# The Fatty Acid Profile of the Initial Oral Biofilm: Characterization and Modification

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# PREFACE

The underlying work of this cumulative dissertation was conducted between Februrary 2010 and April 2014. The development and early work of the study was done at the Department of Environmental Health Sciences (Prof. Dr. K. Kümmerer) in cooperation with the Department of Operative Dentistry and Periodontology (PD Dr. C. Hannig), both located at the University Medical Center of the Albert-Ludwigs University Freiburg at this time. Within the first year, both groups moved to different universities. After the relocation process, which ended up being easier said than done, the work was successfully continued at the Institute of Sustainable and Environmental Chemistry at Leuphana University Lueneburg in cooperation with the Clinic of Operative Dentistry at TU Dresden.

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This cumulative dissertation is based on three scientific papers, all of which are published in international peer-reviewed journals and focus on lipids/fatty acids in the pellicle:

1. Marco Reich, Christian Hannig, Ali Al-Ahmad, Richard Bolek and Klaus Kümmerer (**2012**). A Comprehensive Method for Determination of Fatty Acids in the Initial Oral Biofilm (Pellicle). *Journal of Lipid Research* 56: 2226-2230.
   DOI: 10.1194/jlr.D026260

2. Marco Reich, Klaus Kümmerer, Ali Al-Ahmad and Christian Hannig (**2013**). Fatty Acid Profile of the Initial Oral Biofilm (Pellicle): an in-situ Study. *Lipids* 48: 929-937.
   DOI: 10.1007/s11745-013-3822-2

3. Anna Kensche, Marco Reich, Klaus Kümmerer and Christian Hannig (**2013**). Lipids in Preventive Dentistry. *Clinical Oral Investigations* 17: 669-685.
   Review article
   DOI: 10.1007/s00784-012-0835-9

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In the following extended summary, a general introduction and the background of the work performed during the doctorate are given in chapters 1 and 2. Chapter 3 describes the objectives and hypotheses, and chapter 4 outlines the study's methodical approach. The comprehensive data and results are presented and discussed in chapter 5, followed by the final conclusion and an outlook to future research needs in chapter 6.

## ABSTRACT

Despite the great progress that has been made in the prophylaxis of oral diseases over the past decades, dental caries and periodontal diseases remain major challenges in the field of dentistry. Biofilm formation on dental hard tissues is strongly associated with the etiology of these oral diseases. Therefore, the process of bioadhesion and biofilm formation on tooth surfaces is of particular interest for dental research.

The first stage of bioadhesion on dental surfaces is the formation of the pellicle layer. This mainly acellular film, composed largely of adsorbed proteins, glycoproteins, and lipids, is distinguished from the microbial biofilm (plaque). As the interface between teeth and the oral environment, the pellicle plays a key role in the maintenance of oral health and is of great physiological and pathophysiological importance. On the one hand, the pellicle shows protective properties for the underlying dental hard tissues. On the other hand, it also serves as the basis for dental plaque and therefore, for the development of oral diseases such as caries and periodontitis. Hydrophobic interactions, which are governed by lipophilic substances, are of high relevance for bacterial adherence. Therefore, pellicle lipids, which are a significant constituent of this biological structure, are an interesting target for dental research, as they could modulate oral surfaces, influence microbial interactions, and potentially impede bacterial adherence. Compared to the extensive work on the pellicle's ultrastructure and protein/amino acid composition, little attention has been given to its lipid profile. Knowledge of the lipid composition of the pellicle may provide insight into several oral pathological states, including caries, dental erosion, and periodontal disease processes and could contribute to novel approaches in preventive dentistry.

The principle aim of this thesis was the comprehensive characterization of the fatty acid (FA) profile of the *in situ* formed pellicle layer. This includes the influence of pellicle maturation on the FA profile as well as intra- and interindividual differences. Furthermore, investigations on the effect of rinses with edible oils on the pellicle's FA composition were a focus of this work. For these purposes, an analytical method based on a combination of innovative specimen generation and convenient sample preparation with sensitive mass spectrometric analysis was successfully developed and comprehensively validated within this thesis. Pellicle samples were formed *in situ* on bovine enamel slabs mounted on individual upper jaw splints. After a comprehensive sample preparation, gas chromatography coupled with electron impact ionization mass spectrometry (GC-EI/MS) was used in order to characterize qualitatively and quantitatively a wide range of FA ($C_{12}$-$C_{24}$).

The individual FA profiles of pellicle and saliva samples collected from ten research participants were investigated. The relative FA profiles of the pellicle samples gained from the different subjects were very similar, whereas the amount of FAs showed significant interindividual variability. Compared to the pellicle's characteristic FA profile, higher proportions of unsaturated FAs were detected in the saliva samples, highlighting that FAs available in saliva are not adsorbed equivalently to the pellicle layer. This, in turn, shows that pellicle formation is a highly selective process that does not correlate directly with salivary composition. Additionally, pellicle samples collected after 3, 30, 60, 120, and 240 min of intraoral exposure were analyzed. It could be shown that pellicle maturation has only a minor impact on the FA composition. However, the FA content increased substantially with increasing oral exposure time.

Modifying the pellicle's lipid composition by using edible oils as a mouthwash could alter the physicochemical characteristics of the pellicle and strengthen its protective properties by delaying bacterial adhesion. Therefore, the impact of rinses with safflower oil on the pellicle's FA composition was determined. The application of rinses with safflower oil resulted in an accumulation of its specific FAs in the pellicle, thus representing a possibility for modifying the pellicle's lipid profile.

The present work is the first to apply a validated method that combines *in situ* pellicle formation, sample preparation, and the comprehensive determination of FAs via a sensitive analytical method. The results provide valuable information regarding the pellicle's FA composition which closes an existing knowledge gap in pellicle research. A broader knowledge of the lipid composition of the pellicle contributes to the understanding of oral bioadhesion processes and may help facilitate novel approaches in preventive dentistry.

## Zusammenfassung

Trotz der bedeutenden Fortschritte, die in den letzten Jahrzenten zur Vorbeugung oraler Erkrankungen gemacht wurden, stellen Karies und Parodontitis weiterhin eine große Herausforderung für die Zahnmedizin dar. Maßgebliche Ursache dieser Erkrankungen sind adhärente Biofilme auf den Zahnhartsubstanzen. Eine besondere Determinante für die Ausbildung eines pathogenen Biofilms sind die initialen Bioadhäsionsprozesse, die aus diesem Grund auch für die zahnmedizinische Forschung von großer Bedeutung sind.

Der erste Schritt der Bioadhäsion auf oralen Festkörperoberflächen ist die Ausbildung der Pellikelschicht. Dieser weitgehend zellfreie Film besteht größtenteils aus adsorbierten Proteinen, Glykoproteinen und Lipiden. Dadurch wird die Pellikel vom bakteriellen Biofilm, der sogenannten Plaque, abgegrenzt, die erst sekundär auf Basis der Pellikel entsteht. Als maßgeblicher Mediator zwischen den oralen Festkörperoberflächen und der mikrobiellen Flora der Mundhöhle, nimmt die Pellikel als eigene Entität eine Schlüsselrolle in der Erhaltung der Mundgesundheit ein. Einerseits besitzt die Pellikel eine Vielzahl protektiver Eigenschaften, dient andererseits aber auch als Grundlage für die Ausbildung der Plaque. Somit kommt der Pellikel eine besondere physiologische und pathophysiologische Bedeutung bei der Entstehung von Karies und Parodontopathien zu. Hydrophoben Wechselwirkungen, die naturgemäß durch lipophile Substanzen bestimmt werden, wird eine besondere Bedeutung bei oralen Bioadhäsionsphänomenen zugeschrieben. Dieser Zusammenhang macht Pellikellipide, die einen wesentlichen Bestandteil dieser Schicht ausmachen, zu einem interessanten Forschungsgegenstand der Zahnmedizin. Die Lipidzusammensetzung der Pellikel könnte unter anderem Einfluss auf mikrobielle Wechselwirkungen haben und dadurch die Anheftung von Bakterienzellen an Oberflächen im Mundraum hemmen. Im Vergleich zu den detaillierten Untersuchungen, die zur Ultrastruktur und Proteinzusammensetzung der Pellikel existieren, wurde die Erforschung der Lipidzusammensetzung bisher stark vernachlässigt. Umfassendere Kenntnisse des Lipidprofils der Pellikel könnten Aufschluss über den Zustand verschiedener oraler Erkrankungen geben und dazu beitragen neue Strategien in der Prophylaxe zu entwickeln.

Übergeordnetes Ziel der vorliegenden Arbeit war die umfassende Charakterisierung des Fettsäureprofils der *in situ* gebildeten Pellikel. Dabei sollte der Einfluss der Pellikelbildungszeit ebenso erfasst werden wie inter- und intraindividuelle Unterschiede. Darauf aufbauend lag ein weiterer Fokus auf der Untersuchung der Auswirkung von Spülungen mit Speiseölen auf die Fettsäurezusammensetzung der Pellikel. Es wurde daher zunächst eine neue Analysenmethode entwickelt und umfassend validiert, welche auf der Kombination einer innovativen Probengenerierung mit geeigneter Probenvorbereitung und

einer daran anschließenden empfindlichen massenspektrometrischen Analyse basiert. Mit Hilfe dieser neuen Methode ist es möglich, sowohl Hauptfettsäuren als auch Minorfettsäuren der Pellikel gleichermaßen sicher qualitativ und quantitativ zu erfassen. Die Bildung der Pellikelproben erfolgte *in situ* auf Prüfkörpern aus Rinderzahnschmelz, die auf individuell angefertigten Tiefziehschienen präpariert wurden. Auf die umfassende Probenvorbereitung folgte die Identifizierung und Quantifizierung der einzelnen Fettsäuren ($C_{12}$-$C_{24}$) mittels Gaschromatographie gekoppelt mit Elektronenstoßionisation Massenspektrometrie (GC-EI/MS).

Die Untersuchung der individuellen Fettsäurezusammensetzung der Pellikel bei zehn Probanden zeigte ein charakteristisches Fettsäureprofil, welches sich von Proband zu Proband sehr stark ähnelte. Der Gesamtfettsäuregehalt hingegen variierte erheblich. Beim Vergleich von Pellikelproben mit Speichelproben, die unmittelbar nach der Probenahme der Pellikel genommen wurden, fand sich im Speichel ein deutlich höherer Anteil an ungesättigten Fettsäuren. Diese Beobachtung zeigt, dass die Fettsäuren aus dem Speichel nicht im gleichen Verhältnis an die Pellikelschicht adsorbieren, was wiederum für einen selektiven Prozess der Pellikelbildung spricht. Weitere Pellikelproben wurden nach unterschiedlichen Bildungszeiten (3, 30, 60, 120 und 240 min) auf ihre Fettsäurezusammensetzung untersucht. Es konnte eine Zunahme der Gesamtmenge an Fettsäuren mit steigender Pellikelbildungszeit beobachtet werden. Das Fettsäureprofil hingegen blieb während der unterschiedlichen Expositionszeiten nahezu unverändert. Des Weiteren wurde der Einfluss von Ölspülungen (am Beispiel von Distelöl) auf die Fettsäurezusammensetzung der Pellikel bestimmt. Eine Modifizierung der Pellikellipide durch Mundspülungen mit Speiseölen könnte zu einer Verbesserung der protektiven Eigenschaften der Pellikel führen. Auf diese Weise könnte die Pellikel verstärkt hydrophobisiert werden, was wiederum eine Auswirkung auf die bakterielle Kolonisation der Zahnoberfläche haben könnte. Die untersuchten Proben nach Distelölspülung wiesen einen stark erhöhten Anteil der im Öl enthaltenen Hauptfettsäuren auf. Somit stellen Ölspülungen eine Möglichkeit dar, das Lipidprofil der Pellikel zu modifizieren.

Im Rahmen der vorliegenden Arbeit wurde erstmals die Fettsäurekomposition der *in situ* Pellikel systematisch mit einer Methodenkombination aus *in situ* Probengenerierung, Probenvorbereitung und der umfassenden Fettsäurebestimmung mit einer sensitiven Anlysenmethode untersucht. Die erhaltenen Ergebnisse liefern wertvolle Informationen über die Lipidzusammensetzung der Pellikel und schließen eine bestehende Wissenslücke im Bereich der zahnmedizinischen Forschung. Umfassendere Kenntnisse über die Beschaffenheit und Zusammensetzung der Pellikel tragen dazu bei, orale Bioadhäsionsprozesse besser zu verstehen, was wiederum die Entwicklung neuer Ansätze für die präventive Zahnheilkunde vorantreiben kann.

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# List of Abbreviations

| | |
|---|---|
| **BAME** | Bacterial acid methyl ester |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EI** | Electron impact |
| **ESI** | Electrospray ionization |
| **FA** | Fatty acid |
| **FAME** | Fatty acid methyl ester |
| **FFA** | Free fatty acid |
| **FID** | Flame ionization detector |
| **GC** | Gas chromatography |
| **MALDI** | Matrix-assisted laser desorption/ionization |
| **MS** | Mass spectrometry |
| **m/z** | Mass-to-charge-ratio |
| **IS** | Internal standard |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantification |
| **QC** | Quality control |
| **SD** | Standard deviation |
| **RT** | Retention time |
| **SIM** | Selected ion monitoring |
| **TAG** | Triacylglycerols |
| **TEM** | Transmission electron microscopy |
| **TLC** | Thin layer chromatography |

***Table 1.*** *Nomenclature of fatty acids used in this thesis: Systematic name, trivial name, abbreviated designation, and structural formula according to the LIPID MAPS classification system for lipids (Fahy, 2005; Fahy et al., 2008).*

| Systematic name | Trivial name | Abbreviated designation | Structural formula |
|---|---|---|---|
| dodecanoic | lauric | 12:0 | |
| tridecanoic | | 13:0 | |
| tetradecanoic | myristic | 14:0 | |
| 12-methyl-tetradecanoic | anteiso-pentadecanoic | a15:0 | |
| pentadecanoic | | 15:0 | |
| hexadecanoic | palmitic | 16:0 | |
| cis-9-hexadecenoic | palmitoleic | 16:1n9c | |
| octadecanoic | stearic | 18:0 | |
| cis-9-octadecenoic | oleic | 18:1n9c | |
| cis,cis-9,12-octadecadienoic | linoleic | 18:2n6c | |
| nonadecanoic | | 19:0 | |
| eicosanoic | arachidic | 20:0 | |
| cis-13-docosenoic | erucic | 22:1n9c | |

# 1. INTRODUCTION

Oral health is an integral part of general health and has a decisive influence on one's sense of wellbeing. Therefore, the prevention of oral diseases is an essential part of dentistry and dental research. Although caries and periodontitis are largely preventable and great improvements in the field of prophylaxis have been made, they remain two challenging diseases with extremely high prevalence and considerable economic relevance (Bagramian et al., 2009; Blinkhorn and Davies, 1996; Holtfreter et al., 2010; Marsh, 2005; Petersen, 2003; Petersen et al., 2005). According to the WHO's oral health report from 2003, caries seems to be affected by socioeconomic status, as it is a major health problem not only in industrialized countries, but also increasingly in developing countries worldwide (Petersen, 2003).

Biofilm formation on dental hard tissues, the only non-shedding surface in the human organism, is fundamental for the development of these oral diseases (Marsh, 2004, 2005). Therefore, the process of bioadhesion on tooth surfaces is of particular interest for dental research. The first stage of bioadhesion on solid surfaces exposed to oral fluids is the formation of the pellicle layer. This mainly acellular film, composed largely of adsorbed proteins, glycoproteins, and lipids, is distinguished clearly from the microbial biofilm (plaque) (Hannig and Hannig, 2009a; Hannig and Joiner, 2006; Siqueira et al., 2012). The pellicle represents the interface between the teeth and the oral environment, therefore playing a key role in mediating the process of bioadhesion. Lipids are an important constituent that account for about one quarter of the pellicle's dry weight. Several studies indicate the relevance of hydrophobic interactions and cell hydrophobicity for bacterial adherence (Busscher and van der Mei, 1997; Gibbons and Etherden, 1983; Nyvad et al., 2013; Quirynen et al., 1989). Lipophilic substances potentially govern hydrophobic interactions. Thus, lipids in the pellicle are assumed to have an impact on the process of bioadhesion on dental hard tissues. Despite their high occurrence and assumed importance, only limited information is available on the nature, function and composition of lipids in the pellicle (Paper 3). With that said, lipids in the pellicle are an interesting target for dental research, as they could influence microbial interactions, modulate oral surfaces, and potentially impede bacterial adherence (Hannig et al., 2004; Hannig and Joiner, 2006).

A broader knowledge of the lipid composition of the initial oral biofilm will help in understanding oral bioadhesion processes and could contribute to novel approaches in preventive dentistry. Furthermore, pellicle lipids could serve as valuable biomarkers for diagnostic applications of oral diseases.

# 2. BACKGROUND

## 2.1 Bioadhesion and biofilm formation

Interactions of microorganisms with surfaces were discovered relatively late in the history of microbiology. The importance of sessile growth was first recognized and systematically investigated in the 1930s by Claude E. Zobell, one of the pioneers in the field of biofilm microbiology (Zobell and Allen, 1933; Zobell and Anderson, 1936; Zobell, 1943). Since then, the definition of biofilms evolved over time along with the knowledge accumulated on the structure and function of natural biofilms. However, there is still no universal definition. This can be partially explained by the broad diversity of microbial populations in biofilms. Some common and often cited definitions describe the term "biofilm" as "an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix" (Donlan, 2002), as "a functional consortium of microorganisms organised within an extensive exopolymer matrix" (Elder et al., 1995), or simply as "a community of microbes embedded in an organic polymer matrix, adhering to a surface" (Carpentier and Cerf, 1993).

The phenomenon of bioadhesion, where natural and synthetic materials adhere to a biological surface, and the subsequent development of a biofilm is ubiquitous in different ecosystems. This process represents a survival strategy employed by virtually all bacteria that has evolved over millions of years (Busscher et al., 2012; Costerton et al., 1987; Davey and O'toole, 2000; Dunne, 2002). Basically any surface, biotic or abiotic, is a target for bacterial colonization and biofilm formation, including ship hulls, petroleum pipelines, rocks in rivers, contact lenses, and all varieties of biomedical implants (Bruinsma et al., 2001; Dunne, 2002; Hannig and Hannig, 2009a; Rajasekar et al., 2010; Romaní and Sabater, 2001; Schultz et al., 2011). The main characteristics that are typical for the general bioadhesion process and biofilm formation can be observed very well in the oral cavity (Hannig and Hannig, 2009a). Due to its accessibility and its association with dental caries, the oral microflora has been intensively investigated (Aas et al., 2005; Kolenbrander, 2000; Marsh and Martin, 2009). The oral cavity, a major gateway to the human body, appears as an open ecosystem with a dynamic balance between the entrance, colonization, and removal of microorganisms (Lamont and Jenkinson, 2010). Distinct habitats exist even within the mouth due to the physical nature and biological properties of the various surfaces (Mager et al., 2003). These include mucosal surfaces (lips, palate, tongue) as well as teeth. Due to their hard and non-shedding surface, teeth provide the opportunity for substantial biofilm formation, also known as dental plaque (Marsh and Martin, 2009).

