2% chlorhexidine spray applied once or twice daily on plaque accumulation and gingival inflammation in a geriatric population. Journal of Clinical Periodontology 30:773-777
41. Coelho ASEC, Paula ABP, Carrilho TMP, da Silva MJRF, Botelho MFRR, Carrilho EVVF (2017) Chlorhexidine mouthwash as an anticaries agent: A systematic review. Quintessence International 48: 585-591
42. Colombo APV, Tanner ACR (2019) The role of bacterial biofilms in dental caries and periodontal and peri-implant diseases: A historical perspective. Journal of Dental Research 98:373-385
43. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin- Scott HM (1995) Microbial biofilms. Annual Review of Microbiology 49:711-745
44. Cousido MC, Tomas Carmona I, Garcia-Caballero L, Limeres J, Alvarez M, Diz P (2010) *In vivo* substantivity of 0.12% and 0.2% chlorhexidine mouthrinses on salivary bacteria. Clinical Oral Investigations 14:397-402

45. Davies GE, Francis J, Martin AR, Rose FL, Swain G (1954) 1:6-Di-4'-hlorophenyldiguanidohexane (hibitane); laboratory investigation of a new antibacterial agent of high potency. British Journal of Pharmacology and Chemotherapy 9:192-196
46. de Souza-E-Silva CM, da Silva Ventura TM, de Pau L, la Silva C, de Lima Leite A, Buzalaf MAR (2017) Effect of gels containing chlorhexidine or epigallocatechin-3-gallate on the protein composition of the acquired enamel pellicle. Archives of Oral Biology 82:92-98
47. De Vries J, Ruben J, Arends J (1991) Determination of chlorhexidine in saliva and in aqueous solutions. Caries Research 25:410-414
48. Decker EM, Weiger R, Wiech I, Heide PE, Brecx, M (2003) Comparison of antiadhesive and antibacterial effects of antiseptics on *Streptococcus sanguinis*. European Journal of Oral Sciences 111:144-148
49. Denton GW (2001) Chlorhexidine. In Disinfection Sterilization and preservation, Block SS, ed. (Philadelphia, Lippincott Williams and Wilkins), pp. 321-357.
50. Dever JG (1979) Oral hygiene in mentally handicapped children. A clinical trial using a chlorhexidine spray. Australian Dental Journal 24:301-305
51. Dige I, Nilsson H, Kilian M, Nyvad B (2007) *In situ* identification of *streptococci* and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence *in situ* hybridization. European Journal of Oral Sciences 115:459-467
52. Dige I, Nyengaard JR, Kilian M, Nyvad B (2009) Application of stereological principles for quantification of bacteria in intact dental biofilms. Oral Microbiology and Immunology 24:69-75
53. Dogan AA, Adiloglu AK, Onal S, Cetin ES, Polat E, Uskun E Koksal F (2008) Short-term relative antibacterial effect of octenidine dihydrochloride on the oral microflora in orthodontically treated patients. International Journal of Infectious Diseases 12:E19-E25
54. Dogan AA, Cetin ES, Hüssein E, Adiloglu AK (2009) Microbiological evaluation of octenidine dihydrochloride mouth rinse after 5 days' use in orthodontic patients. Angle Orthodontist 79:766-772
55. Dolles OK, Gjermo P (1980) Caries increment and gingival status during two years of chlorhexidine and fluoride containing dentifrices. Scandinavian Journal of Dental Research 88:22-27
56. Egner W, Helbert M, Sargur R, Swallow K, Harper N, Garcez T, Savic S, Savic L, Eren E (2017) Chlorhexidine allergy in four specialist allergy centers in the United Kingdom, 2009–13: clinical features and diagnostic tests. Clinical & Experimental Immunology 188:380-386
57. Eley BM (1999) Antibacterial agents in the control of supragingival plaque-a review. British Dental Journal 186:286-296

58. Emilson CG, Bowen WH, Robrish SA, Kemp CW (1981) Effect of the antibacterial agents octenidine and chlorhexidine on the plaque flora in primates. Scandinavian Journal of Dental Research 89:384-392
59. Faveri M, Gursky LC, Ferres M, Shibli JA, Salvador SL, de Figueiredo LC (2006) Scaling and root planning and chlorhexidine mouth rinses in the treatment of chronic periodontitis: a randomized placebo-controlled clinical trial. Journal of Clinical Periodontology 33:819-828
60. Feng J, Cheng L, Zhou X, Xu HH, Weir MD, Meyer M, Maurer H, Li Q, Hannig M, Rupf S (2015) *In situ* antibiofilm effect of glass-ionomer cement containing dimethylaminododecyl methacrylate. Dental Materials 31:992-1002
61. Ferretti G, Hansen DMD, Whittenburg K, Brown AT, Lillich TT, Ash RC (1987) Therapeutic use of chlorhexidine in bone marrow transplant patients: case studies. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 63:683-687
62. Fiorentino FAM, Correa MA, Salgado HRN (2010) Analytical methods for the determination of chlorhexidine: a review. Critical Reviews in Analytical Chemistry 40:89-101
63. Fiorillo L (2019) Chlorhexidine gel use in the oral district: A systematic review. Gels 5:31
64. Flemming HC, Neu TR, Wozniak DJ (2007) The EPS matrix: the "house of biofilm cells. Journal of Bacteriology 189:7945-7947
65. Flotra L, Gjermo P, Rölla G, Waerhaug, J (1971) Side effects of chlorhexidine mouth washes. Scandinavian Journal of Dental Research 79:119-125
66. Francetti L, Del Fabbro M, Basso M, Testori T, Taschieri S, Weinstein R (2004) Chlorhexidine spray versus mouthwash in the control of dental plaque after implant surgery. Journal of Clinical Periodontology 31:857-862
67. Francetti L, Del Fabbro M, Testori T, Weinstein RL (2000) Chlorhexidine spray versus chlorhexidine mouthwash in the control of dental plaque after periodontal surgery. Journal of Clinical Periodontology 27:425-430
68. Francis JR, Hunter B, Addy M (1987a) A comparison of three delivery methods of chlorhexidine in handicapped children. I. Effects on plaque, gingivitis, and tooth staining. Journal of Periodontology 58:451-455
69. Francis JR, Addy M, Hunter B (1987b) A comparison of three delivery methods of chlorhexidine in handicapped children. II. Parent and houseparent preferences. Journal of Periodontal Research 8:456-459
70. Gage TW, Pickett FA (2002) Mosby's Dental Drug Reference. St Louis: Mosby 563-565.
71. García-Caballero L, Carmona IT, González MC, Posse JL, Taboada JL, Dios PD (2009) Evaluation of the substantivity in saliva of different forms of application of chlorhexidine. Quintessence International 40:141-144