Dental plaque is one of the best studied biofilms, and it displays all of the characteristics of a typical biofilm. These include the adsorption of salivary and bacterial components to form the pellicle layer, passive transport of bacteria to the pellicle-coated tooth surface, a reversible adherence-phase involving van der Waals forces, hydrophobic- and electrostatic interactions, an irreversible adherence-phase involving interactions between bacterial adhesins and receptors, coadhesion between suspended and already-adhering microorganisms, and matrix synthesis leading to biofilm formation (Buzalaf et al., 2012; Hannig and Joiner, 2006; Leach and Saxton, 1966; Mayhall, 1970; Vacca Smith and Bowen, 2000). During the first 12 h of bacterial colonization, microbial adherence is mainly determined by the pellicle layer (Hannig and Hannig, 2009a; Marsh and Bradshaw, 1995), which highlights the important role of this structure in oral biofilm formation.

## 2.2 The pellicle and its role in oral health

The pellicle is defined as an initial integument on oral surfaces (Dawes et al., 1963), which describes the cuticular structure that is formed on all solid surfaces exposed to oral fluids. This mainly acellular layer is formed instantaneously on the enamel surface after eruption or cleaning and is clearly distinguished from dental plaque (Hannig, 1999; Skjørland et al., 1995; Vacca Smith and Bowen, 2000). It is composed of adsorbed proteins, including several enzymes, glycoproteins, and lipids (Aroonsang et al., 2014; Carlén et al., 1998; Hannig et al., 2005a; Hannig and Joiner, 2006; Lee et al., 2013; Murty et al., 1987; Siqueira et al., 2012).

As the interface between teeth and the oral environment, the pellicle layer plays a key role in the maintenance of oral health and is of great physiological and pathophysiological importance for all interactions at the tooth-saliva surface (Hannig and Joiner, 2006; Siqueira et al., 2012). It serves multiple protective functions such as a reservoir for remineralization, a protective lubricant, a diffusion barrier, and a buffer (Hannig and Balz, 2001; Hannig and Hannig, 2009a; Hannig and Joiner, 2006; Vacca Smith and Bowen, 2000). Furthermore, the pellicle is effective in reducing dental erosion caused by various acids (Hannig et al., 2009b; Hannig et al., 2012; Hannig and Hannig, 2014; Hara et al., 2006; Wiegand et al., 2008). Dental erosion can be described as the loss of dental hard substance as a result of acid exposure without bacterial involvement (Ganss, 2006; Larsen, 1990). The acids involved can be either intrinsic (regurgitated gastric acid) or extrinsic (e.g. dietary components such as soft drinks, acidic fruits) (Lussi, 2006).

Several proteins and enzymes with antibacterial activity, such as lysozyme, peroxidase, and histatins, are present within the pellicle (Hannig et al., 2005a; Hannig and Joiner, 2006; Lee et al., 2013; Vukosavljevic et al., 2014). Nevertheless, several bacteria have

adapted to this protective structure, as certain pellicle components also serve as specific receptors for bacterial adhesion (Hannig and Joiner, 2006; Kolenbrander and London, 1993). Fibrinogen, mucins, glucans, and bacterial glycosyltransferases are examples of important bacterial binding sites that have been detected in the pellicle (Aroonsang et al., 2014; Carlén et al., 2003; Gibbons et al., 1986; Hannig et al., 2005a; Schilling and Bowen, 1992).

The function of the pellicle is rather ambivalent. On the one hand, as stated above, the pellicle shows protective properties for the underlying dental hard tissues. On the other hand, it also serves as the basis for biofilm formation (Marsh and Martin, 2009), and therefore, for the development of oral diseases such as caries and periodontitis.

## 2.3 Pellicle composition - current state of scientific research

Considering its important role in oral health, it is surprising that the pellicle received relatively little attention in dental research when the structure was first discovered (Dawes, C., Jenkins, G.N., Tonge, C.H., 1963). Associated with the advances in the field of microscopy and the progressive developments in analytical chemistry instrumentation (especially in mass spectrometry), activities in the field of pellicle research have increased over the past decades. Even nowadays, however, several aspects and scientific issues concerning the nature and function of the pellicle are still unresolved. Detailed information on the composition of the pellicle is especially lacking. One reason for this is the fact that only minute amounts of pellicle material are available for analytical investigation, which is illustrated by the thickness of the pellicle layer. It ranges between 10 and 20 nm when formed *in situ* within a few minutes (Hannig, 1999; Skjørland et al., 1995). Depending on the intraoral location of the tooth surface, pellicle thickness increases up to 700 nm within 2 h on buccal sites, but only up to 80 nm on palatal sites (Hannig, 1999). The process of pellicle formation is determined by regional differences in salivary composition, salivary flow, and shearing forces (due to tongue movement) (Amaechi et al., 1999; Hannig, 1999). Moreover, the pellicle is a crosslinked biopolymer layer of high tenacity (Hannig, 1999; Hannig and Joiner, 2006; Siqueira et al., 2012). Therefore, direct and complete extraction of pellicle components is difficult to achieve.

Due to the low sample volume, an efficient method for pellicle formation and collection is elementary for the subsequent compositional analysis of this structure. There are three experimental models used for this task that need further differentiation: *in vitro* methods (pellicle formed *in vitro* from collected saliva on different materials) (Carlén et al., 1998; Zahradnik et al., 1976), *in vivo* methods (pellicle harvested by scraping with a curette or sponge from the tooth surface) (Rykke et al., 1990; Skjørland et al., 1995) and *in situ* methods (enamel slabs exposed to the oral cavity with splints) (Hannig et al., 2005b).

*In vitro* studies do not adequately simulate conditions in the oral cavity. Thus, *in vitro* formed pellicle differs significantly from those formed *in vivo* (Carlén et al., 2003; Hannig and Hannig, 2009a; van der Mei et al., 2002). However, mechanical harvesting of the *in vivo* pellicle leads to insufficient amounts of sample material, and the basal structures of the pellicle cannot be removed adequately (Hannig et al., 2005b). Furthermore, a quantitative analysis of pellicle components in practice is very difficult because of the poor repeatability of sample collection *in vivo*. For pellicle formation *in situ*, enamel slabs of standardized size and surface structure are fixed on individual splints and carried in the oral cavity (Hannig et al., 2005b). The slabs can be removed easily and the formed pellicle can be analyzed with various methods, either in the adherent state or after desorption. Accordingly, *in situ* setups are the method of choice in several studies focusing on the protective impact of the pellicle on enamel surfaces (Deimling et al., 2007; Hannig et al., 2012; Hara et al., 2006; Vacca Smith and Bowen, 2000).

Numerous studies have investigated the pellicle's ultrastructure, protein/amino acid composition and enzymatic activity in detail. These studies have shown that the pellicle contains characteristic components such as proline-rich proteins, statherin, histatin, salivary α-amylase, lysozyme, glycosyltransferase, peroxidase, immunoglobulins, and mucins (Aroonsang et al., 2014; Custodio et al., 2014; Deimling et al., 2007; Hannig et al., 2005a; Lee et al., 2013; Siqueira et al., 2012; Vukosavljevic et al., 2014; Yao et al., 2003). Carbohydrates were also detected in the pellicle, with glucose accounting for more than half of the total amount of carbohydrates. It has been suggested that glucose originates from salivary glycoproteins, glucans of bacterial origin, and glycolipids that are adsorbed to the pellicle (Mayhall and Butler, 1976; Slomiany et al., 1986; Sønju et al., 1974).

Compared to the extensive work on the pellicle's ultrastructure and protein/amino acid composition, little attention has been given to the lipid profile of the adsorbed pellicle layer. There is a paucity of studies, and scientific literature provides little information that describes the role and composition of lipids in the pellicle. Thus far, data on the lipid composition of the pellicle derive from three studies carried out in the 1980s that used either *in vivo* (Murty et al., 1987; Slomiany et al., 1986) or *in vitro* setups (Slomiany et al., 1990). The analytical methods predominantly used in these studies were thin layer chromatography (TLC) and gas chromatography coupled with a flame ionization detector (GC-FID). Using these methods, it was shown that lipids account for about one quarter of the pellicle's dry weight (Slomiany et al., 1990). The major lipid classes identified in the pellicle are glycolipids, phospholipids and neutral lipids, which include free fatty acids (FFAs), triacylglycerols (TAG) and cholesterol/cholesterol esters. Furthermore, these studies showed that interindividual differences in the pellicle's lipid composition and amount reflect differences in the caries

activity of the individual. This was explained by a delayed acid diffusion through the pellicle layer, which can be partially attributed to pellicle lipids (Slomiany et al., 1990).

Since all investigations concerning the lipid composition solely refer to these few studies that were performed approximately 25 years ago, additional research with state-of-the-art methods is required to gain further insights into the pellicle's lipid composition.

## 2.4 Lipids and fatty acids

Lipids play a vital role in all organisms, not only for the storage of energy (e.g., neutral lipids) or as structural elements of cell membranes (e.g., phospholipids), but also in signal transduction processes (e.g., isoprenoides/steroids) (Berg et al., 2002). Thus, the term "lipid" comprises a diverse range of compounds varying in characteristics, structure, and functionality. For this reason, there is no widely accepted definition of what is considered a lipid. Over the past decades, many attempts to create a comprehensive classification system for lipids were made (Fahy, 2005; Fahy et al., 2008; Fisher, 1954; Hutt, 1955). Lipids are traditionally described as nonpolar compounds insoluble in water but readily soluble in organic solvents such as alcohols, ethers, hydrocarbons, and chloroform. However, a definition of this kind excludes many substances that are widely regarded as lipids and are almost as soluble in water as in organic solvents (e.g. gangliosides). Therefore, Christie (Christie, 1989) introduced another definition, which describes lipids as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds." When defining lipids in this way, the term "fatty acid" (FA) should also be defined. A FA is a carboxylic acid with a long hydrocarbon side chain. Usually FAs contain even numbers of carbon atoms (commonly $C_{12}$-$C_{24}$) because they are synthesized in nature via condensation of malonyl coenzyme A units (Berg et al., 2002). Although FAs can occur in nature in a free (unesterified) state, they are most often found as esters with glycerol, cholesterol, or long-chain aliphatic alcohols and as amides in sphingolipids (Figure 1). FAs do not only represent an independent lipid class, moreover, they are the basic building blocks of most of the components that are classified as lipids (Figure 1). Therefore, FAs are considered to be largely responsible for the defining characteristics of these lipids. Due to their diversity in terms of chain length, degree of unsaturation, geometry, and position of double bonds, the FA composition of a lipid has a distinct influence on its chemical and physical properties. This is the reason why FAs are such an important target for lipid analyses. Figure 1 gives a brief overview of different lipid classes. Due to their amphiphilic and hydrophobic properties, most of these substances are of potential interest for the purpose of biofilm management from a theoretical point of view.

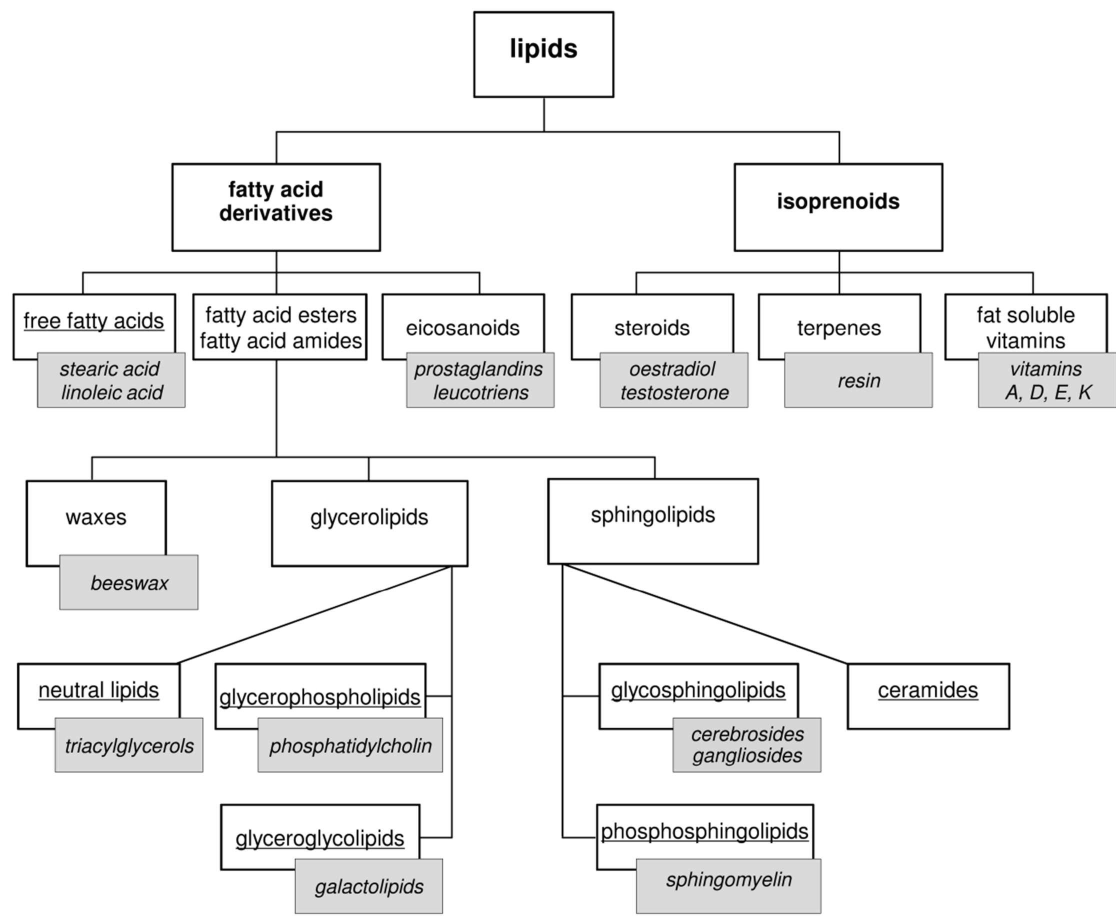


***Figure 1.*** *Overview of various lipid classes with examples. The major FA containing lipid classes that are relevant for the present work are underlined. Figure summarizes data presented in textbooks by Berg (Berg et al., 2002) and Lottspeich (Lottspeich and Engels, 2006).*

## 2.5 From lipid analysis to lipidomics

Analytical methods for the determination of lipids are as various as the lipid classes themselves (Carrasco-Pancorbo et al., 2009; Christie and Xianlin Han, 2010; Fuchs et al., 2011; Fuchs and Schiller, 2008; Milne et al., 2006; Peterson and Cummings, 2006). Nowadays, the ability to profile the lipid composition of biological samples is crucial for a wide range of scientific disciplines such as microbiology, disease diagnostics, drug discovery and health and nutrition research. However, this has not always been the case. In the beginning of modern biochemistry research, which was mainly characterized by the progressive development and discovery of new MS ionization techniques (ESI and MALDI), little attention was devoted to lipids. Peptides and proteins were by far the more interesting molecules, whereas lipids were restricted to their simple role as a source of energy or as a determinant for the fluidity of cell membranes (Christie, 2009). Until the early 2000s, there

was something like a “lipid-phobia” in biochemistry research. After the introduction of the “omics”-sciences such as genomics, proteomics, and metabolomics, however, it was only a matter of time until the “new” science of lipidomics as a subcategory of metabolomics joined this group. This was followed by a sudden increase of interest in lipid molecules and in techniques for lipid analysis. From then on, scientific publications that use the term “lipidomics” have increased substantially.

But what exactly is lipidomics? There are several definitions that try to answer this question. Wenk (Wenk, 2005), for example, describes lipidomics as the “systems-level analysis of lipids and factors that interact with lipids.” According to this definition, the aim of lipidomics is more than just analyzing lipids in a biological system. It is crucial to relate the lipid composition (analytical data) of a biological sample to physical properties and biological functions in general.

# 3. OBJECTIVES AND HYPOTHESES

The pellicle plays an important role not only as a physiological protective layer, but also as a conditioning film in the development of the pathogen biofilm (plaque) (Marsh and Martin, 2009). Despite their high occurrence and assumed importance, only preliminary information is available on the nature and composition of pellicle lipids (Paper 3). Knowledge of the lipid composition of the pellicle and how this correlates with its protective functions may provide insight into several oral pathological states, including caries, dental erosion, and periodontal disease. Until now, however, no validated method for a comprehensive investigation of the pellicle's lipid profile existed that includes *in situ* pellicle formation, sample preparation, and the comprehensive determination of FAs via a current analytical method (Paper 1).

A limited number of studies indicate that topical application of edible oils increases the hydrophobicity of oral biofilms (Hannig et al., 2012; van der Mei et al., 2002). This leads to the hypothesis that rinses containing lipids (in the simplest case edible oils) might change the amount and composition of lipid components in the pellicle. Furthermore, hydrophobization of the oral surfaces might result in an enhancement of the protective effects since hydrophobic interactions have been shown to influence the adherence of microorganisms on oral surfaces *in vitro* and *in vivo* (Busscher and van der Mei, 1997; Gibbons and Etherden, 1983; Quirynen et al., 1989).

In order to close existing knowledge gaps in pellicle research and to evaluate the aforementioned hypotheses, this thesis addresses the following objectives:

(1) Development and validation of an analytical method suitable for the determination of FAs in the *in situ* pellicle

(2) Qualitative and quantitative characterization of the FA composition of the *in situ* formed pellicle layer, including a screening for bacterial lipids

(3) Comparison of the FA profiles of saliva and pellicle

(4) Assessment of the influence of pellicle formation time on the FA composition

(5) Investigation into the effect of oil rinses on the FA composition of the pellicle

# 4. METHODS

The following chapters briefly describe the study design and methods on which this thesis is based. More detailed information on preparation steps, single parameters, and materials used in this study are provided in papers 1 and 2, which are attached at the end of this cumulative thesis.

## 4.1 Study design

To get a detailed overview of the current state of research of lipids in the pellicle and saliva, an extensive literature search was conducted, focusing on methods used for determination of lipids in biofilms and saliva. The results of this literature search were the basis of the analytical method development and contributed to the preparation of a review article about lipids in preventive dentistry (Paper 3).

Preparatory work for method development (sample preparation and GC-MS analysis) was performed in order to test the suitability of pellicle formation and collection methods for the subsequent analytical procedures. After the successful development and validation of the analytical method for the determination of minute amounts of FAs in the pellicle layer (Paper 1), the individual FA profiles (pellicle and saliva) of ten research participants were investigated (Paper 2). Pellicle samples from ten subjects formed after 3, 30, 60, 120, and 240 min of intraoral exposure were analyzed in order to investigate the FA profile at different pellicle maturation states. Furthermore, the impact of rinses with safflower oil on the pellicle's FA composition was investigated (Paper 2).

In addition to the work steps presented in this thesis, further *in situ* studies have been carried out in the group around C. Hannig concerning the lipase activity in the pellicle and saliva, the influence of oil rinses on the bacterial colonization of enamel, (Hannig et al., 2013) and the impact of oil rinses on the protective properties of the pellicle layer against erosive attacks (Hannig et al., 2012).

## 4.2 Subjects and sample collection

*Subjects*

The *in situ* formed pellicle samples were collected from ten healthy research participants (6 female, 4 male) between 26 and 57 years old. After being examined by an experienced dentist, it was determined that the subjects, all members of the laboratory staff, showed no signs of untreated carious lesions, and plaque and gingivitis indices were close to zero.

Ethical approval of the study design was granted by the Ethics Committees of the University of Freiburg (# 222/08) and the TU Dresden Medical Faculty (EK 275092012).

*Preparation of enamel specimens*

Bovine enamel was used as a substrate for pellicle formation, which shows considerable structural similarities to that of human enamel, and is thereby a highly suitable substitute for application in *in situ* experiments (Deimling et al., 2007; Nakamichi et al., 1983; Wegehaupt et al., 2008). Bovine incisors were extracted from 2-year-old cattle (BSE-negative) and stored in a thymol solution (0.1 %). A trepan bur was used to prepare enamel disks (5 mm diameter, 19.635 $mm^2$ surface area, 1.5 mm height) from the labial surfaces of the teeth (Figure 2). These disks were subsequently subjected to further preparation procedures including polishing, cleaning, and disinfection processes (Paper 1 and 2). Prior to oral exposure, the processed enamel disks were stored in deionized water for 24 h in order to form a hydration layer (Deimling et al., 2007; Hannig, 1999).

*In situ pellicle formation*

For *in situ* exposure of the enamel specimens, the disks were fixed into small cavities on individual upper jaw splints with polyvinyl siloxane impression material (Figure 2). Thus, only the enamel surface was exposed to oral fluids. In total, 12 disks per splint were fixed on buccal and palatal sites (6 each) of the premolars and the 1st molar. Before insertion and during oral exposure of the prepared splints, the subjects had to carry out specified instructions. These included cleaning their teeth thoroughly without tooth paste before inserting the splints and refraining from eating and drinking 120 min prior to and during exposure of the samples in the oral cavity.

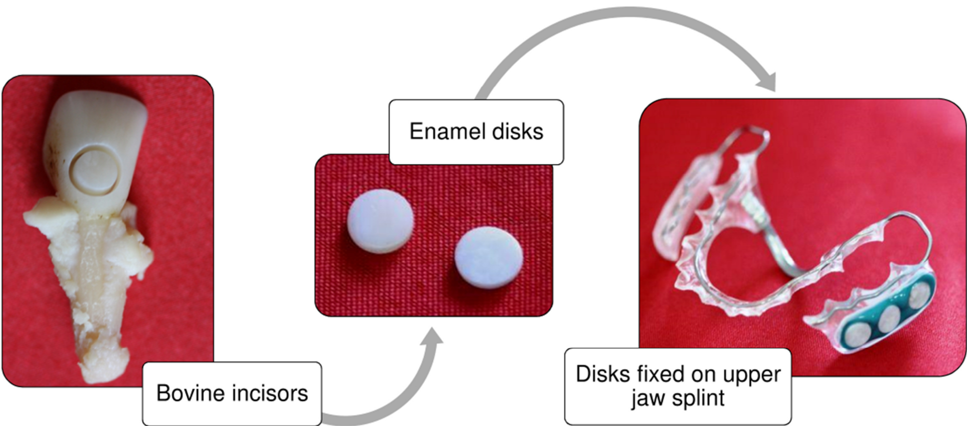


***Figure 2.*** *Preparation of enamel disks from bovine incisors and attachment to upper jaw splint. (These steps were carried out at the Clinic of Operative Dentistry; TU Dresden)*

The splints were carried intraorally for different exposure times to allow pellicle formation on the surfaces of the specimens. After the respective pellicle formation periods, the disks were rinsed with saline solution to remove loosely attached salivary fractions. The enamel samples were carefully removed from the splints using a dental probe and underwent an ultrasound-supported pellicle desorption procedure (Paper 1 and 2). A previous study indicated that the applied treatment allows for quantitative detachment and collection of the *in situ* formed pellicle (Hannig et al., 2005b). To ensure that enough sample material is available for the subsequent analysis, pellicle samples formed on the 12 enamel disks (2.356 $cm^2$ surface area) were pooled. The desorbed pellicle sample was pipetted into 1.5 mL amber screw vials, covered with nitrogen, and stored at -20 °C until analysis.

For the experiments on the influence of oil rinses on the pellicle's composition, the splints were carried intraorally for 3 min to form the basal pellicle layer on the enamel surfaces. Afterwards, the subjects rinsed thoroughly with 8 mL safflower oil for 10 min. The samples remained in the oral cavity for a further 107 min, providing a total intraoral exposure time of 120 min. After intraoral exposure, the enamel slabs were removed immediately from the splints and rinsed thoroughly with water. Then the disks underwent the same procedure for pellicle desorption as mentioned above (Paper 1 and 2).

Additionally, unstimulated saliva samples were obtained prior to the respective pellicle formation time points. These samples were centrifuged at 6,000 *g* for 10 min and sterile-filtered (0.2 µm) before analysis.

## 4.3 Sample preparation - extraction and derivatization

As with any analytical procedure, the validity of results also depends on proper sampling and preservation of the sample prior to analysis. The importance of sample preparation is often underestimated and therefore carried out hurriedly and incorrectly. It should be kept in mind that errors during sample preparation can render even the best analytics worthless. The protocols for the extraction and derivatization procedures are outlined below. For a more detailed version of these protocols, please refer to paper 1. Prior to all sample preparation steps tridecanoic and nonadecanoic acid, which are used as internal standards (IS), were added to the desorbed pellicle samples.

*Extraction*

Independent of the specific analytical method, the first and crucial step of FA analysis is the extraction of lipids from the matrix. After the formation and desorption of the pellicle samples, the lipid content has to be separated from other pellicle components (e.g. proteins, carbohydrates), which otherwise could interfere in the following derivatization step and the subsequent analysis via GC-EI/MS. The Folch extraction procedure for isolation and purification of total lipids (Folch et al., 1957) was modified and applied in order to isolate the lipid fractions of the pellicle (Paper 1).

*Derivatization*

In their free underivatized form, FAs are difficult to analyze because these polar compounds tend to form hydrogen bonds, leading to adsorption issues in chromatographic separation. To determine the complete FA composition of a sample via GC-MS, the FA components of lipids have to be converted into more volatile and thermally stable derivatives. In this study, the FA components of lipids were converted to more volatile fatty acid methyl esters (FAMEs). The primary reason for analyzing FAs as FAMEs is the reduction in polarity, as derivatizing the carboxyl functional groups makes them more amenable for analysis.

Rapid transesterification (1 h at 100 °C) of all FA containing lipids (plus esterification of FFAs) into FAMEs was carried out in methanol using concentrated HCl (35 %, w/w) as an acidic catalyst (Paper 1). Transesterification was checked for completeness by analyzing the reaction products via TLC on silica gel (appendix p.37). After quantitative methylation, the reaction mixture was cooled to room temperature. Then, 2 mL deionized water and 2 mL hexane were added and FAMEs were extracted by vortexing the mixture. After phase separation, the hexane phase was isolated and then evaporated under a gentle stream of nitrogen. The residue was redissolved in 0.1 mL of hexane. For the subsequent GC-MS analyses, 1 µL of this solution was injected directly into the split-/splitless injector of the GC-MS.

## 4.4 Analytical method development

Since an appropriate method for the determination of FAs in the pellicle was lacking, an important task within this thesis was the development, validation, and application of a GC-EI/MS-based analytical method. The methodical approach is described below (Figure 3). For more detailed information, see paper 1.

(1) In order to determine the optimal parameters for the GC-MS system (column, oven temperature program, MS parameters, etc.), commercially available FAME standard mixtures were used for test runs. Identification of the analytes via MS was carried out in scan-mode. Characteristic molecule and fragment ions were recorded and a spectral library containing the 49 FAMEs of the standard mixtures was generated.

(2) In a pilot study, pellicle and saliva samples of three subjects were used to develop and evaluate quantitative sample extraction, cleanup, and derivatization methods. Additionally, the suitability of the *in situ* sample generation and desorption treatment for the subsequent analytical procedure was checked.

(3) The 49 different FAMEs included in the standard mixtures were used as reference standards to screen the pellicle and saliva samples for the most abundant FAs. Identification of FAs (in form of their FAMEs) was achieved by using full-scan mass spectral data and retention time (RT) information. An individual FA calibration standard mix, containing the previously characterized FAs, was prepared and used for quantitative analysis.

(4) Method optimization was performed to provide a more sensitive and robust analysis. This includes the development of a selected ion monitoring (SIM) method to allow for detection of target compounds with increased sensitivity relative to the full scan mode. Two internal standards (IS) were introduced to improve the precision of quantitative analysis and to correct fluctuations in concentration due to analyte loss during sample preparation.

(5) Method validation was done according to the guidelines of the Society of Toxicological and Forensic Chemistry (Peters et al., 2009). The main performance characteristics evaluated were selectivity, linearity of response, closeness to the true value, precision of the results, and detection and quantification limits (Paper 1).

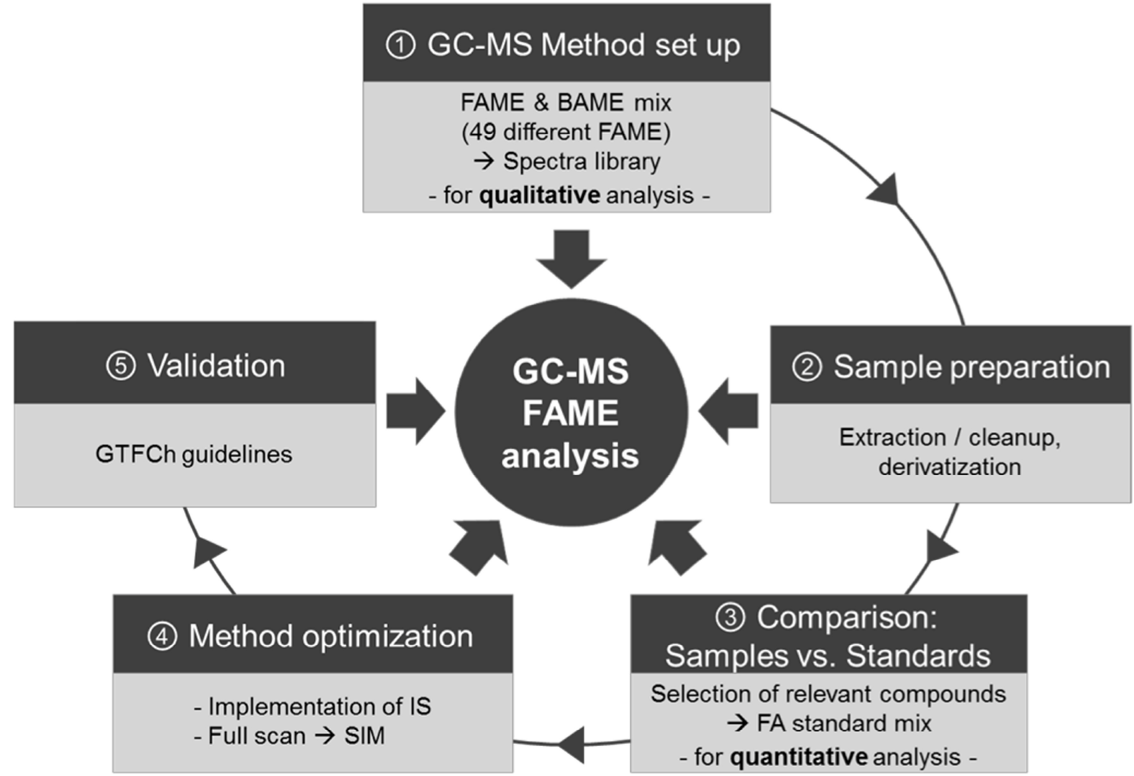


***Figure 3.*** *Approach to the development of the GC-EI/MS based analytical method.*

## 4.5 Data evaluation

Retention times of the separated FAMEs as well as the respective mass spectra gained from full scan measurements were used for qualitative analysis. Although EI-ionization was applied, the molecular ion ($M^+$) of each FAME was visible in the mass spectrum. Quantification of data obtained from SIM mode measurements was performed using the peak area ratios relative to that of the IS. The odd-numbered FAs tridecanoic and nonadecanoic acid were used as IS, since they occur only marginally in the human organism (mostly as components of bacteria) and were not detected in the pellicle. Least squares regression analysis was implemented, using the peak area ratios against increasing standard concentrations to obtain calibration linearity. Peak area ratios of the unknown samples were referred to this calibration curve. Prior to the sample run, a blank sample and the seven calibration standards were measured. Measurements of the pellicle samples were bracketed by injections of quality control (QC) samples to validate the results (Paper 1).

# 5. Results and Discussion

Regarding literature that deals with the compositional analysis of the pellicle, the aforementioned “lipid-phobia” in biochemical analysis becomes clearly evident. A plethora of studies is available that involve the investigation of the pellicle’s ultrastructure, protein/amino acid composition, and enzymatic activity in detail (Deimling et al., 2007; Hannig, 1999; Hannig et al., 2005a; Hannig et al., 2010; Lee et al., 2013; Schüpbach et al., 2001; Yao et al., 2003), whereas research on lipids in the pellicle was previously neglected. As shown in this thesis, the current state of research did not provide a complete determination of the FA composition of the pellicle, even though this parameter may be important to better understand the pellicle’s protective properties and oral bioadhesion processes in general (Paper 3).

*Methodology*

This thesis is the first to study the FA profile of the *in situ* pellicle. The present study demonstrates a procedure based on a combination of innovative specimen generation and convenient sample preparation with sensitive GC-EI/MS analysis.

A precise sample collection technique is essential for obtaining sufficient amounts of pellicle samples that are free of contamination (e.g. saliva, plaque or other elements present in the oral cavity). Previous studies indicate that the applied sample generation and desorption treatments allow for quantitative collection and detachment of the *in situ* formed pellicle (Deimling et al., 2007; Hannig et al., 2005b). The major benefit of this *in situ* method is that it combines the advantages of mimicking the *in vivo* oral exposure with the possibility of completely detaching the pellicle structure from the enamel surface. This provides the basis for a reliable evaluation of the pellicle’s FA profile as determined by GC-EI/MS.

Due to the high chromatographic resolution (narrow peaks, high peak capacity) and the great sensitivity, the GC-EI/MS approach particularly suits the chromatographic separation and identification of the trace quantities of FAs (ng-levels) in the pellicle, a multi-component mix. Analysis of FAs can be complicated due to cross-contamination since lipids are omnipresent in nature and are constituents of commercial plastics, surfactants, and lubricants (Christie, 1989). Therefore, special attention was paid to proper sample preparation and preservation. The developed analytical method is reliable when plastic products are avoided whenever possible and all required glassware is cleaned thoroughly (e.g. rinsed with methanol). Additionally, precaution should be taken to minimize autoxidation of unsaturated FAs. Therefore, nitrogen was used to flush air out of glass containers, reaction vessels, and sample vials prior to the extraction, derivatization, and storage of samples.

This entire analytical approach was specifically developed and validated to allow for reliable evaluation of the FA profile of the *in situ* pellicle (Paper 1). For method validation, parameters such as accuracy, precision, selectivity, and the analytical limits (limit of detection, LOD and limit of quantification, LOQ) were evaluated. The GC-MS analysis in SIM mode provided LOQs ranging from 7.6 to 91.8 ng/mL whereas those of most FAs ranged from 7.6 to 28.8 ng/mL, except for 18:0 (83.9 ng/mL) and 22:1n9c (91.8 ng/mL). The calibration curve obtained from a blank sample and seven calibration standards was linear over a 20-fold concentration range with coefficients of determination $R^2 > 0.995$ for all analyzed FAs. Precision and accuracy were determined by analyzing the QC samples acquired for the intra- and interday assays. The intraday (n = 5) precision ranged from 1.1 to 12.0 % (% CV), and accuracies ranged from 90.0 to 107.1 % (% bias). Interday (n = 5) precision and accuracy were between 1.2 to 13.4 % and 84.4 to 106.3 %. Bias values within an interval of ± 15 % (± 20 % for concentrations close to the LOQ) of the nominal value were accepted as a tolerance (Peters et al., 2009). Method validation showed that the developed analytical method is a reliable, robust, and accurate procedure, which meets the requirements for achieving the objectives addressed in this thesis. Although the focus lies on the FA analysis of lipids in pellicle samples, lipids in saliva samples can also be analyzed using the developed protocol.

*FA profile of the pellicle*

Eleven FAs (in the form of their methyl ester derivatives) were identified and quantified in the pellicle samples of the ten subjects. Palmitic (16:0), stearic (18:0), oleic (18:1n9c), and erucic acid (22:1n9c) are the major FAs and account for more than 80 % of the pellicle's FAs (Figure 4). The minor FAs include lauric (12:0), myristic (14:0), palmitoleic (16:1n9c), linoleic (18:2n6c), arachidic (20:0), and the two bacterial FAs pentadecanoic (15:0) and anteiso-pentadecanoic acid (a15:0).