72. García-Caballero L, Quintas V, Prada-López I, Seoane J, Donos N, Tomas I (2013) Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an *in situ* model. PLoS One 8:e83522
73. Ghannoum MA, Abuelteen K, Ellabib M, Whittaker PA (1990) Antimycotic effects of octenidine and pirtenidine. Journal of Antimicrobial Chemotherapy 25:237-245
74. Gilbert P, Moore LE (2005) Cationic antiseptics: diversity of action under a common epithet. Journal of Applied Microbiology 99:703-715
75. Greenstein G, Berman C, Jaffin R (1986) Chlorhexidine. An adjunct to periodontal therapy. Journal of Periodontology 57:370-377
76. Guggenheim B, Giertsen E, Schüpbach P, Shapiro S (2001). Validation of an *in vitro* biofilm model of supragingival plaque. Journal of Dental Research 80:363-370
77. Hamerly T, Butler MH, Fisher ST, Hilmer JK, JamesGA, Bothner B (2015) Mass spectrometry imaging of chlorhexidine and bacteria in a model wound. AIMS Medical Science 2:150-161
78. Hannig C, Basche S, Burghardt T, Al-Ahmad A, Hannig M (2013a) Influence of a mouthwash containing hydroxyapatite microclusters on bacterial adherence *in situ*. Clinical Oral Investigations 17:805-814
79. Hannig C, Gaeding A, Basche S, Richter G, Helbig R, Hannig M (2013b) Effect of conventional mouthrinses on initial bioadhesion to enamel and dentin *in situ*. Caries Research 47:150-161
80. Hannig C, Hannig M (2009) The oral cavity-a key system to understand substratum-dependent bioadhesion on solid surfaces in man. Clinical Oral Investigations 13:123-139
81. Hannig C, Hannig M, Rehmer O, Braun G, Hellwig E, Al-Ahmad A (2007) Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel *in situ*. Archives of Oral Biology 52:1048-1056
82. Hannig M (1999) Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period. Clinical Oral Investigations 3:88-95
83. Hannig M, Joiner A (2006) The structure, function and properties of the acquired pellicle. Monographs in Oral Science 9:29-64
84. Hannig M, Khanafer AK, Hoth-Hannig W, Al-Marrawi F, Acil Y (2005) Transmission electron microscopy comparison of methods for collecting *in situ* formed enamel pellicle. Clinical Oral Investigations 9:30-37
85. Harke HP (1989) Octenidine dihydrochloride, properties of a new antimicrobial agent. Zentralblatt für Hygiene und Umweltmedizin 188:188-193
86. Hegde MN, Sajnani AR (2017) Salivary proteins—a barrier on enamel demineralization: An *in vitro* study. International Journal of Clinical Pediatric Dentistry 10:10-13
87. Hubner NO, Siebert J, Kramer A (2010) Octenidine dihydrochloride, a modern antiseptic for skin, mucous membranes and wounds. Skin Pharmacology and Physiology 23:244-258

88. Hugo WB, Longworth AR (1965) Cytological aspects of the mode of action of chlorhexidine diacetate. Journal of Pharmacy and Pharmacology 17:28-32
89. Hugo WB, Longworth AR (1966) The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*. Journal of Pharmacy and Pharmacology 18:569-578
90. Hulsemann W, Habenicht R (2009) Severe side effects after octenisept irrigation of penetrating wounds in children. Handchirurgie - Mikrochirurgie - Plastische Chirurgie 41:277-282
91. Hunter ML, Addy M (1987) Chlorhexidine gluconate mouthwash in the management of minor aphthous ulceration. A double-blind, placebo-controlled cross-over trial. British Dental Journal 162:106-110
92. Jeffcoat MK, Bray KS, Ciancio SG, Dentino AR, Fine DH, Gordon JM, Gunsolley JC, Killoy WJ, Lowenguth RA, Magnusson NI, Offenbacher S, Palcanis KG, Proskin HM, Finkelman, R.D, Flashner M (1998) Adjunctive use of a subgingival controlled-release chlorhexidine chip reduces probing depth and improves attachment level compared with scaling and root planning alone. Journal of Periodontology 69:989-997.
93. Jensen JE, Christensen F (1971) A study of the elimination of chlorhexidine from the oral cavity using a new spectrophotometric method. Journal of Periodontal Research 6:306-311
94. Johnson SA, Goddard PA, Iliffe C, Timmins B, Rickard AH, Robson G, Handley PS (2002) Comparative susceptibility of resident and transient hand bacteria to para-chloro-meta-xylenol and triclosan. Journal of Applied Microbiology 93:336-344
95. Joiner A, Elofsson UM, Arnebrant T (2006) Adsorption of chlorhexidine and black tea onto *in vitro* salivary pellicles, as studied by ellipsometry. European Journal of Oral Sciences 114:337-342
96. Joiner A, Schwarz A, Philpotts CJ, Cox TF, Huber K, Hannig M (2008) The protective nature of pellicle towards toothpaste abrasion on enamel and dentine. Journal of Dentistry 36:360-368
97. Jones G (1997) Chlorhexidine: is it still the gold standard? Periodontology 2000 15:55-62
98. Jones RP (1987) Measures of yeast death and deactivation and their meaning: parts 1 and 2. Process Biochemistry 22:118-128
99. Junka A, Bartoszewicz M, Smutnicka D, Secewicz A, Szymczyk P (2014) Efficacy of antiseptics containing povidone-iodine, octenidine dihydrochloride and ethacridine lactate against biofilm formed by *Pseudomonas aeruginosa* and *Staphylococcus aureus* measured with the novel biofilm-oriented antiseptics test. International Wound Journal 11:730-734
100. Kalaga A, Addy M, Hunter B (1989a) Comparison of chlorhexidine delivery by mouthwash and spray on plaque accumulation. Journal of Periodontology 60:127-130
101. Kalaga A, Addy M, Hunter B (1989b) The use of 0.2% chlorhexidine spray as an adjunct to oral hygiene and gingival health in physically and mentally handicapped adults. Journal of Periodontology 60:381-385