Compared to a study carried out by Slomiany et al. (Slomiany et al., 1986), which is the only reference on the FA composition of the pellicle, a broader range of FAs was observed in the present study. In particular, more minor FAs were detected, reflecting the high sensitivity of the new method that was developed within the framework of this thesis. However, the major FAs were identical in both studies. Furthermore, the odd-numbered FAs 15:0 and a15:0 were detected in the current study, which indicate the presence of bacterial components in the pellicle layer. The presumed bacterial biomarkers correspond well with results of recent studies on bacterial colonization on enamel *in situ* (Al-Ahmad et al., 2009; Hannig et al., 2007). These studies showed that the earliest bacterial colonizers of an erupted or freshly cleaned tooth surface appear within minutes.

The FA patterns of the analyzed pellicle samples showed no considerable differences among the ten study subjects (Figure 4). As compared to other pellicle parameters, the natural variability is rather low (Hannig et al., 2005a). The FA profile of the pellicle seems to be characteristic for this biological structure. In contrast to the very stable relative FA composition, the total amount of FAs in the pellicle varied substantially among the subjects. No significant female-male differences were observed. However, based on the number of subjects participating in this study, no precise statement can be made concerning a gender-specific impact on the pellicle's FA profile.

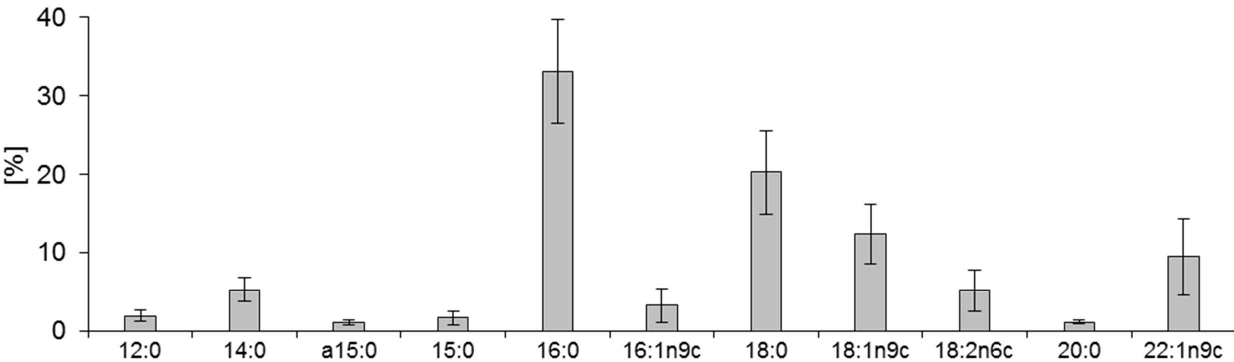


***Figure 4.*** *FA composition of the pellicle (in-situ formation time 120 min). Values represent the means ± standard deviation (SD) (n=10) of ten subjects expressed as a percentage of the investigated FAs. This profile seems to be characteristic for the biological composition of the pellicle*

*FA profile - pellicle vs. saliva*

Pellicle formation is largely determined by adsorption of salivary components from the oral environment (Bennick et al., 1983; Rykke et al., 1990; Vacca Smith and Bowen, 2000). Salivary lipids can potentially modulate oral bioadhesion processes and add hydrophobic characteristics to dental surfaces (Grivet et al., 2000; Tomita et al., 2008; van der Mei et al., 2002). Therefore the comparison of the FA profiles of saliva and pellicle is of great interest.

The FA profile of the examined saliva samples was dominated by 18:1n9c, 16:0, 18:2n6c, and 18:0, (Figure 5). The major FAs of the pellicle samples and the saliva samples are very similar, but their relative ratios differ distinctly. Compared to the pellicle's FA profile, higher proportions of unsaturated FAs, particularly 18:1n9c (two times higher) and 18:2n6c (three-four times higher), were detected in saliva. As shown before for the pellicle samples, considerable interindividual variations in the total FA contents were also noticed in the saliva samples from the ten study subjects (Paper 2).

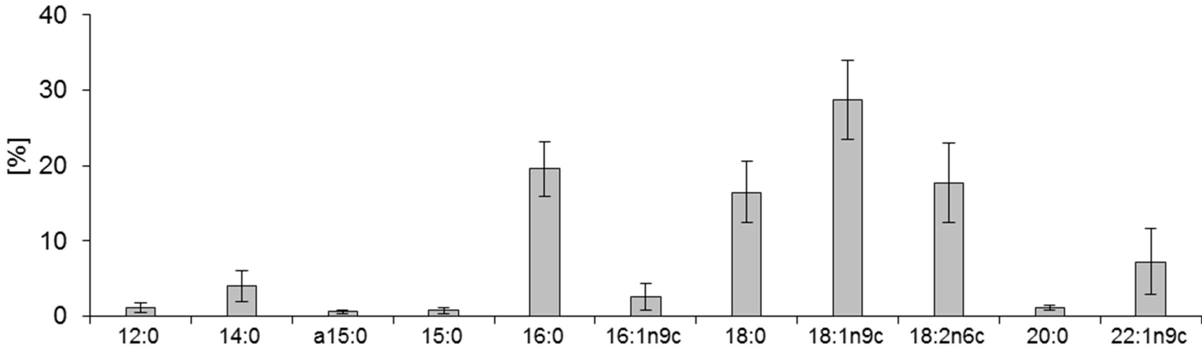


***Figure 5.*** *FA composition of saliva. Each value represents the mean ± SD (n=10) of ten subjects expressed as percentage of the investigated FAs.*

The major salivary FAs identified in the present study are comparable to those detected in recent studies on the lipid composition of saliva (Actis et al., 2005; Kulkarni et al., 2012; Neyraud et al., 2013; Tomita et al., 2008) (Table. 2). Because of the different methods that are used in these studies, a direct comparison of the presented data is only partially possible. Nevertheless, it is particularly noticeable that the major FAs detected in all studies are identical and represent almost 90% of total investigated FAs in the saliva samples.

***Table 2.*** *Comparison of major salivary FAs detected in recent studies. Direct comparison of data is only possible with caution because of the different methods that are applied in the respective studies.*

| FA | Actis *et al.* (2005) (n=10) | Tomita *et al.*[a] (2008) (n=44) | Neyraud *et al.* (2013) (n=48) | Reich *et al.* (present work) (n=10) | Kulkarni *et al.*[b] (2012) (n=15) |
|---|---|---|---|---|---|
| | % of total investigated FAs ± SD | | | | major FAs |
| **14:0** | 2.8 ± 0.7 | 6.1 | 6.7 ± 4.8 | 4.1 ± 1.4 | |
| **16:0** | 28.7 ± 3.2 | 24.5 | 27.1 ± 4.5 | 20.8 ± 3.6 | major |
| **16:1** | 5.2 ± 1.3 | 3.5 | 1.6 ± 1.4 | 2.5 ± 1.5 | |
| **18:0** | 21.3 ± 4.5 | 30.3 | 16.6 ± 5.0 | 16.8 ± 3.8 | major |
| **18:1** | 21.1 ± 3.7 | 11.6 | 23.2 ± 6.3 | 28.1 ± 4.5 | major |
| **18:2** | 10.2 ± 2.1 | 11.6 | 8.2 ± 3.5 | 16.5 ± 4.9 | major |
| ∑ [c] | 89.3 % | 87.6 % | 83.4 % | 88.8 % | |

[a] data on SD was not available.
[b] data refers to non-esterified fatty acids (“major” corresponds to a concentration ≥ 1 µM).
[c] since only the major FAs are included in the table, total does not correspond to 100 %.

Furthermore, the FA profile of saliva showed notable differences compared to that of the pellicle, highlighting that FAs available in saliva are not adsorbed equivalently to the pellicle layer. This finding infers that the formation of the pellicle is a highly selective process that does not correlate directly with salivary composition, as shown elsewhere for protein fractions found in the pellicle layer (Yao et al., 2003).

*Influence of pellicle formation time on FA composition*

Pellicle formation proceeds in two stages: (1) initial adsorption of a dense basal layer followed by (2) a slower adsorption of a globular and less dense layer (Hannig, 1999). Ultrastructural observations showed that important changes occur during formation of the pellicle, which modify its structure and thickness (Hannig, 1999). Therefore, the investigation of the FA composition at different time points of pellicle formation is of particular interest and an objective addressed in this thesis.

***Table 3.*** *FA composition of pellicle samples after different oral exposure times (splints carried in situ for 3-240 min). Values represent the means (n=10) of ten subjects, expressed as the percentage of total investigated FAs, as well as ng per cm² enamel surface.*

| | 3 min | | 30 min | | 60 min | | 120 min | | 240 min | |
|---|---|---|---|---|---|---|---|---|---|---|
| **FA** | % | ng/cm² | % | ng/cm² | % | ng/cm² | % | ng/cm² | % | ng/cm² |
| **12:0** | 2.1 | 19.0 | 2.2 | 24.4 | 2.2 | 22.5 | 1.7 | 22.2 | 1.9 | 28.5 |
| **14:0** | 5.6 | 51.3 | 5.7 | 62.3 | 5.5 | 59.6 | 4.6 | 61.4 | 5.1 | 74.2 |
| **a15:0** | 0.8 | 7.8 | 1.0 | 10.9 | 0.9 | 9.8 | 1.3 | 16.8 | 1.3 | 19.0 |
| **15:0** | 1.2 | 11.3 | 1.7 | 18.5 | 1.4 | 13.9 | 1.4 | 18.0 | 1.4 | 20.3 |
| **16:0** | 30.7 | 283.2 | 33.5 | 365.9 | 31.7 | 339.8 | 35.4 | 471.7 | 34.9 | 533.4 |
| **16:1n9c** | 2.6 | 23.9 | 2.9 | 31.8 | 3.3 | 35.2 | 3.0 | 40.5 | 3.2 | 48.4 |
| **18:0** | 21.8 | 201.4 | 22.3 | 244.1 | 23.0 | 246.4 | 20.0 | 265.4 | 23.3 | 357.6 |
| **18:1n9c** | 14.5 | 133.4 | 14.7 | 160.5 | 14.0 | 140.1 | 14.7 | 195.9 | 14.2 | 240.1 |
| **18:2n6c** | 4.9 | 45.0 | 5.0 | 54.6 | 5.4 | 60.3 | 6.1 | 81.1 | 4.6 | 82.2 |
| **20:0** | 1.0 | 9.0 | 1.1 | 11.7 | 1.0 | 10.8 | 1.3 | 16.7 | 1.1 | 15.7 |
| **21:1n9c** | 14.8 | 137.9 | 9.9 | 108.1 | 11.6 | 118.8 | 10.5 | 139.5 | 9.0 | 142.5 |
| **Total** | | 923.3 | | 1092.7 | | 1057.3 | | 1329.1 | | 1561.8 |

An overview of the pellicle's FA composition for the different oral exposure times is given in Table 3. Within the first 60 min, the total amount of FAs stayed nearly constant, whereas steady accumulation of FAs and FA-containing lipids was observed between 60 min and 2 h of oral exposure. This is mainly due to an increase in the total amount of adsorbed pellicle components associated with the increase in pellicle thickness in general. This conclusion is in accordance with the results of a study that showed a constant growth of the pellicle layer between 60 min and 2 h (Hannig, 1999).

Despite the accumulation of FAs, the pellicle's FA profile remained remarkably constant during the examined pellicle formation periods (Table 3; % values for the single FAs from left to right). This result strengthens the assumption that the increasing amounts of FAs over time correlate with the growth of pellicle material in general.

*Effect of oil rinses on the FA composition of the pellicle*

The evaluation of the effect of rinses with edible oils (e.g. safflower oil) on the pellicle's FA composition is another objective addressed in this thesis. Modifying the pellicle's FA composition by using natural, biological products could strengthen the protective properties of the pellicle and lead to a promising approach in preventive dentistry. In this context, the targeted enrichment of lipids in the pellicle by using edible oils as a mouthwash has come into focus, as this might be an efficient method for modifying the pellicle's physicochemical properties and delaying bacterial adhesion (Paper 3) (Hannig et al., 2012; Hannig et al., 2013). The use of edible oils in this context has many advantages, as they are readily accessible worldwide, and undesirable side effects are not to be expected.

Using edible oils as a mouthwash, a process known as "oil pulling," has long been practiced in traditional Indian folk medicine (Asokan, 2008; Asokan et al., 2011). This procedure involves taking one tablespoon (~ 15 mL) of edible oil, usually sesame or sunflower oil, into the mouth and pushing and pulling it between the teeth for 10–20 min. Benefits of this treatment for oral health, especially the prophylaxis of periodontitis and gingivitis, have been reported and discussed in literature (Paper 3) (Asokan et al., 2008; Asokan et al., 2009; Asokan et al., 2011; Wiegand et al., 2007). However, the scientific background of the described effects has mainly been explained theoretically and not yet investigated in detail. It is presumed that oil pulling modifies the pellicle's lipid composition resulting in hydrophobization of the oral surfaces. This may explain its protective effects since hydrophobic interactions have been shown to be of importance for the adherence of microorganisms on oral surfaces (Busscher and van der Mei, 1997; Gibbons and Etherden, 1983; Quirynen et al., 1989). According to investigations of biofilm formation on different surfaces in the oral cavity (polysiloxane pretreated crowns, dental restorative and implant

materials), less plaque formation was observed on hydrophobic surfaces compared to hydrophilic ones (Busscher et al., 2010; Hannig and Hannig, 2009a; Olsson et al., 1992; Quirynen et al., 1989). The authors explained this result by a decreased binding force between bacteria and the hydrophobic surface. In contrast, other scientific studies indicate that hydrophobically modified structures in the oral cavity could either hamper, but possibly also facilitate the attachment of certain microorganisms (Hannig et al., 2013; Hannig and Hannig, 2009a; Schachtele et al., 1978). Apart from these divergent results, a study published by van der Mei et al. showed that the application of dietary lipids from salad oil increased the hydrophobicity of the pellicle layer *in vitro* (van der Mei et al., 2002). However, it must be noted that no analysis of the pellicles' specific lipid content was carried out in the few studies investigating the relevance of hydrophobic interactions on oral bioadhesion.

The comprehensive method that was developed within this study was used to investigate whether rinses with safflower oil can modify the pellicle's FA composition. Reasons for using safflower oil in this study were rather simple. They include its neutral taste as well as its availability as a common edible oil. There are two types of safflower that produce oils with different FA compositions: one with a high content of monounsaturated FA (oleic acid) and another high in polyunsaturated FA (linoleic acid). The high-oleic safflower oil, which was used in this study, is predominantly used as edible oil because of the higher oxidative stability and therefore increased shelf life.

Figure 6 shows enlarged sections of two GC-MS chromatograms of pellicle samples analyzed after a total oral exposure time of 120 min with (A) and without (B) using safflower oil (high oleic, FA composition see appendix, p. 38) as an initial mouthrinse. An accumulation of the safflower oil's specific FAs (18:1n9c, 18:2n6c) in the pellicle is clearly visible, even 107 min after the application of the oil mouthrinse. Compared to the pellicle samples treated without an oil rinse, the peak area ratios of the associated FAs increased by a factor of 6 and 2 for oleic acid and linoleic acid, respectively (Paper 2).

The present study showed that rinses with edible oils (as shown for safflower oil) can change the FA composition and content of the pellicle, thus representing one possibility for modifying and increasing the hydrophobicity and thereby the functional properties of the pellicle layer. Accumulation of FAs was observed after rinses with safflower oil (Figure 6). This is in good accordance with latest information gained by transmission electron microscopy (TEM) evaluations of *in situ* formed pellicle samples after rinses with various edible oils (Hannig et al., 2012; Hannig et al., 2013). These TEM images indicated that the lipids are not completely integrated in the microstructure of the pellicle. Lipid micelles adhering to the pellicle were observed directly after the rinses.

Furthermore, less electron-dense pellicle structures were found 109 min after rinses with edible oils (Hannig et al., 2013). This appears to be in good agreement with recent findings that rinses with edible oils have no influence on the protective properties of the pellicle (Hannig et al., 2012). These results show that the combination of the presented analytical procedure with TEM methods is necessary to obtain conclusive results from studies on the modification of the pellicle layer.

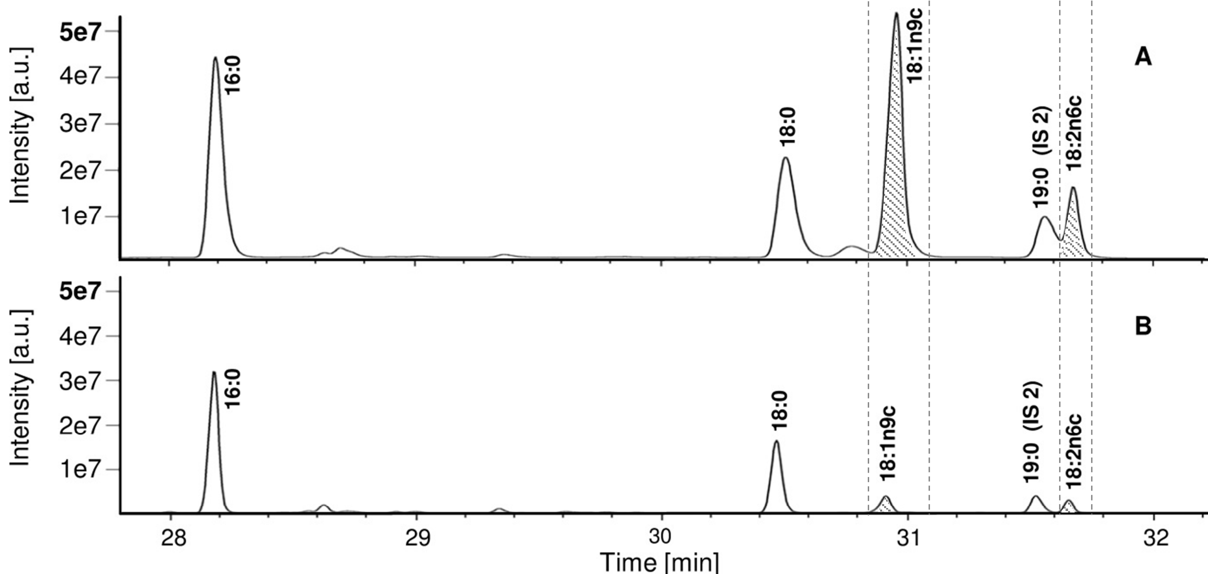


***Figure 6.*** *Enlarged sections of GC-MS chromatograms of pellicle samples analyzed after a total oral exposure time of 120 min with (A) and without (B) using safflower oil (high oleic) as initial mouthrinse (8 mL; 10 min)*

# 6. FINAL CONCLUSION AND FUTURE RESEARCH NEEDS

The principle aim of this thesis was the comprehensive characterization of the FA profile of the *in situ* pellicle. For this purpose, a chemical-analytical method based on a combination of innovative specimen generation and convenient sample preparation with sensitive GC-EI/MS analysis was successfully developed, validated, and applied. With the aid of this method, the objectives addressed in this thesis (Chapter 3) have been achieved and the following conclusions can be drawn:

- The present study shows, for the first time, the qualitative and quantitative characterization of FAs in the *in situ* pellicle. The investigations proved that a characteristic FA profile exists in healthy adults. Furthermore, FAs of bacterial origin were detected, which indicates the presence of bacterial components in the pellicle layer.