102. Keijser BJ, Zaura E, Huse SM, van der Vossen JM, Schuren FH, Montijn RC, ten Cate JM, Crielaard W (2008) Pyrosequencing analysis of the oral microflora of healthy adults. Journal of Dental Research 87:1016-1020
103. Kempka M (2005) Improved mass accuracy in MALDI-TOF-MS analysis. Department of Chemistry Division of Analytical Chemistry. Stockholm: Royal Institute of Technology1-44
104. Kendall MJ (2002) London British medical association and royal pharmaceutical society of Great Britain 538
105. Kensche A, Reich M. Kümmerer K, Hannig M, Hannig C (2013) Lipids in preventive dentistry. Clinical Oral Investigations 17: 669-685
106. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis, K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiology Letters 230:13-18
107. Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, Tonetti MS, Wade WG, Zaura E (2016) The oral microbiome - an update for oral healthcare professionals. British Dental Journal 221:657-666
108. Kinane FD, Berglundh T, Lindhe J (2008) Pathogenesis of Periodontitis. In Clinical Periodontology and Implant Dentistry, Lindhe J, Lang NP, Karring K, eds. (Oxford, Blackwell Publishing, Ltd), pp. 285-306.
109. Koburger T, Hubner NO, Braun M, Siebert J, Kramer A (2010) Standardized comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine digluconate. Journal of Antimicrobial Chemotherapy 65:1712-1719
110. Kocak MM, Ozcan S, Kocak S, Topuz O, Ertend H (2009) Comparison of the Efficacy of Three Different Mouthrinse Solutions in Decreasing the Level of *Streptococcus Mutans* in Saliva. European Journal of Dentistry 3:57-61
111. Kolahi J, Soolari A (2006) Rinsing with chlorhexidine gluconate solution after brushing and flossing teeth: a systematic review of effectiveness. Quintessence International 37:605-612
112. Kour K, Kaur S (2019) Short term side effects of 0.2% and 0.12% chlorhexidine mouthwash. IP International Journal of Periodontology and Implantology 4:138-140
113. Kramer A, Assadian O (2013) SP19-4 Octenidine dihydrochloride – Characteristics and clinical use. International Journal of Antimicrobial Agents 42: S21
114. Kuroiwa M, Kodaka T, Kuroiwa M, Abe M (1992) Acid resistance of human enamel by brushing with and without abrasive dentifrice. Journal de biologie buccale 20:175-180
115. Lachapelle JM (2014) A comparison of the irritant and allergenic properties of antiseptics. European Journal of Dermatology 24:3-9
116. Lam YWF, Chan DCN, Rodriguez SY, Lintakoon JH, Lam TH (1993) Sensitive high-performance liquid chromatographic assay for the determination of chlorhexidine in saliva. Journal of Chromatography B: Biomedical Sciences and Applications 612:166-171

117. Lamkin MS, Arancillo AA, Oppenheim FG (1996) Temporal and compositional characteristics of salivary protein adsorption to hydroxyapatite. Journal of Dental Research 75:803-808
118. Lang PN, Mombelli A, Attström R (2008) Oral Biofilms and Calculus. In Clinical Periodontology and Implant Dentistry, Lindhe J, Lang NP, Karring K, eds. (Oxford, Blackwell Publishing, Ltd), pp. 183-206
119. Langner I, Kramer A, Matthes R, Rebert F, Kohler C, Koban I, Hübner NO, Kohlmann T, Patrzyk M (2019) Inhibition of microbial growth by cold atmospheric plasma compared with the antiseptics chlorhexidine digluconate, octenidine dihydrochloride, and polyhexanide. Plasma Processes and Polymers 16:e1800162
120. Larsen T, Freihn NE (2017) Dental biofilm infections–an update. APMIS 125:376-384
121. Lee YH, Zimmerman JN, Custodio W, Xiao Y, Basiri T, Hatibovic-Kofman S, Siqueira WL (2013) Proteomic evaluation of acquired enamel pellicle during *in vivo* formation. PLoS One 8:e67919
122. Lendenmann U, Grogan J, Oppenheim FG (2000) Saliva and dental pellicle-a review. Advances in Dental Research 14: 22-28
123. Lewis JK, Wei J, Siuzdak G (2000) Matrix-assisted Laser Desorption/Ionization Mass Spectrometry in Peptide and Protein Analysis. In Encyclopedia of Analytical Chemistry, Meyers RA, ed. pp. 5880-5894.
124. Lie T (1977) Scanning and transmission electron microscope study of pellicle morphogenesis. Scandinavian Journal of Dental Research 85:217-231
125. Lindhe J, Koch G (1967) The effect of supervised oral hygiene on the gingivae of children. Journal of Periodontal Research 2:215-220
126. Lindquist B, Edward S, Torell P, Krasse B (1989) Effect of different caries preventive measures in children highly infected with *mutans streptococci*. Scandinavian Journal of Dental Research 97:330-337
127. Löe H, Schiott CR (1970) The effect of mouth rinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. Journal of Periodontol Research 5:79-83
128. Löe H, Theilade E, Jensen SB (1965) Experimental gingivitis in man. Journal of Periodontology 36:177-187
129. Louise FB, Pauline JF, Anne LS (2017) Periodontal disease and the special needs patient. Periodontolgy 2000 74:182-193
130. MacFarlane TW, Ferguson MM, Mulgrew CJ (1984) Post extraction bacteremia: role of antiseptics and antibiotics. British Dental Journal 156:179-181
131. Macpherson LM, MacFarlane TW, Weetman DA, Stephen KW (1991) Comparison of the plaque microflora from natural and appliance-borne enamel surfaces. Caries Research 25:58-64

132. Magaz VR, Llovera BF, Martí M, Garre A (2018) Clinical impact and cosmetic acceptability of chlorhexidine-enriched toothpaste and mouthwash application on periodontal disease: A randomized clinical study. The Journal of Contemporary Dental Practice 19:1295-1300
133. Makkar S, Aggarwal A, Pasricha S, Kapur I (2015) Comparative evaluation of octenidine hydrochloride and chlorhexidine as antibacterial root canal irrigant. Indian Journal of Oral Sciences 6:10-13
134. Malhotra A, Bali A, Bareja R (2016) Anti-bacterial efficacy of octenidine as a mouth wash. International Journal of Pharmaceutical Sciences and Research 7:340-344
135. Mandel ID (1988) Chemotherapeutic agents for controlling plaque and gingivitis. Journal of Clinical Periodontology 15:488-498
136. Marsh PD (2004) Dental plaque as a microbial biofilm. Caries Research 38:204-211
137. Martínez-Hernández M, Reda B, Hannig M (2020) Chlorhexidine rinsing inhibits biofilm formation and causes biofilm disruption on dental enamel *in situ*. Clinical Oral Investigations 24:843–3853
138. McDonnell G, Russell AD (1999) Antiseptics and disinfectants: Activity, action, and resistance. Clinical Microbiology Reviews 12:147-179
139. Medlicott NJ, Ferry DG, Tucker IG, Rathbone MJ, Holborow DW, Jones DS (1994) High performance liquid chromatographic (HPLC) assay for the determination of chlorhexidine in saliva film. Journal of Liquid Chromatography & Related Technologies 17:1605-1620
140. Meurman JH, Frank RM (1991) Scanning electron microscopic study of the effect of salivary pellicle on enamel erosion. Caries Research 25:1-6
141. Mirzakhani H, Heidari H, Hasanpour A, Deris F (2017) Effect of chlorhexidine and orthodentol mouthwash on oral hygiene of patients who underwent mechanical ventilation, hospitalized in intensive care unit. Journal of Client-Centered Nursing Care 3:161-166
142. Müller G, Kramer A (2005) Effect of selected wound antiseptics on adult articular cartilage (bovine sesamoid bone) in the presence of *Escherichia coli* and *Staphylococcus aureus*. Journal of Orthopaedic Research 23:127-133
143. Müller G, Kramer A (2007) Interaction of octenidine and chlorhexidine with mammalian cells and the resulting microbicidal effect (remanence) of the combinations. GMS Hygiene and Infection Control 2:46-49
144. Müller G, Kramer A (2008) Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. Journal of Antimicrobial Chemotherapy 61:1281-1287
145. Musteata FM, Pawliszyn J (2005) Assay of stability, free and total concentration of chlorhexidine in saliva by solid phase microextraction. Journal of Pharmaceutical and Biomedical Analysis 37:1015-1024