- Distinct differences were found between the pellicle's FA profile and that of corresponding saliva samples. This strengthens the hypothesis that the formation of the pellicle is a highly selective process that does not correlate directly with salivary composition.

- It was shown that formation time has only a minor impact on the FA composition of the pellicle layer. However, the FA content of the pellicle increased substantially with increasing formation time, which can be mainly explained by pellicle growth in general.

- The experiments described in this thesis proved that it is possible to modify the pellicle's FA composition using edible oils (as shown for safflower oil) as a mouthrinse. Targeted accumulation of the safflower oil's specific FAs resulted in the modification of the pellicle's "natural" FA profile as well as in an increase of the total FA amount. However, further TEM investigations showed that the lipids are not completely integrated in the microstructure of the pellicle, but rather adhere in micelles to the pellicle surface.

The present work contributes valuable information on the pellicle's FA composition which closes an existing knowledge gap in pellicle research. A broader knowledge of the lipid composition of the pellicle contributes to the understanding of oral bioadhesion processes and may help facilitate novel approaches in preventive dentistry. Furthermore, pellicle lipids could serve as biomarkers useful for diagnostic applications of oral diseases.

The experiments reported herein indicate that the developed method is suitable for evaluating the impact of different dentifrices and mouthrinses on the lipid composition of the pellicle. It was postulated that edible oils could add hydrophobic and therewith anti-adhesive properties to the pellicle, hampering bacterial adhesion. As shown in this study, a modification of the pellicle's FA profile and thus hydrophobization of the pellicle layer can be achieved by rinses with edible oils. Nevertheless, the modulated pellicle structure could either hamper or facilitate the attachment of certain microorganisms due to enforced hydrophobic interactions. Further research into the value of lipid-containing mouthwashes and edible oils in the context of bioadhesion and preventive strategies is necessary. The methods developed within this thesis may be helpful to conduct such studies.

A further research direction that may result from the outcome of this thesis is the investigation of the correlation between the lipid composition of the pellicle and caries or dental erosion. Since a characteristic FA profile of the pellicle seems to exist in healthy adults, structured analysis and comparison of the lipid composition of caries-resistant and caries-susceptible subjects could give further information on the protective properties of lipophilic key components in the pellicle.

The highly selective formation of the pellicle layer is only one example for the ubiquitous process of bioadhesion. Understanding conditioning biofilms remains a great challenge in life sciences. The described methods could also be transferred to bioadhesion processes in general and be helpful when characterizing FAs in any adherent biofilm.

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# APPENDIX

## Quantitative Transesterification

Quantitative formation of FAMEs using the developed method was investigated with cholesteryl palmitate because sterol esters are the most resistant to transesterification of lipid classes with ester linkages (Ichihara et al., 2003). Transesterification of cholesteryl palmitate results in the formation of cholesterol and methyl palmitate (Figure 7).

$H_3C(H_2C)_{13}H_2C$–COO–Cholesteryl → (MeOH, 1,5% HCl, Δ T) → HO–Cholesterol + $H_3C(H_2C)_{13}H_2C$–CO–O–$CH_3$

Cholesteryl palmitate — Cholesterol — Methyl palmitate

***Figure 7.*** *Transesterification of cholesteryl palmitate to cholesterol and methyl palmitate.*

Figure 8 shows that the transesterification of cholesteryl palmitate proceeded quanitatively (reaction products only methyl palmitate and cholesterol) and no major artefacts were produced when the described method (Paper 1) was used.

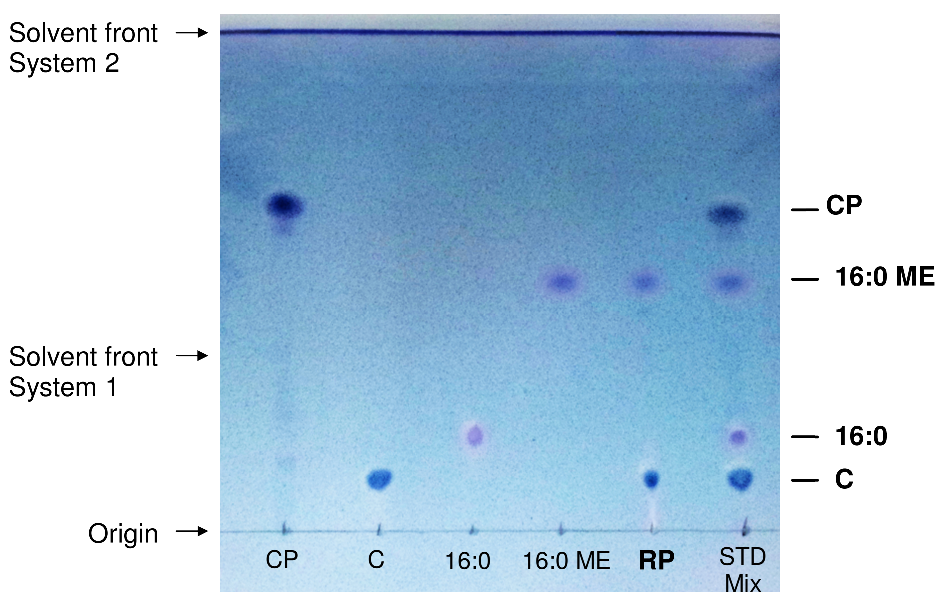


***Figure 8.*** *TLC screening of the methanolysis of cholesteryl palmitate.*
*Two micrograms of each standard and two microliters of the RP mixture were applied to the TLC plate. Abbreviations: CP, cholesteryl palmitate; C, cholesterol; 16:0, palmitic acid; 16:0 ME, methyl palmitate; RP, reaction products; STD mix, mixture of standard substances.*

The reaction mixture (100 µg CP in 2 mL methanol, 1.5% HCl) was incubated at 100°C for 60 min. Reaction products and standard substances were analyzed by two-stage TLC development:

- First stage: 3.0 cm from the origin with hexane / diethyl ether / acetic acid (60:40:0.5; v/v/v).
- Second stage: Redeveloped to 7.0 cm from the origin with hexane / diethyl ether / acetic acid (95:5:0.5; v/v/v).

Staining of Lipids was done by immersing the developed TLC plate for 15 min in the staining solution (0.2 % Amido black 10B in 1 M NaCl).

## Fatty acid composition of safflower oil

***Table 4.*** *Fatty acid composition of safflower oil (high oleic) (Fiebig, 2011)*

| Fatty acid | Weight percentage [a] |
|---|---|
| 12:0 | n.d.-0.2 |
| 14:0 | n.d.-0.2 |
| 16:0 | 3.6-6.0 |
| 16:1 | n.d.-0.2 |
| 17:0 | n.d.-0.1 |
| 17:1 | n.d.-0.1 |
| 18:0 | 1.5-2.4 |
| 18:1 | 70.0-83.7 |
| 18:2 | 9.0-19.9 |
| 18:3 | n.d.-1.2 |
| 20:0 | 0.3-0.6 |
| 20:1 | 0.1-0.5 |
| 20:2 | n.d. |
| 22:0 | n.d.-0.4 |
| 22:1 | n.d-0.3 |
| 22:2 | n.d. |
| 24:0 | n.d.-0.3 |

[a] % values with reference to investigated FAs
n.d.: not detectable.

# Curriculum vitae

**Marco Reich**, born 14 October 1983 in Weingarten, Germany

## Education

Nov 2010 - present

**PhD student and research fellow**
Leuphana Universität Lüneburg, Germany
- Project management and academic tutoring
- Thesis/Project: *The Fatty Acid Profile of the Initial Oral Biofilm-Characterization and Modification*

Oct 2004 – Apr 2009

**Dipl.-Ing., Pharmaceutical Chemistry**
NTA Isny - University of Applied Sciences
- Focus on analytical chemistry
- Diploma thesis in cooperation with synlab Services GmbH, Stuttgart, Germany: *Implementation of MALDI-TOF Mass Spectrometry for the Rapid Identification of Microorganisms in Clinical Routine*

Sept 1994 – Aug 2003

**Abitur** (higher education entrance qualification)
Studienkolleg St. Johann, Blönried

## Professional Experience

Jan 2010 – Oct 2010

**Research fellow**
Albert-Ludwigs-Universität Freiburg im Breisgau, Germany
- Analytical chemistry
- Planning, implementation and evaluation of analyses

Apr 2009 – Dec 2009

**Analytical chemist** (Clinical chemistry and microbiology)
synlab Services GmbH, Stuttgart, Germany
- Managing and working on research projects
- Clinical chemistry (drug/metabolite monitoring)

Mar 2007 – Sept 2007

**Full-time internship**
Roche, Basel, Switzerland
Department of preclinical pharmaceutical research
(Subgroup:Metabolic diseases)
- Synthesis of new active ingredients and intermediates in the field of Arteriosclerosis research

## Publications

Reich M., Kümmerer K., Al-Ahmad A. and Hannig C. (**2013**).
Fatty Acid Profile of the Initial Oral Biofilm (Pellicle): an in-situ Study.
*Lipids* 48: 929-937.
DOI: 10.1007/s11745-013-3822-2

Reich M., Bosshard P.P., Stark M., Beyser K. and Borgmann S. (**2013**)
Species Identification of Bacteria and Fungi from Solid and Liquid Culture Media by MALDI-TOF Mass Spectrometry.
*Journal of Bacteriology and Parasitology* S5: 002.
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Lipids in Preventive Dentistry.
*Clinical Oral Investigations* 17: 669-685.
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Reich M., Hannig C., Al-Ahmad A., Bolek R. and Kümmerer K. (**2012**).
A Comprehensive Method for Determination of Fatty Acids in the Initial Oral Biofilm (Pellicle).
*Journal of Lipid Research* 56: 2226-2230.
DOI: 10.1194/jlr.D026260

## Reprint of articles included in this thesis

**PAPER 1:**

Marco Reich, Christian Hannig, Ali Al-Ahmad, Richard Bolek and Klaus Kümmerer (**2012**).
A Comprehensive Method for Determination of Fatty Acids in the Initial Oral Biofilm (Pellicle).
*Journal of Lipid Research* 56: 2226-2230
DOI: 10.1194/jlr.D026260

**PAPER 2:**

Marco Reich, Klaus Kümmerer, Ali Al-Ahmad and Christian Hannig (**2013**).
Fatty Acid Profile of the Initial Oral Biofilm (Pellicle): an *in-situ* Study.
*Lipids* 48: 929-937
DOI: 10.1007/s11745-013-3822-2

**PAPER 3:**

Anna Kensche, Marco Reich, Klaus Kümmerer and Christian Hannig (**2013**).
Lipids in Preventive Dentistry.
*Clinical Oral Investigations* 17: 669-685
DOI: 10.1007/s00784-012-0835-9

# PAPER 1

## A Comprehensive Method for Determination of Fatty Acids in the Initial Oral Biofilm (Pellicle)

Marco Reich, Christian Hannig, Ali Al-Ahmad, Richard Bolek and Klaus Kümmerer

(**2012**)

*Journal of Lipid Research* 56: 2226-2230
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methods

# A comprehensive method for determination of fatty acids in the initial oral biofilm (pellicle)

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Institute of Sustainable and Environmental Chemistry,* Faculty of Sustainability, Leuphana University Lüneburg, Lüneburg, Germany; Clinic of Operative Dentistry,[†] Faculty of Medicine Carl Gustav Carus, Technical University of Dresden, Dresden, Germany; and Department of Operative Dentistry and Periodontology, [§] Albert-Ludwigs University Freiburg, Freiburg, Germany

**Abstract The acquired pellicle is a tenacious organic layer covering the surface of teeth, protecting the underlying dental hard tissues. Lipids account for about one quarter of the pellicle's dry weight and are assumed to be of considerable importance for their protective properties. Nevertheless, only preliminary information is available about the nature of lipids in the pellicle. Gas chromatography coupled with electron impact ionization mass spectrometry was used to establish a convenient analytical protocol in order to obtain a qualitative and quantitative characterization of a wide range of FAs ($C_{12}$–$C_{22}$). In situ biofilm formation was performed on bovine enamel slabs mounted on individual splints carried by 10 subjects. A modified Folch extraction procedure was adopted to extract the lipids from the detached pellicle, followed by transesterification to fatty acid methyl esters using methanol and concentrated hydrochloric acid. Tridecanoic and nonadecanoic acid were used as internal standards suitable and reliable for robust, precise and accurate measurements. The present study demonstrates, for the first time, a procedure based on a combination of innovative specimen generation and convenient sample preparation with sensitive GC-MS analysis for the determination of the fatty acid profile of the initial oral biofilm.**—Reich, M., C. Hannig, A. Al-Ahmad, R. Bolek, and K. Kümmerer. **A comprehensive method for determination of fatty acids in the initial oral biofilm (pellicle).** ***J. Lipid Res.*** **2012.** 53: **2226–2230.**

**Supplementary key words** clinical trials • fatty acid • derivatization • saliva • mass spectrometry • in situ

Biofilm formation on dental hard tissues is fundamental for caries and periodontitis, two diseases with extremely high prevalence and considerable economic relevance (1–6). Dental hard tissues are the only nonshedding surfaces in the human organism. Accordingly, the process of bioadhesion at tooth surfaces is of particular significance for oral diseases (7). The first step is the formation of the pellicle layer, which is mainly composed of adsorbed proteins and other macromolecules from the oral environment (saliva, crevicular fluids) and is clearly distinguished from the microbial biofilm (plaque) (7, 8). The selective process of pellicle formation is driven by physicochemical interactions such as van der Waals forces as well as electrostatic and hydrophobic interactions (7, 9). Serving as a protective lubricant, diffusion barrier, and buffer, the pellicle layer participates in all interfacial events taking place in the oral cavity (8). Furthermore, several antibacterial proteins and enzymes are present in this proteinaceous layer of high tenacity (10, 11). Nevertheless, several bacteria have adapted to this protective structure, as certain pellicle components provide specific receptors for bacterial adhesion to the tooth surface, making the pellicle a conditioning film for bacterial biofilm formation (12, 13). All in all, the pellicle is a key structure, mediating the process of bioadhesion at the tooth surface and the interaction between bacteria, saliva, and teeth. Some properties of the pellicle, such as ultrastructure, amino acid composition, and enzyme activity, have been investigated in detail (7, 8, 13). Therefore, three types of studies have to be differentiated: in vitro studies (pellicle formed in vitro from collected saliva on different materials), in vivo studies (pellicle harvested by scraping with a curette from the tooth surface), and in situ approaches (samples exposed to the oral cavity with splints) (7, 14). In vitro studies do not adequately mimic the situation in the oral cavity due to lacking maturation processes; thus, the in vitro pellicle differs considerably from the in vivo situation (15). Harvesting the in vivo pellicle yields only very small amounts of sample material and the basal structures of the pellicle are not removed sufficiently (14). Accordingly, in situ setups with

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Abbreviations: BAME, bacterial acid methyl ester; CV, coefficient of variation; FAME, fatty acid methyl ester; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; RSD, relative standard deviation; RT, retention time; SIM, selected ion monitoring; S/N ratio, signal-to-noise ratio; TEM, transmission electron microscopy.

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enamel slabs are preferable and allow evaluation of the pellicle with many elaborate methods. However, there is only limited information on the nature, function, and composition of lipids in the pellicle. Data is predominantly derived from studies carried out in the 1980s and refers exclusively to the workgroup around Slomiany (16, 17). Therefore, further research is required to get a wider understanding of their biological effect in the oral cavity. Lipids in the pellicle are assumed to hamper bacterial adhesion and to protect the tooth surface against erosive noxae. Methods such as GC-MS offer the opportunity to analyze the lipid composition of the pellicle layer more precisely than in previous studies. The aim of the present study was to establish and validate a precise method for the evaluation of the FA pattern of the in situ formed pellicle. Harvesting of the pellicle and the small amount of sample material represents considerable challenges (14).

## MATERIALS AND METHODS

### Chemicals and standards

A Supelco 37-component fatty acid methyl ester (FAME) mix, a Supelco 23-component Bacterial Acid Methyl Ester (BAME) mix, as well as additional standards of single FA target compounds (12:0, 14:0, a15:0, 15:0, 16:0, 16:1n-9, 18:0, 18:1n9c, 18:2n-6, 20:0, 22:1n-9) and the two internal standards (IS) (13:0, 19:0) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Hydrochloric acid, chloroform, methanol, and n-hexane were purchased from Carl Roth (Karlsruhe, Germany) in GC ultra grade and LC-MS grade. Water utilized for preparation of standard and extraction solutions was deionized with a Milli-Q purification system (Millipore, Schwalbach/Ts, Germany).

### Instrumental conditions

Gas chromatography/electron impact ionization-mass spectrometry (GC/EI-MS) analyses were performed with a Fisons GC 8065 gas chromatograph interfaced with a single-quadrupole Fisons 800 MSD. The samples (1 µl) were injected via a CTC A200S autosampler (splitless, split open after 90 s). The injector and transfer line temperatures were kept at 260°C. A Select FAME fused silica capillary column (50 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Waldbronn, Germany) was used for separation of the target compounds. Helium (purity 5.0) was used as carrier gas with a constant pressure of 100 kPa. The GC temperature program started at 50°C (hold time 5 min) and was increased to 260°C (hold time 8 min) at a ramp rate of 6.5°C/min. A solvent delay of 8 min was applied. The electron energy was 70 eV and the temperature of the ion source was set to 250°C. In the GC/EI-MS full scan mode, $m/z$ 60-400 was recorded. For GC/EI-MS in the selected ion monitoring (SIM) mode, fragment ions including $m/z$ 74, $m/z$ 87, $m/z$ 81, and $m/z$ 79 for FAME were recorded throughout the run (18).