146. Nasmyth A (1839) On the structure, physiology, and pathology of the persistent capsular investments and pulp of the tooth. Royal Medical and Chirurgical Society of London 22:310-328
147. Netuschil L, Hoffmann T, Brecx M (2003) How to select the right mouthrinses in periodontal prevention and therapy. Part 1. Test systems and clinical investigations. International Journal of Dental Hygiene 1:143-150
148. Netuschil L, Reich E, Unteregger G, Sculean A, Brecx M (1998) A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography. Archives of Oral Biology 43:277-285
149. Newcombe RG, Addy M, McKeown S (1995) Residual effect of chlorhexidine gluconate in 4-day plaque regrowth crossover trials, and its implications for study design. Journal of Periodontal Research 30:319-324
150. Okada EMP, Ribeiro LNS, Stuani MBS, Borsatto MC, da Silva Fidalgo TK, de Paula-Silva FWG, Küchler EC (2016) Effects of chlorhexidine varnish on caries during orthodontic treatment: A systematic review and meta-analysis. Brazilian Oral Research 30:e115
151. Olsson H, Asklöw B, Johansson E, Slotte C (2012) Rinsing with alcohol-free or alcohol-based chlorhexidine solutions after periodontal surgery. A double-blind, randomized, cross-over, pilot study. Swedish Dental Journal 6:91-99
152. Pai MR, Acharya LD, Udupa N (2004) The effect of two different dental gels and a mouthwash on plaque and gingival scores: a six-weeks clinical study. International Dental Journal 54:219-223
153. Papaioannou W, Vassilopoulos S, Vrotsos I, Margaritis V, Panis V (2016) A comparison of a new alcohol-free 0.2% chlorhexidine oral rinse to an established 0.2% chlorhexidine rinse with alcohol for the control of dental plaque accumulation. International Journal of Dental Hygiene 14:272-277
154. Patters MR, Anerud K, Trummel CL, Kornman KS, Nalbandian J, Robertson PB (1983) Inhibition of plaque-formation in humans by octenidine mouthrinse. Journal of Periodontal Research 18:212-219
155. Patters MR, Nalbandian J, Nichols FC, Niekrash CE, Kennedy JE, Kiel RA, Trummel CL (1986) Effects of octenidine mouthrinse on plaque-formation and gingivitis in humans. Journal of Periodontal Research 21:154-162
156. Pesonem J, Holmalahti J, Pohjola J (1995) Determination of chlorhexidine in saliva using high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 665:222-225
157. Pitten FA, Werner HP, Kramer A (2003) A standardized test to assess the impact of different organic challenges on the antimicrobial activity of antiseptics. Journal of Hospital Infection 55:108-115.

158. Prada-López I, Quintas V, Vilaboa C, Suárez-Quintanilla D, Tomás I (2016) Devices for *in situ* development of non-disturbed oral biofilm. A systematic review. Frontiers in Microbiology 7:1055

159. Pratten J, Barnett P, Wilson M (1998) Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. Applied and Environmental Microbiology 64:3515-3519

160. Puig-Silla M, Montiel-Company JM, Almerich-Silla JM (2008) Use of chlorhexidine varnishes in preventing and treating periodontal disease. A review of the literature. Medicina Oral, Patologia Oral, Cirugia Bucal 13:257-360

161. Raj D, Chakrabarty H, Badr J, Das G, Pal TK. A (2017) comparative evaluation between chlorhexidine chip and diode laser as an adjunct to scaling and root planning in patients with chronic periodontitis. Journal of Indonesian Dental Association 11:16-22

162. Reda B, Dudek J, Martínez-Hernández M, Hannig M (2021a) Effects of octenidine on the formation and disruption of dental biofilms: An exploratory in situ study in healthy subjects. Journal of Dental Research 1–10

163. Reda B, Hollemeyer K, Trautmann S, Hannig M Volmer DA (2020) Determination of chlorhexidine retention in different oral sites using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Archives of Oral Biology 110:104623

164. Reda B, Hollemeyer K, Trautmann S, Volmer DA, Hannig M (2021b) First insights into chlorhexidine retention in the oral cavity after application of different regimens. Clinical Oral Investigations

165. Reich M, Kummerer K, Al-Ahmad A, Hannig C (2013). Fatty acid profile of the initial oral biofilm (pellicle): an *in-situ* study. Lipids 48:929-937

166. Rohrer N, Widmer AF, Waltimo T, Kulik EM, Weiger R, Filipuzzi-Jenny E, Walter C (2010) Antimicrobial efficacy of 3 oral antiseptics containing Octenidine, polyhexamethylene biguanide, or citroxx: Can chlorhexidine be replaced? Infection Control & Hospital Epidemiology 31:733-739

167. Rölla G, Löe H, Schiott CR (1970) The affinity of chlorhexidine for hydroxyapatite and salivary mucins. Journal of Periodontal Research 5:90-95

168. Rölla G, Melsen B (1975) On the mechanism of plaque inhibition by chlorhexidine. Journal of Dental Research (special issue B) 54:57-62

169. Rupf S, Balkenhol M, Sahrhage TO, Baum A, Chromik JN, Ruppert K, Wissenbach DK, Maurer HH, Hannig M (2012) Biofilm inhibition by an experimental dental resin composite containing octenidine dihydrochloride. Dental Materials 28:974-984

170. Sachdeva S, Grover V, Malhotra R, Kapoor A, Mohanty K (2018) Comparison of clinical effectiveness of single and multiple applications of 1% chlorhexidine varnish (Cervitec Plus)

along with scaling and root planning in patients with chronic periodontitis. Journal of Indian Society of Periodontology 22:523-528