### Calibration standards and quality control samples

The Supelco FAME mix and the Supelco BAME mix, including 49 different FAMEs, were used as reference standards to identify the FAs of the pellicle samples. After screening the pellicle samples for the most abundant FAs, a stock solution containing 11 FAs of two levels of concentration (1 mg/ml each of 12:0, 14:0, a15:0, 15:0, 16:1n-9, 18:2n-6, 20:0; 5 mg/l each of 16:0, 18:0, 18:1n-9, 22:1n-9; in methanol) was prepared from the individual FA standards for quantitative analysis. Calibration standards were made up of seven different concentrations, depending on the particular FA, ranging from 12.5 ng/ml to 250 ng/ml and 62.5 ng/ml to 1250 ng/ml, respectively. The final concentrations were yielded by diluting the stock solution with methanol. Quality control (QC) samples were prepared at four different concentrations (30, 175 ng/ml and 150, 875 ng/ml; in 0.4% EDTA solution). The FA stock solution and the QC samples were aliquoted and stored at −20°C under nitrogen.

### Subjects and sample collection

Bovine incisors were acquired from two-year-old cattle (BSE-negative). After extraction, the teeth were stored in thymol solution. For sample generation, round enamel slabs (5 mm diameter) were gained from the labial surface of the teeth with a trepan bur. The surface of the enamel slabs was wet-ground with up to 4,000-grit abrasive paper. Afterwards, the samples were disinfected in a sequential procedure in an ultrasonic bath. After 3 min in sodium hypochlorite (2%), the slabs were washed twice in deionized water for 5 min each followed by ultrasonication in ethanol (70%) for 10 min and final cleaning in deionized water for another 10 min. Before exposure to the oral fluids, the slabs were stored in deionized water for 24 h to form a hydration layer (19, 20).

For pellicle formation, the slabs were fixed into small cavities on individual upper jaw splints with silicon impression material (Aquasil, Dentsply De Tray, Konstanz, Germany), so that only the surface was exposed to the oral fluids. 12 slabs per splint were fixed on buccal and palatal sites of the premolars and the first molar (14, 19). After oral exposure for 30 min, the slabs were rinsed for 10 s with saline solution to remove loosely attached salivary fractions. Then the slabs were removed from the splints with a dental probe and transferred to 15 ml Falcon tubes. For the purpose of desorption, the samples were incubated in an ultrasonic bath with 1 ml 0.4% EDTA (pH 7.4) for 60 min (14). The pellicle is a biofilm of high tenacity; therefore, direct and complete extraction of pellicle components is difficult. A previous study indicates that the adopted desorption procedure allows complete and quantitative detachment of the in situ formed pellicle as validated by transmission electron microscopy (TEM) (14). The desorbed pellicle was pipetted into 1.5 ml amber screw vials and stored at −20°C until analysis.

The age of the subjects participating in this study ranged between 26 and 57 (4 male, 6 female). The subjects showed no signs of caries and periodontitis and the plaque indices were near zero. The study protocol was approved by the ethics committee of the medical faculty of the University of Freiburg (# 222/08).

### Sample preparation

The pellicle sample, dissolved in 1 ml 0.4% EDTA solution, was spiked with 30 µl of tridecanoic and nonadecanoic acid (25 µM each in methanol) as IS prior to extraction. A modified Folch extraction procedure (21) was applied in which 3.9 ml of a $CHCl_3$/MeOH (2:1, v/v) solution were added to the desorbed pellicle sample. After vortexing, the mixture was centrifuged at 900 $g$ for 5 min. The lower phase, containing virtually all the lipids, was isolated in a screw-capped glass test tube (16.5 × 105 mm), and the solvent was evaporated under a gentle stream of nitrogen. Transesterification was carried out based on the method of Ichihara and Fukubayashi (22) and adapted to the pellicle matrix. The sample was dissolved in 0.2 ml of chloroform, 2 ml of methanol, and 0.1 ml of concentrated hydrochloric acid (35%, w/w), which were added in this order to the lipid solution. The final HCl concentration was 1.5% (w/v) in a total volume of 2.3 ml. The solution was overlaid with nitrogen and the tube was tightly closed. After vortexing, the tube was heated at 100°C for 1 h. Once cooled to room temperature, 2 ml of hexane and 2 ml of water were added for



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TABLE 1. Validation results of the overall method in the intra- and interday assays

| FA | RT min | Linearity[a] $r^2$ | LOD[b] ng/ml | LOQ[c] ng/ml | Intraday $QC_{low}$ (n = 5) CV[d] % | Intraday $QC_{low}$ (n = 5) Accuracy[d] % | Intraday $QC_{high}$ (n = 5) CV % | Intraday $QC_{high}$ (n = 5) Accuracy % | Interday $QC_{low}$ (n = 5) CV % | Interday $QC_{low}$ (n = 5) Accuracy % | Interday $QC_{high}$ (n = 5) CV % | Interday $QC_{high}$ (n = 5) Accuracy % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12:0 | 22.7 | 0.9994 | 5.7 | 12.1 | 5.3 | 94.8 | 1.8 | 102.1 | 13.4 | 87.2 | 3.5 | 99.6 |
| 14:0 | 25.6 | 0.9993 | 6.6 | 12.4 | 5.7 | 90.0 | 6.3 | 96.0 | 12.3 | 84.4 | 5.9 | 93.4 |
| a15:0 | 26.5 | 0.9999 | 2.2 | 7.6 | 1.1 | 101.5 | 3.2 | 99.9 | 5.1 | 101.2 | 3.3 | 99.0 |
| 15:0 | 26.9 | 0.9998 | 4.6 | 10.6 | 1.6 | 97.1 | 4.7 | 97.4 | 2.6 | 96.1 | 3.5 | 98.0 |
| 16:0 | 28.1 | 0.9997 | 8.4 | 28.8 | 12.0 | 102.8 | 3.2 | 101.5 | 12.2 | 99.1 | 2.5 | 101.5 |
| 16:1n9c | 28.9 | 0.9991 | 8.1 | 13.3 | 5.9 | 104.5 | 3.8 | 105.6 | 7.8 | 102.3 | 4.7 | 104.0 |
| 18:0 | 30.4 | 0.9988 | 26.4 | 83.9 | 2.7 | 107.1 | 2.2 | 106.9 | 11.2 | 99.4 | 1.9 | 106.3 |
| 18:1n9c | 30.8 | 0.9998 | 8.3 | 28.6 | 2.2 | 101.0 | 1.9 | 100.1 | 6.8 | 95.9 | 1.2 | 99.9 |
| 18:2n6c | 31.6 | 0.9998 | 2.6 | 9.2 | 4.6 | 103.2 | 1.8 | 101.0 | 5.0 | 100.5 | 1.6 | 101.1 |
| 20:0 | 32,4 | 0.9997 | 4.5 | 10.6 | 6.2 | 102.9 | 2.1 | 99.8 | 9.0 | 94.6 | 1.9 | 100.9 |
| 22:1n9c | 34.7 | 0.9979 | 29.1 | 91.8 | 9.7 | 102.2 | 2.8 | 101.0 | 6.3 | 93.9 | 4.3 | 100.7 |

[a] Calibration range from 12.5 ng/ml to 250 ng/ml and 62.5 ng/ml to 1250 ng/ml.
[b] The limit of detection was measured at S/N ratio > 3.
[c] The limit of quantification was measured at S/N ratio > 10.
[d] Precision and accuracy were expressed as the mean values of data obtained from QC samples ($QC_{low}$: 30, 150 ng/ml and $QC_{high}$: 175, 875 ng/ml, depending on the particular FA) through intra- and interday assays.

extraction of FAMEs. The tube was vortexed and after phase separation, the hexane phase was isolated and evaporated under a gentle stream of nitrogen. The residue was redissolved in 0.1 ml of hexane and 1 µl of this solution was injected for GC-MS analysis.

### Data evaluation

Retention times (RTs) of the separated FAs as well as the respective mass spectra gained from full scan measurement were used for qualitative analysis. Although EI-ionization was applied, the molecular ion ($M^+$) of each FA was visible in the mass spectrum. Quantification of data obtained from SIM mode measurements was performed using the peak area ratios relative to that of the IS. Least squares regression analysis was implemented, using the peak area ratios against increasing standard concentrations to obtain calibration linearity. Peak area ratios of the unknown samples were referred to this calibration curve. Prior to the sample run, a blank sample and the seven calibration standards were measured. Measurements of the pellicle samples were bracketed by injections of QC samples to validate the results.

### Method validation

Statistical analysis was done referring to the guidelines for method validation of the Society of Toxicological and Forensic Chemistry (23). The main performance characteristics evaluated were selectivity over the analyte, linearity of the response, closeness to the true value, precision of the obtained results, and detection and quantification limits. The limit of detection (LOD) and limit of quantification (LOQ) were defined to be the lowest concentration with a signal-to-noise (S/N) ratio > 3 for LOD and 10 for LOQ. The precision expressed as the coefficient of variation (% CV) and the accuracy as the percentage relative error (% bias) were determined from the QC samples at two different concentrations based on the calibration range of each FA. For intraday repeatability, five replicates were analyzed, whereas the interday reproducibility was measured from samples run over 5 nonconsecutive days.

## RESULTS AND DISCUSSION

### Method validation

The characterization of lipids and their FA profiles via GC-MS is a widely accepted practice (24, 25). Nevertheless, analysis of FAs can be complicated due to cross-contamination because lipids are omnipresent in nature and

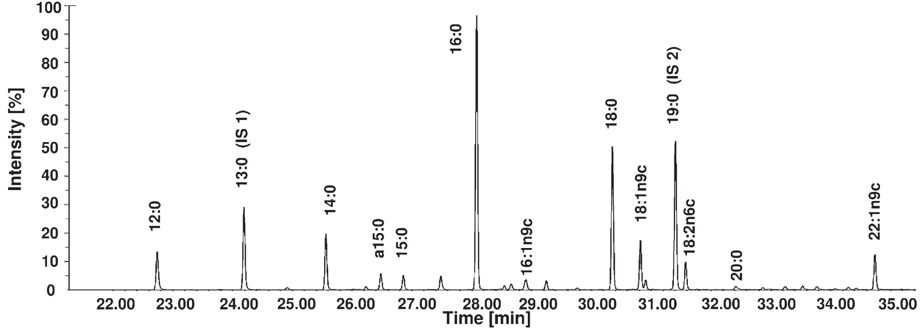


**Fig. 1.** GC/MS chromatogram of a pellicle sample (formation time 30 min) acquired in SIM mode. The sample was separated through a thermally stable Select FAME capillary column (50 m × 0.25 mm ID, 0.25 µm film thickness). The GC oven program started at 50°C (hold time 5 min) and was increased to 260°C (hold time 8 min) at a ramp rate of 6.5°C/min. Characteristic fragment ions (*m/z* 74, *m/z* 87, *m/z* 81, and *m/z* 79) were monitored throughout the run.



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are constituents of commercial plastics, surfactants, and lubricants (24). As with any analytical procedure, the validity of the results depends on proper sampling and preservation of the sample prior to analysis. The importance of sample preparation is often underestimated and therefore carried out hurriedly and incorrectly. It must be kept in mind that in case of errors occurring during the extraction procedure, even the best analytics is worthless. The described analytical method is reliable when plastic products are avoided whenever possible and all the glassware used is cleaned (e.g., rinsed with methanol) prior to use.

For method validation, parameters such as accuracy, precision, selectivity, and the analytical limits (LOD, LOQ) were evaluated (**Table 1**). The GC/EI-MS analysis in the SIM mode provided LOQs ranging from 7.6 to 91.8 ng/ml whereas those of most FAs ranged from 7.6 to 28.8 ng/ml, except for 18:0 (83.9 ng/ml) and 22:1n9c (91.8 ng/ml).

The calibration curve obtained from a blank sample and seven calibration standards was linear over a 20-fold concentration range with coefficients of determination $r^2 > 0.995$ for all analyzed FAs.

Precision and accuracy were determined by analyzing the QC samples acquired for the intra- and interday assays. The intraday (n = 5) precision ranged from 1.1 to 12.0% (% CV), and accuracies ranged from 90.0 to 106.9% (% bias). Interday (n = 5) precision and accuracy were between 1.2 to 13.4% and 84.4 to 106.3% (Table 1). Bias values within an interval of ± 15% of the nominal value are accepted as a tolerance limit except for compounds with concentrations close to the LOQ, where 20% is acceptable (23). With respect to the nature and available sample volume of the matrix, these results demonstrate the applicability of the method.

### FA profile of the initial oral biofilm (pellicle)

The lipid content of the pellicle has not been investigated thoroughly, even though lipids seem to be a significant constituent of the pellicle formed in vivo (16). Regarding the nature, function, and composition of lipids in the acquired pellicle, the current state of research provides only preliminary information. Studies on pellicle composition are hampered by the fact that only limited amounts of pellicle material can be harvested and recovered from human teeth in vivo for analytical investigation. The thickness of the pellicle layer is variable and depends on the oral exposure time as well as the localization in the oral cavity. It ranges between 10 to 20 nm after 3 min and up to 500 nm on buccal sites after 2 h (8, 20, 26). Despite these limitations, precise analysis of FAs in the pellicle is possible with the presented procedure. The chromatographic separation of the 13 FAs as their methyl ester derivatives was achieved with excellent peak shapes and high responses (**Fig. 1**).

Although FA analysis of the lipid classes in pellicle samples was reported here, saliva samples can also be analyzed using this protocol as well as other biofilms relevant for the pathogenesis of certain diseases. Examples are contact lenses or bypass due to coronary heart disease.

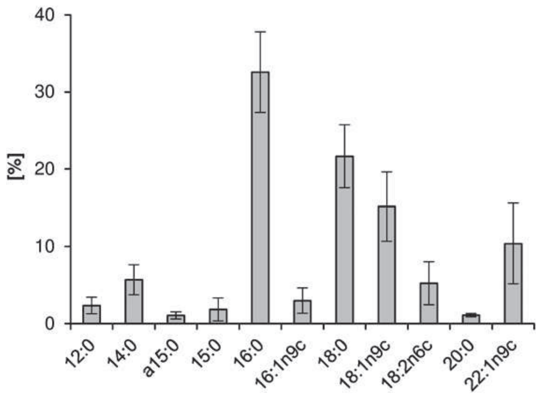


**Fig. 2.** FA composition of pellicle sample (formation time 30 min). Values represent the means ± SD of 10 subjects expressed as percent of the investigated FAs. This profile seems to be characteristic for the biological structure of the pellicle.

Using the devised method, 11 FAs (12:0, 14:0, a15:0, 15:0, 16:0, 16:1n9c, 18:0, 18:1n9c, 18:2n6c, 20:0, 22:1n9c) were detected and quantified by GC-MS analysis of the pellicle samples. Among these, palmitic- (16:0) (32%), stearic- (18:0) (21%), oleic- (18:1n9c) (14%), erucic- (22:1n9c) (10%), and linoleic acids (18:2n6c) (5%) account for the majority of FAs in the pellicle. The FA profile of the pellicle seems to be characteristic for this biological structure (**Fig. 2**). The composition is very stable. However, the total amount of investigated FAs shows distinctive interindividual differences among the 10 study subjects (**Fig. 3**). Values vary from 680 to 1600 ng per $cm^2$ pellicle formation surface. As compared with other pellicle parameters, the natural variability is rather low (11). Further research based on the presented method is necessary to evaluate the influence of saliva, oral localization, and pellicle formation time on the FA composition of the pellicle layer. Thereafter, epidemiological studies on the lipid composition of the pellicle in patients suffering from diseases such as xerostomia, periodontitis, dental erosions, or caries are possible. This offers further insight into the respective pathological mechanisms and new approaches in dental

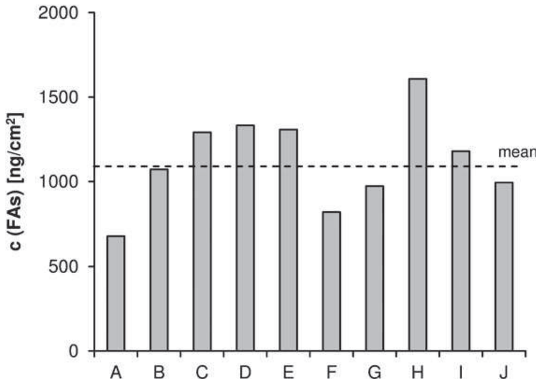


**Fig. 3.** Amount of total investigated FAs of the 30 min pellicle of the 10 study subjects (A–J). Values vary from 680 to 1600 ng per $cm^2$ pellicle formation surface, illustrating interindividual differences (dashed line marks the mean amount of total fatty acids of the 10 subjects).



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prophylaxis are conceivable. This is of considerable relevance as hydrophobic interactions are essential for the process of pellicle formation and bacterial adhesion as well as for the protective properties of the pellicle layer (7). Furthermore, the method allows for investigation of potential effects of rinses with mouthwashes or edible oils on the composition and functional properties of the pellicle layer.

In conclusion, a comprehensive GC-MS method was developed as a practical and feasible assay, which allows the quantification of pellicle FAs and helps to understand the initial process of bioadhesion in the oral cavity, which is also governed by hydrophobic interactions. The presented study demonstrates, for the first time, a procedure based on a combination of innovative specimen generation and convenient sample preparation with sensitive GC-MS analysis for the determination of the FA profile of the initial oral biofilm.

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# PAPER 2

## Fatty Acid Profile of the Initial Oral Biofilm (Pellicle): an In-Situ Study

Marco Reich, Klaus Kümmerer, Ali Al-Ahmad and Christian Hannig

(**2013**)

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ORIGINAL ARTICLE

# Fatty Acid Profile of the Initial Oral Biofilm (Pellicle): an In-Situ Study

**Marco Reich · Klaus Kümmerer · Ali Al-Ahmad · Christian Hannig**

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**Abstract** The first step of bioadhesion on dental surfaces is the formation of the acquired pellicle. This mainly acellular layer is formed instantaneously on all solid surfaces exposed to oral fluids. It is composed of proteins, glycoproteins and lipids. However, information on the lipid composition is sparse. The aim of the present study was to evaluate the fatty acid (FA) profile of the in-situ pellicle for the first time. Furthermore, the impact of rinses with safflower oil on the pellicle's FA composition was investigated. Pellicles were formed in situ on bovine enamel slabs mounted on individual upper jaw splints. The splints were carried by ten subjects over durations of 3–240 min. After comprehensive sample preparation, gas chromatography coupled with electron impact ionization mass spectrometry (GC–EI/MS) was used in order to characterize qualitatively and quantitatively a wide range of FA ($C_{12}$–$C_{24}$). The relative FA profiles of the pellicle samples gained from different subjects were remarkably similar, whereas the amount of FA showed significant interindividual variability. An increase in FA in the pellicle was observed over time. The application of rinses with safflower oil resulted in an accumulation of its specific FA in the pellicle. Pellicle formation is a highly selective process that does not correlate directly with salivary composition, as shown for FA.

**Keywords** Fatty acid composition · GC–MS · Extraction · Transesterification · Biofilm · Pellicle · In situ · Safflower oil · Bioadhesion · Saliva

**Abbreviations**

| | |
|---|---|
| BAME | Bacterial acid methyl ester |
| EDTA | Ethylenediaminetetraacetic acid |
| EI | Electron impact |
| FA | Fatty acid(s) |
| FAME | Fatty acid methyl ester(s) |
| GC | Gas chromatography |
| IS | Internal standard |
| LC | Liquid chromatography |
| LOQ | Limit of quantification |
| MS | Mass spectrometry |
| MSD | Mass selective detector |
| QC | Quality control |
| RT | Retention time |
| SIM | Selected ion monitoring |
| TEM | Transmission electron microscopy |

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## Introduction

The prevention of caries and periodontitis, two diseases with high prevalence and significant economic relevance, are ongoing challenges in dentistry [1–3]. Biofilm formation on dental hard tissues, the only non-shedding surface in the human organism, is fundamental for the development of these oral diseases [4, 5]. Therefore, the process of bioadhesion on tooth surfaces is of particular interest.

The first stage of bioadhesion on solid surfaces exposed to the oral fluids is the formation of the pellicle layer, which is a highly selective and dynamic process [6–9]. This acellular biofilm is composed largely of adsorbed proteins, glycoproteins and lipids, and is distinguished clearly from the microbial biofilm (plaque) [7, 8, 10]. The pellicle represents the interface between the teeth and the oral environment, therefore playing a key role in mediating the process of bioadhesion. Furthermore, it serves multiple functions such as a reservoir for remineralization, a protective lubricant, a diffusion barrier, and a buffer [7, 8, 10].

There is a large number of studies investigating the pellicle's ultrastructure, protein/amino acid composition and enzymatic activity in detail [6, 7]. However, little information exists concerning lipids, an important constituent that accounts for about one quarter of the pellicle's dry weight [11]. Lipophilic substances potentially govern hydrophobic interactions and are therefore assumed to have an impact on the process of bioadhesion on dental hard tissues, as well as on the composition and ultrastructure of the pellicle [12]. Several studies have indicated the relevance of hydrophobic interactions and cell hydrophobicity for bacterial adherence [13–15]. Despite their high occurrence and assumed importance, only limited information is available on the nature, function and composition of lipids in the pellicle.

The pellicle is a crosslinked biopolymer layer of high tenacity, therefore direct and complete extraction of pellicle components is difficult to achieve. Harvesting the minute amounts of pellicle material for analytical investigations is a challenging task that hampers studies on the lipid composition of the pellicle [7]. The thickness of the initial oral biofilm formed in situ within a few minutes ranges between 10 and 20 nm and remains stable for about 30 min [16, 17]. Depending on the intraoral location of a tooth surface, the thickness of the pellicle layer increases up to 500 nm within 2 h [17]. Because of the low availability of sample material, an efficient method for pellicle formation and collection is elementary for the subsequent compositional analysis of the structure. There are three approaches used for this task that need to be differentiated: in-vitro methods (oral biofilm formed in vitro from collected saliva on different materials), in-vivo methods (pellicle harvested by scrapping with a curette from the tooth surface) and in-situ methods (enamel slabs exposed to the oral cavity with splints) [8, 18]. In-vitro studies do not simulate adequately conditions in the oral cavity. Thus, in-vitro formed pellicle differs significantly from the in-vivo situation [8, 12, 19, 20]. However, mechanical harvesting the in-vivo pellicle leads to insufficient amounts of sample material and the basal structures of the pellicle cannot be removed adequately [18]. Furthermore, a quantitative analysis of pellicle components in practice is very difficult, because of the poor repeatability of sample collection in vivo. For the pellicle formation in situ, enamel slabs of standardized size and surface structure are fixed on individual splints and carried in the oral cavity. The slabs can be removed easily and the formed pellicle can be analyzed with various methods, either in the adherent state, or after desorption. Accordingly, in-situ setups are the method of choice in recent studies focusing on the protective impact of the pellicle on enamel surfaces [9, 21–23].

Thus far, data on the lipid composition of the pellicle derive from three studies carried out in the 1980s that used either in-vivo [11, 24] or in-vitro setups [25]. Since all investigations concerning the lipid composition solely refer to these few studies carried out 25 years ago, additional research of lipids in the in-situ pellicle is required to gain further insights into the pellicle's composition.

The aim of the present study was to characterize the fatty acid composition of an in-situ formed pellicle layer for the first time. FA are the basic building blocks of most of the components that are classified as lipids. Therefore, FA are considered to be in large part responsible for the defining characteristics of these lipids. Furthermore, certain FA are not only lipid components, but also have specific functions themselves, such as interacting with receptors [26, 27].

A broader knowledge of the lipid composition of the initial oral biofilm will help us to better understand oral bioadhesion processes and the phenomenon of bioadhesion in general.

## Materials and Methods

### Chemicals and Standards

A Supelco 37-component fatty acid methyl ester (FAME) mix, a Supelco 23-component bacterial acid methyl ester (BAME) mix, as well as additional standards of single FA target compounds (12:0, 14:0, a15:0, 15:0, 16:0, 16:1n9c, 18:0, 18:1n9c, 18:2n6c, 20:0, 22:1n9c) and the two internal standards (IS) (13:0, 19:0) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Hydrochloric acid, chloroform, methanol, and *n*-hexane were purchased from Carl Roth (Karlsruhe, Germany) in GC ultra or LC-MS grades. Water utilized for the preparation of standard and extraction solutions was deionized with a Milli-Q purification system (Millipore, Schwalbach/Ts, Germany). The safflower oil (*Carthamus tinctorius* L., Brölio Distelöl, Brökelmann & Co. Ölmühle GmbH & Co., Hamm, Germany) used as a mouthrinse contained 18:1n9c (70–80 %) and 18:2n9c (10–20 %) as major FA.

The 49 different FAME, included in the FAME and BAME mixes, were used as reference standards to screen the pellicle samples for the most abundant FA. For quantitative analysis, a stock solution containing 11 FA of two concentration levels (1 mg/ml each of 12:0, 14:0, a15:0, 15:0, 16:1n9c, 18:2n6c, 20:0; 5 mg/l each of 16:0, 18:0, 18:1n9c, 22:1n9c; in methanol) was prepared. Calibration standards were made up of seven different concentration levels depending on the particular FA, ranging from 12.5 to 250 ng/ml or from 62.5 to 1,250 ng/ml. The final concentrations were achieved by diluting the stock solution with methanol. Quality control (QC) samples were prepared at four concentrations (30, 175 ng/ml and 150, 875 ng/ml; in 0.4 % EDTA solution).

## Subjects

Ten healthy subjects (6 female, 4 male) aged between 26 and 57 years participated in this study. The subjects, all members of the laboratory staff, showed no signs of untreated carious lesions, and plaque and gingivitis indices were close to zero. This was approved after examination by an experienced dentist. Ethical approval of the study design was granted by the Ethics Committees of the Freiburg University (# 222/08) and the TU Dresden, Medical Faculty (EK 275092012).

## Preparation of Enamel Specimens

Bovine incisors were extracted from 2-year-old cattle (BSE-negative) and stored in a thymol solution (0.1 %) [17, 23, 28]. A trepan bur was used to prepare round enamel slabs (5 mm diameter) from the labial surfaces of the teeth. The surface of the slabs was polished with abrasive paper (400–4,000 grit). Afterwards, the specimens were disinfected in a sequential procedure using ultrasonication: After 3 min in sodium hypochlorite (2 %), the slabs were washed twice with deionized water for 5 min each, followed by disinfection in ethanol (70 %) for 10 min and final cleaning in deionized water for another 10 min. All four steps were supported by ultrasonication. Prior to oral exposure, the processed enamel slabs were stored in deionized water for 24 h in order to form a hydration layer [17, 21, 23, 28, 29].

## In-Situ Pellicle Formation

For in-situ exposure of the enamel specimens, the slabs were fixed into small cavities on individual upper jaw splints with polyvinyl siloxane impression material (Aquasil, Dentsply De Tray, Konstanz, Germany), thus only the enamel surface was exposed to oral fluids. In total, 12 slabs per splint were fixed on buccal and palatal sites (6 each) of the premolars and the 1st molar [18]. Before insertion and during oral exposure of the prepared splints, the subjects had to carry out specified instructions [17, 23, 29]. The test persons were instructed to clean their teeth thoroughly without tooth paste before inserting the splints. Eating and drinking was prohibited 120 min prior and during exposure of the samples in the oral cavity. The splints were carried intraorally for 3, 30, 60, 120 and 240 min, to allow for increasing pellicle formation on the surfaces of the specimens. After the respective pellicle formation periods, the slabs were rinsed with saline solution to remove loosely attached salivary fractions. The enamel samples were carefully removed from the splints using a dental probe and transferred to a 15 ml falcon tube. The pellicle structures were desorbed from the enamel specimens in an ultrasonic bath in 1 ml 0.4 % EDTA solution (pH 7.4) for 60 min [18]. The desorbed pellicle was pipetted into 1.5 ml amber screw vials and stored at 20 °C until analysis. A previous study indicated that the described treatment allows for quantitative detachment and collection of the in-situ formed pellicle [18].

For the experiments on the influence of oil rinses on the pellicle's composition, the splints were carried intraorally for 3 min to form the basal pellicle layer on the enamel surfaces. Afterwards, the subjects rinsed thoroughly with 8 ml safflower oil for 10 min. The samples remained in the oral cavity for further 107 min in order to give a total intraoral exposure time of 120 min. After intraoral exposure, the enamel slabs were removed immediately from the splints and rinsed thoroughly with water. Then the slabs underwent the same procedure for pellicle desorption as described above.

Additionally, unstimulated saliva samples were obtained prior to the respective pellicle formation time points. These samples were centrifuged at 6,000*g* for 10 min and sterile-filtered (0.2 µm) before analysis.

## Analytical Method

A validated method, specifically developed for the determination of fatty acids in the pellicle, was used in this study. Only a brief description of the analytical method is given here since it is described in detail elsewhere [28].

### *Sample Preparation*

Tridecanoic and nonadecanoic acid were used as internal standards and were added to the desorbed pellicle samples (in 1 ml 0.4 % EDTA solution) prior to all sample preparation steps. A modified Folch extraction procedure was applied in order to isolate the lipid fractions of the pellicle. Rapid transesterification (1 h; 100 °C) of all FA containing lipids (plus esterification of FFA) into FAME was carried

out in methanol using concentrated HCl (35 %, w/w) as an acidic catalyst. After methylation, FAME were extracted by adding deionized water and hexane. The hexane phase was isolated, evaporated under a gentle stream of nitrogen and the residue was redissolved in 0.1 ml of hexane. For GC-MS analyses, 1 µl of this solution was injected.

*Instrumental Conditions*

FAME were analyzed with a Fisons 8065 gas chromatograph interfaced with a single-quadrupole MSD. The injector was operated in splitless mode and kept at 260 °C. A Select FAME fused silica capillary column (50 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Waldbronn, Germany) was used for separation of the target compounds with helium as carrier gas. The oven temperature was programmed at 50 °C for 5 min, followed by an increase of 6.5 °C/min to reach the final temperature of 260 °C and then held for 8 min. For qualitative characterization, the range of *m/z* 60–400 was recorded in the full scan mode. Quantitative analysis was performed using selected ion monitoring (SIM), recording fragment ions including *m/z* 74, *m/z* 79, *m/z* 81 and *m/z* 87 throughout the run [30].

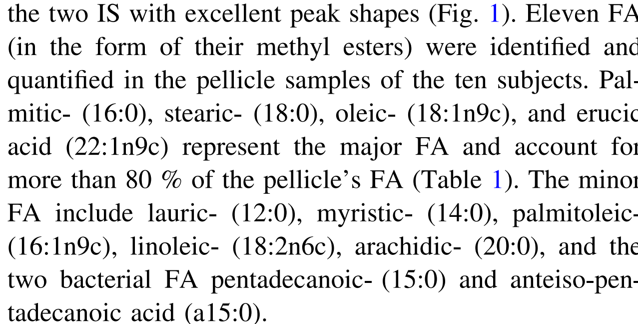
*Data Evaluation*

Retention times (RT) of the separated FAME as well as the respective mass spectra gained from full scan measurements were used for qualitative analysis. Quantification of data obtained from SIM mode measurements was performed using the peak area ratios (sum of the four recorded *m/z* values) relative to that of the IS. Tridecanoic acid was used as IS for the quantification of lauric acid. All other FA were referred to nonadecanoic acid. Least squares regression analysis was applied, using the peak area ratios against increasing standard concentrations in order to obtain calibration linearity. Peak area ratios of the samples were referred to this calibration curve. A blank sample and the seven calibration standards were measured prior to the sample runs, which were bracketed by injections of QC samples to validate the results. The quantitative analysis provided LOQ values ranging from 7.6 to 91.8 ng/ml [28].

## Results

### FA Profile of the Pellicle

The FA composition of the pellicle samples was analyzed using a GC-EI/MS method specially developed for this purpose [28]. Chromatographic baseline separation was obtained for all signals of the FAME target compounds and



the two IS with excellent peak shapes (Fig. 1). Eleven FA (in the form of their methyl esters) were identified and quantified in the pellicle samples of the ten subjects. Palmitic- (16:0), stearic- (18:0), oleic- (18:1n9c), and erucic acid (22:1n9c) represent the major FA and account for more than 80 % of the pellicle's FA (Table 1). The minor FA include lauric- (12:0), myristic- (14:0), palmitoleic- (16:1n9c), linoleic- (18:2n6c), arachidic- (20:0), and the two bacterial FA pentadecanoic- (15:0) and anteiso-pentadecanoic acid (a15:0).

The FA pattern of the pellicle showed no considerable differences among the ten study subjects (Fig. 2). In contrast to the very stable relative FA composition, the total amount of FA in the pellicle varied substantially among the subjects.

### FA Profile of Saliva

The FA profile of the examined saliva samples was dominated by 18:1n9c, 16:0, 18:2n6c and 18:0, in that order (Fig. 3). The major FA of the pellicle samples and the saliva samples are almost the same, but their ratios to one another differ distinctly. Compared to the pellicle's FA profile, higher proportions of the unsaturated FA 18:1n9c (two times higher) and 18:2n6c (four times higher) were detected. As shown before for the pellicle samples, considerable interindividual variations in the total FA contents were also noticed for the saliva samples from the ten study subjects (Fig. 4).

### Influence of Pellicle Formation Time

An overview of the pellicle FA composition for the different oral exposure times is given in Table 1. Within the 1st hour, the total amount of FA stayed nearly constant, whereas an accumulation of FA (FA containing lipids) was clearly evident over a formation period of 60–240 min. Despite the accumulation of the FA, the profile remained almost unchanged over increasing periods of oral exposure (Table 1; % values for the single FA from left to right).

### Effect of Rinses with Safflower Oil

Figure 5 shows enlarged sections of the GC-MS chromatograms of pellicle samples analyzed after a total oral exposure time of 120 min with (A) and without (B) using safflower oil (high oleic) as the initial mouthrinse. An increase in the safflower oil's specific FA (18:1n9c, 18:2n6c) in the pellicle is clearly visible, even 107 min after the application of the oil mouthrinse. Compared to the pellicle samples treated without an oil rinse, the peak area ratios of the associated FA increased by a factor of 6 and 2 for oleic acid and linoleic acid, respectively.

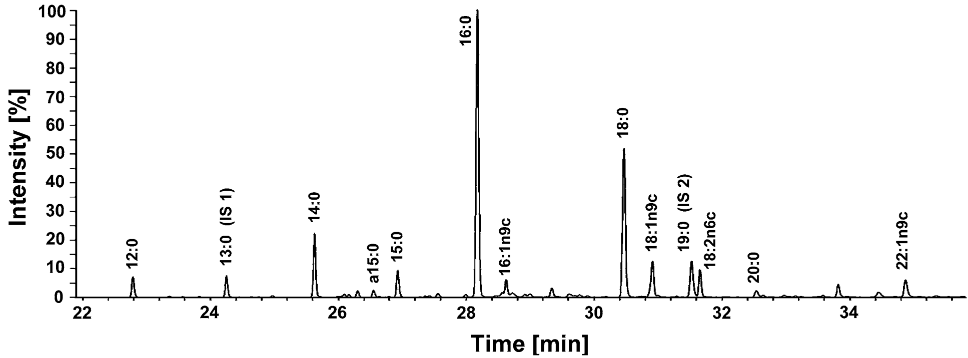



**Fig. 1** GC-MS chromatogram of a pellicle sample (in-situ formation time 120 min) acquired in SIM mode. The sample was separated through a thermally stable Select FAME capillary column (50 m × 0.25 mm ID, 0.25 μm film thickness). The GC oven temperature started at 50 °C (hold time 5 min) and was increased to 260 °C (hold time 8 min) at a ramp rate of 6.5 °C/min. Characteristic fragment ions (*m/z* 74, *m/z* 79, *m/z* 81, and *m/z* 87) were monitored throughout the run

**Table 1** FA composition of pellicle samples after different oral exposure times (splints carried in situ for 3-240 min)

| FA | 3 min | | 30 min | | 60 min | | 120 min | | 240 min | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % | ng/cm$^2$ | % | ng/cm$^2$ | % | ng/cm$^2$ | % | ng/cm$^2$ | % | ng/cm$^2$ |
| 12:0 | 2.1 | 19.0 | 2.2 | 24.4 | 2.2 | 22.5 | 1.7 | 22.2 | 1.9 | 28.5 |
| 14:0 | 5.6 | 51.3 | 5.7 | 62.3 | 5.5 | 59.6 | 4.6 | 61.4 | 5.1 | 74.2 |
| a15:0 | 0.8 | 7.8 | 1.0 | 10.9 | 0.9 | 9.8 | 1.3 | 16.8 | 1.3 | 19.0 |
| 15:0 | 1.2 | 11.3 | 1.7 | 18.5 | 1.4 | 13.9 | 1.4 | 18.0 | 1.4 | 20.3 |
| 16:0 | 30.7 | 283.2 | 33.5 | 365.9 | 31.7 | 339.8 | 35.4 | 471.7 | 34.9 | 533.4 |
| 16:1n9c | 2.6 | 23.9 | 2.9 | 31.8 | 3.3 | 35.2 | 3.0 | 40.5 | 3.2 | 48.4 |
| 18:0 | 21.8 | 201.4 | 22.3 | 244.1 | 23.0 | 246.4 | 20.0 | 265.4 | 23.3 | 357.6 |
| 18:1n9c | 14.5 | 133.4 | 14.7 | 160.5 | 14.0 | 140.1 | 14.7 | 195.9 | 14.2 | 240.1 |
| 18:2n6c | 4.9 | 45.0 | 5.0 | 54.6 | 5.4 | 60.3 | 6.1 | 81.1 | 4.6 | 82.2 |
| 20:0 | 1.0 | 9.0 | 1.1 | 11.7 | 1.0 | 10.8 | 1.3 | 16.7 | 1.1 | 15.7 |
| 21:1n9c | 14.8 | 137.9 | 9.9 | 108.1 | 11.6 | 118.8 | 10.5 | 139.5 | 9.0 | 142.5 |
| Total | | 923.3 | | 1,092.7 | | 1,057.3 | | 1,329.1 | | 1,561.8 |

The FA profile is remarkably stable whereas the amount of investigated FA increases after 60 min. Values represent the means of ten subjects, expressed as the percentage of total investigated FA, as well as ng per cm$^2$ enamel surface

## Discussion

### Methodology

The current state of research undoubtedly does not provide a complete determination of the composition of the pellicle. Especially for lipids, which are assumed to have a major impact on the process of bioadhesion and the ultrastructure of the pellicle [12], only little information is available. The present study used, for the first time, an in-situ approach for the compositional analysis of fatty acids in the pellicle. Our method combines the advantages of mimicking the in-vivo oral exposure and the possibility to completely detach the pellicle structure from the enamel surface. A previous study confirmed complete desorption and collection of the in-situ pellicle when using the described method [18]. Bovine enamel was used as pellicle formation surface, which shows considerable structural similarities compared to that of human enamel, and is thereby a highly suitable substitute that can be applied for in-situ experiments [23, 31, 32]. To ensure that enough sample material was available for the subsequent analysis, pellicle samples formed on 12 enamel slabs (six buccal, six palatal) per subject and time period were pooled. However, it has to be noted that the pellicle thickness might differ, depending on the intraoral location [17].