171. Sanz M, Newman MG, Anderson L, Matoska W, Otomo-Corgel J, Saltini C (1989) Clinical enhancement of post-periodontal surgical therapy by a 0.12% chlorhexidine gluconate mouthrinse. Journal of Periodontology 60:570-576
172. Schmidt J, Zyba V, Jung K, Rinke S, Haak R, Mausberg RF, Ziebolz D (2016) Cytotoxic effects of octenidine mouth rinse on human fibroblasts and epithelial cells – an *in vitro* study. Drug and Chemical Toxicology 39:322-330
173. Sedlock DM, Bailey M (1985) Microbicidal activity of octenidine hydrochloride, a new alkanediylbis (pyridine) germicidal agent. Antimicrobial Agents and Chemotherapy 28:786-790
174. Sennhenn-Kirchner S, Nadine W, Klaue S, Mergeryan H, Zepelin M (2009) Decontamination efficacy of antiseptic agents on *in vivo* grown biofilms on rough titanium surfaces. Quintessence International 10:e80-e88
175. Shah R, Donde R, Mitra D, Rodrigues S (2019) Chlorhexidine-different forms in dentistry - A review. World Journal of Advanced Scientific Research 2:225-231
176. Shani S, Friedman M, Steinberg D (2000) The anticariogenic effect of amine fluorides on *streptococcus sobrinus* and glucosyltransferase in biofilms. Caries Research 34:260-267
177. Shapiro S, Giertsen E, Guggenheim B (2002) An *in vitro* oral biofilm model for comparing the efficacy of antimicrobial mouthrinses. Caries Research 36:93-100
178. Slee AM, O'Connor JR (1983) *In vitro* antiplaque activity of octenidine dihydrochloride (WIN 41464-2) against preformed plaques of selected oral plaque-forming microorganisms. Antimicrobial Agents and Chemotherapy 23:379-384
179. Slee AM, Cimijotti E, Rothstein S (1985) The effect of daily treatments with an octenidine dentifrice formulation on gingival health in cynomolgus monkeys. Journal of Periodontal Research 20:542-549
180. Slomiany BL, Murty VLN, Mandel ID, Sengupta S, Slomiany A (1990) Effect of lipids on the lactic-acid retardation capacity of tooth enamel and cementum pellicles formed *in vitro* from saliva of caries-resistant and caries-susceptible human adult. Archives of Oral Biology 35:75-80
181. Slomiany BL, Murty VLN, Zdebska E, Slomiany A, Gwozdzinski K, Mandel ID (1986) Tooth surface-pellicle lipids and their role in the protection of dental enamel against lactic-acid diffusion in man. Archives of Oral Biology 31:187-191
182. Slot DE, Berchier CE, Addy M, Van der Velden U, Van der Weijden GA (2014) The efficacy of chlorhexidine dentifrice or gel on plaque, clinical parameters of gingival inflammation and tooth discoloration: a systematic review. International Journal of Dental Hygiene 12:25-35
183. Slot DE, Rosema NA, Hennequin-Hoenderdos NL, Versteeg PA, Van der Velden U, Van der Weijden GA (2010) The effect of 1% chlorhexidine gel and 0.12% dentifrice gel on plaque accumulation: a 3-day non-brushing model. International Journal of Dental Hygiene 8:294-300

184. Socransky SS, Haffajee AD (2002) Dental biofilms: Difficult therapeutic targets. Periodontology 2000 28:12-55

185. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1998) Microbial complexes in subgingival plaque. Journal of Clinical Periodontology 25:134-144

186. Socransky SS, Haffajee AD (2008) Periodontal Infections. In Clinical Periodontology and Implant Dentistry, Lindhe J, Lang NP, Karring K, eds. (Oxford, Blackwell Publishing, Ltd), pp. 207-267.

187. Sonju T, Rölla G (1973) Chemical analysis of the acquired pellicle formed in two hours on cleaned human teeth *in vivo*. Rate of formation and amino acid analysis. Caries Research 7:30-38

188. Soskolne WA, Heasman PA, Stabholz A, Smart GJ, Palmer M, Flashner M, Newman HN (1997) Sustained local delivery of chlorhexidine in the treatment of periodontitis: a multi-center study. Journal of Periodontology 68:32-38

189. Stanley A, Wilson M, Newman HN (1989) The *in vitro* effects of chlorhexidine on subgingival plaque bacteria. Journal of Clinical Periodontology 16:259-264

190. Stewart S, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358:135-138

191. Stirrups DR, Laws E, Honigman JL (1981) The effect of a chlorhexidine mouthrinse on oral health during fixed appliance orthodontic treatment. British Dental Journal 151:84-86

192. Stocklin R, Arrighi JF, Hoang-Van K, Vu L, Cerini F, Gilles N, Genet R, Markussen J, Offord RE. Rose K (2000) Positive and negative labelling of human proinsulin, insulin, and C-peptide with stable isotopes. New tools for *in vivo* pharmacokinetic and metabolic studies. Methods in Molecular Biology 146:293-315

193. Stocklin R, Vu L, Vadas L, Cerini F, Kippen AD, Offord RE, Rose K (1997) A stable isotope dilution assay for the *in vivo* determination of insulin levels in humans by mass spectrometry. Diabetes 46:44-50

194. Stoeken JE, Versteeg PA, Rosema NA, Timmerman MF, Van der Velden U, Van der Weijden GA (2007) Inhibition of "de novo" plaque formation with 0.12% chlorhexidine spray compared to 0.2% spray and 0.2% chlorhexidine mouthwash. Journal of Periodontology 78:899-904

195. Storhaug K (1977) Habitant in oral disease in handicapped patients. Journal of Clinical Periodontology 4:102-107

196. Supranoto SC, Slot DE, Addy M, Van der Weijden GA (2015) The effect of chlorhexidine dentifrice or gel versus chlorhexidine mouthwash on plaque, gingivitis, bleeding and tooth discoloration: a systematic review. International Journal of Dental Hygiene 13:83-92

197. Taira EA, Ventura TMS, Cassiano LPS, Silva CMS, Martini T, Leite AL, Rios D, Magalhães AC, Buzalaf MAR (2018) Changes in the proteomic profile of acquired enamel pellicles as a function of their time of formation and hydrochloric acid exposure. Caries Research 52:367-377

198. Takahashi N, Nyvad B (2008) Caries ecology revisited: microbial dynamics and the caries process. Caries Research 42:409-418
199. Takenaka S, Ohsumi T, Noiri Y (2019) Evidence-based strategy for dental biofilms: Current evidence of mouthwashes on dental biofilm and gingivitis. Japanese Dental Science Review 55:33-40
200. Tandjung L, Waltimo T, Hauser I, Heide P, Decker EM, Weiger R (2007) Octenidine in root canal and dentine disinfection *ex vivo*. International Endodontic Journal 40:845-851
201. Tawakoli PN, Al-Ahmad A, Hoth-Hannig W, Hannig M, Hannig C (2013) Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm. Clinical Oral Investigations 17:841-50
202. ten Cate JM, Marsh PD (1994) Procedures for establishing efficacy of antimicrobial agents for chemotherapeutic caries prevention. Journal of Dental Research 73:695-703
203. Tinanoff N, Glick PL, Weber DF (1976) Ultrastructure of organic films on the enamel surface. Caries Research 10:19-32
204. Tirali RE, Bodur H, Sipahi B, Sungurtekin E (2013) Evaluation of the antimicrobial activities of chlorhexidine gluconate, sodium hypochlorite and octenidine hydrochloride *in vitro*. Australian Dental Journal 39:15-18
205. Tirali RE, Turan Y, Akal N, Karahan ZC (2009) *In vitro* antimicrobial activity of several concentrations of NaOCl and Octenisept in elimination of endodontic pathogens. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 108:E117-E120
206. Tomás I, Cousido MC, Garcia-Caballero L, Rubido S, Limeres J, Diz P (2010) Substantivity of a single chlorhexidine mouthwash on salivary flora: influence of intrinsic and extrinsic factors. Journal of Dentistry 8:541-546
207. Tomás I, Cousido MC, Tomás M, Limeres J, García-Caballero L, Diz P (2008) *In vivo* bactericidal effect of 0.2% chlorhexidine but not 0.12% on salivary obligate anaerobes. Archives of Oral Biology 53:1186-1191
208. Tomás I, García-Caballero L, Cousido MC, Limeres J, Alvarez M, Diz P (2009) Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy. Oral Diseases 15:428-433
209. Trautmann S, Barghash A, Fecher-Trost C, Schalkowsky P, Hannig C, Kirsch J, Rupf S, Keller A, Helms V, Hannig M (2019) Proteomic analysis of the initial oral pellicle in caries-active and caries-free individuals. Proteomics Clinical Applications 13:e1800143
210. Trautmann S, Künzel N, Fecher-Trost C, Barghash A, Schalkowsky P, Dudek J, Delius J, Helms V, Hannig M (2020) Deep proteomic insights into the individual short-term pellicle formation on enamel-an *in situ* pilot study. Proteomics Clinical Applications 14:e1900090
211. Tsuchiya H, Miyazaki T, Ohmoto S (1999) High-performance liquid chromatographic analysis of chlorhexidine in saliva after mouthrinsing. Caries Research 33:156-163

212. Tsuchiya H, Sato M, Iinuma M, Yokoyama J, Ohyama M, Tanaka T, Takase I, Namikawa I (1994) Inhibition of the growth of cariogenic bacteria *in vitro* by plant flavanones. Experientia 50:846-849
213. Valm AM (2019) The structure of dental plaque microbial communities in the transition from health to dental caries and periodontal disease. Journal of Molecular Biology 431:2957-2969
214. Van Strydonck DA, Timmerman MF, van der Velden U, van der Weijden GA (2005) Plaque inhibition of two commercially available chlorhexidine mouthrinses. Journal of Clinical Periodontology 32:305-309
215. Van Strydonck DAC, Slot DE, Velden U, Weijden F (2012) Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a systematic review. Journal of Clinical Periodontology 39:1042-1055
216. Vanscheidt W, Baer M, May TW, Siebert J (2005) Beeinflussung der Wundheilung bei chronischen Beinulzera durch ein lokales Octenidindihydrochloridhaltiges Wundantiseptikum. Hygiene & Medizin 30:153-158
217. Viana GR, Teiltelbaum AP, dos Santos FA, Sabbagh-Haddad A, Guare RO (2014) Chlorhexidine spray as an adjunct in the control of dental biofilm in children with special needs. Special Care in Dentistry 34:286-290
218. Vitkov L, Hermann A, Krautgartner WD, Herrmann M, Fuchs K, Klappacher M, Hannig M (2005) Chlorhexidine-induced ultrastructural alterations in oral biofilm. Microscopy Research and Technique 68:85-89
219. von Ohle C, Gieseke A, Nistico L, Decker EM, DeBeer D, Stoodley P (2010) Real-time microsensor measurement of local metabolic activities in *ex vivo* dental biofilms exposed to sucrose and treated with chlorhexidine. Applied and Environmental Microbiology 76:2326-2334
220. Wade W, Addy M (1989) *In vitro* activity of a chlorhexidine containing mouth-rinse against subgingival bacteria. Journal of Periodontology 60:521-525
221. Weiger R, Netuschil L, von Ohle C, Brecx M (1995) Microbial vitality of supragingival dental plaque during initial stages of experimental gingivitis in humans. Journal of Periodontal Research 30:204-209
222. Welk A, Zahedani M, Beyer C, Kramer A, Müller G (2016) Antibacterial and antiplaque efficacy of a commercially available octenidine-containing mouthrinse. Clinical Oral Investigations 20:1469-1476
223. Worral SF, Knibbs PJ, Glenwright HD (1987) Methods of reducing contamination of the atmosphere from use of an air polisher. British Dental Journal 163:118-119
224. Zaura-Arite E, van Marle J, ten Cate JM (2001) Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. Journal of Dental Research 80:1436-1440

7 Publications and Acknowledgements

7.1 Publications

Published works:

Reda B, Hollemeyer K, Trautmann S, Hannig M Volmer DA (2020) Determination of chlorhexidine retention in different oral sites using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Archives of Oral Biology 110:104623

Martínez-Hernández M, Reda B, Hannig M (2020) Chlorhexidine rinsing inhibits biofilm formation and causes biofilm disruption on dental enamel *in situ.* Clinical Oral Investigations 24:3843–3853

Reda B, Hollemeyer K, Trautmann S, Volmer DA, Hannig M (2021) First insights into chlorhexidine retention in the oral cavity after application of different regimens. Clinical Oral Investigations

Liua Y, Zhao R, Reda B, Yangb W, Hannig M, Qu B (2021) Profiling of cytokines, chemokines and growth factors in saliva and gingival crevicular fluid. Cytokine

Reda B, Dudek J, Martínez-Hernández M, Hannig M (2021) Effects of octenidine on the formation and disruption of dental biofilms: An exploratory in situ study in healthy subjects. Journal of Dental Research 1–10

Published abstracts:

Reda B, Planz V, Yazdani BN, Dudek J, Windbergs M, Hannig M (2019) PD151: Local delivery of antimicrobial plant extracts using electrospun fibers – an *in situ* study. Clinical Oral Investigations 23:2564

Reda B, Martínez-Hernández M, Hannig M (2018) PD035: First-time evaluation of octenidine mouth rinsing effects on the oral bacteria and biofilm ultrastructure *in situ.* Journal of Clinical Periodontology 45:52-52

Short lectures and poster presentations:

Reda B, Martínez-Hernández M, Hannig M. Einfluss einer Octenidin-Mundspülung auf die *in situ*-Biofilmbildung und den reifen mikrobiellen oralen Biofilm 52 Jahrestagung der Arbeitsgemeinschaft für Grundlagenforschung (AfG). (2020). Mainz, Deutschland.

Reda B, Planz V, Yazdani BN, Dudek J, Windbergs M, Hannig M. Local delivery of antimicrobial plant extracts using electrospun fibers – an *in situ* study. CONSEURO 9. (2019). Berlin, Germany.

Rada B, Yazdani BN, Günday-Türeli N, Hannig M. Wirkung von verschiedenen Pflanzenextrakten auf orale Bakterien – eine *ex vivo* und *in situ* Studie. 51 Jahrestagung der Arbeitsgemeinschaft für Grundlagenforschung (AfG). (2019). Mainz, Deutschland.

Reda B, Martínez-Hernández M, Hannig M. Octenidine rinsing inhibits biofilm formation and causes biofilm disruption on dental enamel *in situ* Cell Physics. (2019). Saarbrücken, Germany.

Reda B, Martínez-Hernández M, Hannig M. First-time evaluation of octenidine mouth rinsing effects on the oral bacteria and biofilm ultrastructure *in situ.* EuroPerio9. (2018) Amsterdam, Netherlands.

Reda B, Hollemeyer K, Trautmann S, Volmer DA, Hannig M. Detektion von Chlorhexidin in der Mundhöhle mittels MALDI-TOF MS nach der Anwendung verschiedener Chlorhexidin-Produkte. 32. Jahrestagung der DGZ mit der DGPZM und der DGR²Z. (2018). Dortmund, Deutschland.

Reda B, Martínez-Hernández M, Hannig M. Effect of chlorhexidine on the oral bacteria adhesion and biofilm ultrastructure. CED-IADR/NOF Oral Health Research Congress (2017). Vienna, Austria.

Reda B, Martínez-Hernández M, Hannig M. Analyse der Octenidin Wirkung auf die bakterielle Viabilität und orale Biofilm-Ultrastruktur *in situ.* 3. Gemeinschaftstagung der DGZ mit der DGPZM, DGR²Z und DGET. (2017). Berlin, Deutschland.

Reda B, Hollemeyer K, Trautmann S, Hannig M, Volmer DA. Determination of chlorhexidine by MALDI-TOF MS after application of different chlorhexidine formulations. Cell Physics. (2017). Saarbrücken, Germany.

Reda B, Hollemeyer K, Trautmann S, Volmer DA, Hannig M, Detektion von Chlorhexidin an verschiedenen Stellen der Mundhöhle mittels MALDI-TOF Massenspektrometrie. 30. DGZ-Jahrestagung | DGZ-Tag der Wissenschaften/Universitäten. (2016). Leipzig, Deutschland.

7.2 Acknowledgements

Foremost, I would like to express my gratitude and respect to my supervisor Prof. Dr. Matthias Hannig for his continuous support during my Ph.D. study. I am grateful to him for his motivation, valuable discussions, and outstanding guidance in accomplishing this work.

Special thanks to Dr. Johanna Dudek, Dr. Lilia Lemke, and Dr. Simone Trautman for their daily supportive guidance, discussions, and suggestions in the methodological and laboratory work and for their emotional support during the last few years.

In addition, I thank Prof. Dr. Markus Hoth for the supportive discussions and suggestions for this work.

Besides, I would like to thank all my fellow lab-mates and especially Mrs. Cíntia Mirela Guimarães Nobre, Mrs. Nazife Yazdani Badabi, Dr. Miryam Martínez-Hernández, and Mr. Norbert Putz for their kindness and help with laboratory related work and for the lovely moments we shared together.

I am also very grateful to Dr. Klaus Hollemeyer for the MALDI-TOF analysation as well as for teaching me how to interpret the MALDI-TOF data. As well as special thanks to Mrs. Birgit Leis and Mrs. Belinda König for technical assistance with the processing of the samples for TEM analysis and the excellent images. I am thankful to Mrs. Bettina Neisius-Paul, who prepared the acrylic splints. I appreciate also the help and assistance from Mrs. Silvia Klein.

In addition, I am grateful to my teachers and colleagues at Aleppo University, especially Prof. Dr. Mohammad Mazen Kabbani for all the help and emotional support during studying and working in the faculty of dentistry.

Lastly, my deepest gratitude to my family, my beloved wife, and my angels for their love, kindness, and support throughout my life. To them, I dedicate this dissertation.