This in-situ approach combined with comprehensive sample preparation and sensitive GC-MS analysis allowed for a reliable evaluation of the FA profile of the in-situ pellicle. The whole analytical procedure, including sample preparation and the GC-MS method, was specially developed and validated for this purpose [28]. Although the

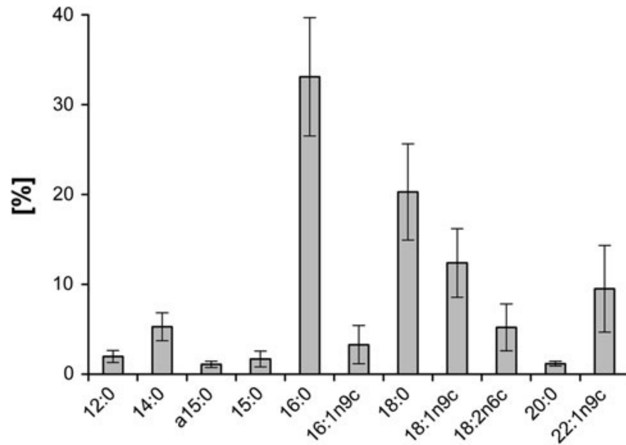



**Fig. 2** FA composition of the pellicle (in-situ formation time 120 min). Values represent the means ± SD of ten subjects expressed as a percentage of the investigated FA. This profile seems to be characteristic for the biological composition of the pellicle

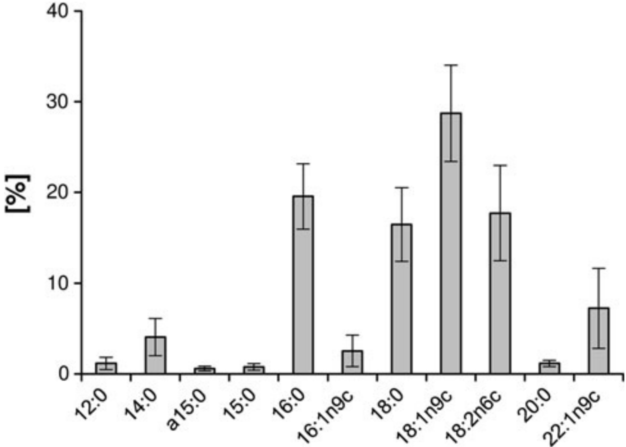



**Fig. 3** FA composition of saliva. Each value represents the mean ± SD of ten subjects expressed as percentage of the investigated FA

focus of this study lies on the FA analysis of lipids in pellicle samples, lipids in saliva samples can also be analyzed using the described methods.

Fatty Acid Profile

The ability to profile the lipid composition of biological samples has become an important element in human diagnostics and is, among others, an important part of the research field of lipidomics [33, 34]. The term "lipid" comprises a diverse range of compounds varying in characteristics, structure, and functionality. FA are one of the defining constituents of lipid structures and are in large part responsible for their distinctive physical and metabolic properties. The present study evaluated the fatty acid profile of the in-situ pellicle with contemporary analytical techniques. Comprehensive knowledge of the pellicle's FA profile can contribute important information on the selectivity and dynamics of biofilm formation and gives further insight into the role of lipophilic substances on bacterial adhesion.

Compared to a study carried out by Slomiany et al. [11], which represents the only reference on the FA composition of the pellicle, a broader range of FA was observed in the present study. In particular, more minor FA were detected, reflecting the high sensitivity of our method. The major FA found were identical in both studies. Additionally, we detected FA 15:0 and a15:0, which infers the presence of bacterial components in the pellicle layer. Presumed bacterial biomarkers correspond well with results of recent studies on bacterial colonization on enamel in situ [35, 36].

The FA profiles of the pellicle samples of all ten study subjects were very similar (Fig. 2). As compared to other pellicle parameters, the natural variability is rather low [37]. The FA profile of the pellicle seems to be characteristic for this biological structure, whereas the total amount of FA showed distinctive interindividual differences. No significant female-male differences were observed. However, based on the number of subjects participating in this study, no precise statement can be made concerning a gender-specific impact.

Additionally, the influence of pellicle formation time on the FA composition and amount was investigated. A steady accumulation of FA was observed after 60 min of oral exposure (Table 1), which can be mainly explained by an increase of the total amount of adsorbed pellicle components associated with the increase in pellicle thickness in general. This conclusion is in accordance with the results of a study that demonstrated a distinct augmentation of the pellicle layer between 60 min and 2 h [17]. Despite the increase in the total FA amount, the FA profile was remarkably constant during the examined pellicle formation periods (Table 1), reinforcing the suggestion that the increasing amounts of FA over time correlate with the growth of pellicle material.

Pellicle formation is largely determined by adsorption of salivary components from the oral environment [7, 9, 38, 39]. Therefore the comparison of the FA profiles of saliva and pellicle is of great interest. The major salivary FA identified in the present study are comparable to those detected in recent studies on the lipid composition of saliva [40, 41]. Furthermore, the FA profile of saliva showed noticeable differences compared to that of the pellicle, highlighting that FA available in saliva are not adsorbed equivalently to the pellicle layer. This finding infers that the formation of the pellicle is a highly selective process, as shown earlier for protein fractions found in the pellicle layer [42].

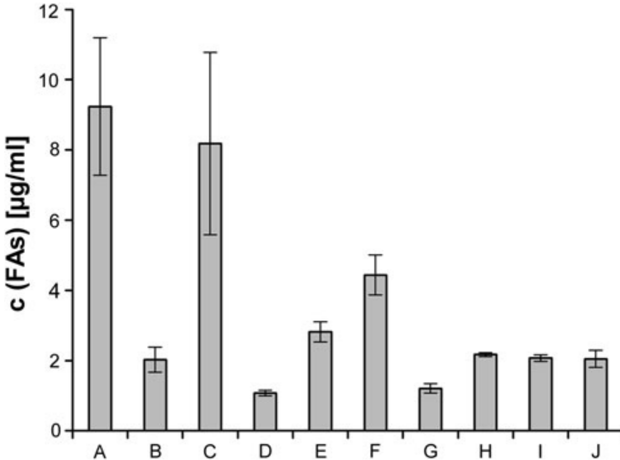



**Fig. 4** Amount of total investigated FA in saliva given for the ten study subjects (A–J). The fatty acid content differs considerably. Each value represents the mean ± SD ($n = 3$)

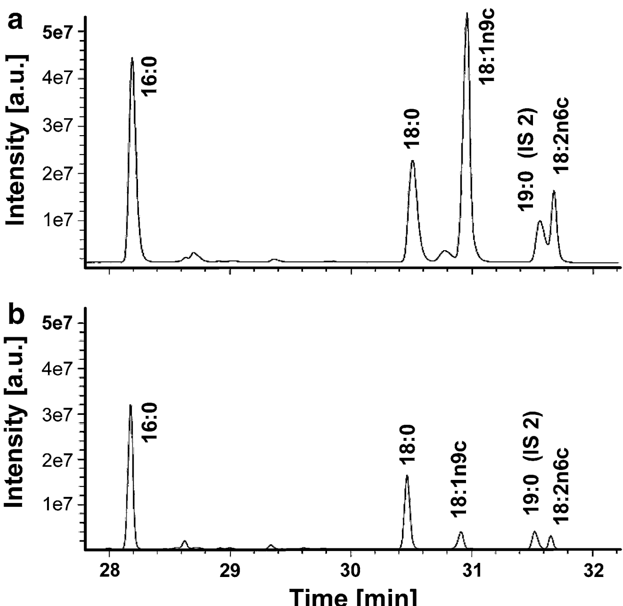



**Fig. 5** Enlarged sections of GC-MS chromatograms (SIM) of pellicle samples analyzed after a total oral exposure time of 120 min with (**a**) and without (**b**) using safflower oil (high oleic) as initial mouthrinse (8 ml; 10 min)

## Effects of Rinses with Safflower Oil

Rinses with edible oils or oil pulling derive from traditional folk medicine. Benefits of this treatment for oral hygiene, especially the prophylaxis of periodontitis and gingivitis, have been reported and discussed in the literature [12, 43–46]. However, the physicochemical background of the described effects has mainly been explained theoretically and not yet investigated adequately. It is presumed that hydrophobization of the oral surfaces might be an explanation for the protective effects since hydrophobic interactions have been shown to be of importance for the adherence of microorganisms on oral surfaces [13–15]. A limited number of studies indicate that the application of edible oils increases the hydrophobicity of oral biofilms [19, 21]. The present study showed that rinses with edible oils (as shown for safflower oil) can change the FA composition of the pellicle, and thus represents one possible way to modify the hydrophobicity and therewith the functional properties of the pellicle layer. Successful accumulation of FA (FA containing lipids) was observed after rinses with safflower oil (Fig. 5). This is in good accordance with previous transmission electron microscopic (TEM) evaluations of pellicle samples after rinses with various edible oils [12, 29]. Apparently, these TEM images indicated that the lipids are not completely integrated in the microstructure of the pellicle. Lipid micelles adhering to the pellicle were observed directly after the rinses [21, 29]. Furthermore, a lower density of the pellicle's ultrastructure was noticed 120 min after rinses with edible oils [29]. This appears to be in good agreement with recent findings that rinses with edible oils impair the protective properties of the pellicle [21]. These findings show that the combination of the presented analytical procedure with TEM methods is necessary to obtain conclusive results from studies on the modification of the pellicle layer.

Rinses with edible oils containing characteristic FA that are not part of the physiological pellicle, such as linseed oil (18:3 as major FA), can help us to understand better the biodynamics and turnover of pellicle formation.

## Future Studies

The pilot experiments herein reported, indicate that our method is suitable for evaluating the impact of different dentifrices and mouthrinses on the FA and lipid composition of the pellicle. This methodology might be helpful to understand the possible impact of other agents on hydrophobic interactions and bacterial adhesion. Furthermore, the kinetics of pellicle formation could be monitored. Studies that investigate the correlation between the lipid composition of the pellicle and caries or dental erosion can follow. Knowing the pellicle's FA profile is valuable, but in order to investigate the whole lipid composition, further research based on the presented results is necessary. For example, screening of individual lipid classes and their specific FA profiles after lipid class separation can give additional, more detailed information about the composition of the pellicle.

## Conclusion

FA are a lipophilic key component of the in-situ pellicle and show a characteristic profile in healthy adults. The highly selective formation of the pellicle layer is only one example for the ubiquitous process of bioadhesion. Understanding conditioning biofilms remains a great challenge in life sciences. The described methods are helpful when characterizing FA in lipids in any adherent biofilm.

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# PAPER 3

Lipids in Preventive Dentistry

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REVIEW

# Lipids in preventive dentistry

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**Abstract**

*Objectives* There is still a great demand for the improvement of oral prophylaxis methods. One repeatedly described approach is rinsing with edible oils. The aim of the present review paper was to analyze the role of lipids in bioadhesion and preventive dentistry.

*Materials and methods* Despite limited sound scientific data, extensive literature search was performed to illustrate possible effects of lipids in the oral cavity.

*Results* It is to be assumed that lipophilic components modulate the process of bioadhesion to the oral hard tissues as well as the composition and ultrastructure of the initial oral biofilm or the pellicle, respectively. Thereby, lipids could add hydrophobic characteristics to the tooth surface hampering bacterial colonization and eventually decreasing caries susceptibility. Also, a lipid-enriched pellicle might be more resistant in case of acid exposure and could therefore reduce the erosive mineral loss. Furthermore, anti-inflammatory effects on the oral soft tissues were described. However, there is only limited evidence for these beneficial impacts. Neither the lipid composition of saliva and pellicle nor the interactions of lipids with the initial oral biofilm and the pellicle layer have been investigated adequately until now.

*Conclusion* Edible oils might qualify as mild supplements to conventional strategies for the prevention of caries, erosion, and periodontal diseases but further research is necessary.

*Clinical relevance* Against the background of current scientific and empirical knowledge, edible oils might be used as oral hygiene supplements but a decisive benefit for the oral health status is questionable.

**Keywords** Lipids · Oral cavity · Pellicle · Bioadhesion · Erosion

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## Introduction

Over the past decades, the prevention of oral diseases has become an essential component of dentistry and dental research [1]. But despite the great improvements in the field of prophylaxis, caries and periodontitis remain two challenging diseases [2, 3].

Recent publications call attention to a global increase in dental caries prevalence, affecting children as well as adults worldwide [4]. Even though many countries exhibit a promising decrease of decayed, missing, filled teeth values throughout the last years [5, 6], further analyses reveal a skewed distribution of caries prevalence occurring due to shifts in populations [2, 7, 8] whereby the status of oral health seems to be affected by the socioeconomic situation [6]. This, in turn, implies the urgent need for reasonable and worldwide accessible preventive methods.

But not only caries can lead to loss of tooth tissue. Dental erosion, induced by the presence of intrinsic and extrinsic acids originating from the environment, the diet, eating disorders, and others, is a severe problem of nonbacterial origin, widely spread throughout the population [9–12]. And there are more challenges to be faced in the future.

As a result of advanced medical knowledge and refined therapies, life expectancy increases, causing a demographic change. According to the Federal Statistical Office, the percentage of people in Germany over 65 will raise to 23 % in 2020 and 33 % in 2050 [13, 14]. Elderly people can be physically restricted, which affects their mechanical oral hygiene and additional systemic diseases could aggravate the risk of developing oral health complications [15]. Additionally, the *Periodontal Country Profiles* by the World Health Organization present further information regarding the occurrence of periodontitis in different age cohorts worldwide. Taking the middle aged and old people in Germany, there are around 50 % with probing depths over 4 mm and 21 % or else 41 % with pocket depths of 6 mm and more [3]. Alteration of oral tissue following severe operations, chemotherapy, and radiotherapy often evokes special requirements. Induced by hyposalivation, the mucosa can be very dry and inflamed [16], the healing of wounds is restricted and there is a high risk of developing radiation caries [17]. Several mouthwashes containing antiseptic or analgesic agents have been developed; however, they are often slightly efficient and the acceptance of conventional chemical solutions is low as they are associated with mouth burn, bad taste, a tainting effect, and dental stains [18–20]. Therefore, the establishment of biological preventive solutions as adjuvant methods could be a sensible attempt to reduce the incidence of oral diseases.

Considering the impact of microbial interaction and physicochemical dynamics onto the health of hard and soft oral tissues, it would be desirable to either strengthen the tissue against extrinsic degradation or modulate the process of bioadhesion. With that said, the characteristics of lipids appear promising as they could influence the microbial interaction, modulated oral surfaces might impede bacterial adherence [21] and a hydrophobic layer could protect against tooth demineralization or dry mouth [5]. Therefore, the aim of the present review was to investigate the value of lipid-containing mouthwashes and edible oils in the context of bioadhesion and preventive strategies.

## Bioadhesion on the dental hard tissues

### Pellicle formation

In the oral cavity, bacterial adhesion evolves on the basis of a proteinaceous layer, the acquired pellicle [22, 23], which is the first step of bioadhesion on solid surfaces exposed to the oral fluids (Figs. 1 and 2).

As a tenaciously absorbed bacteria-free coating of the tooth surface, the pellicle is the result of a highly selective adsorption of proteins, glycoproteins, lipids, and other macromolecules from the oral fluids (Figs. 1, 2, and 3) [24]. The initial formation process is determined by ionic interactions between the enamel surface and certain salivary proteins like statherin, histatin, and proline-rich proteins as well as by thermodynamically driven forces such as van der Waals forces and hydrophobic interactions [5, 22]. Subsequently, the composition becomes more complex by the adsorption of heterotypic structures and protein aggregates [24]. The pellicle serves as a protective lubricant and a diffusion barrier for the tooth, a nonshedding surface [21, 24]. Thereby, the electron-dense basal pellicle layer is especially resistant against acid attack and its semipermeable properties allow ion exchange of calcium and phosphate at the tooth surface (Figs. 2 and 3) [25].

### Bacterial colonization

Nevertheless, components of the pellicle also serve as bacterial receptors and promote microbial biofilm formation [22, 24]. Microorganisms are transported passively to the tooth surface by the saliva flow where an initially nonspecific reversible attraction provoked by physicochemical forces occurs [26]. Several studies have proven the relevance of hydrophobic interactions and cell hydrophobicity for bacterial adherence. The experimental extraction of lipids from pellicles in vitro resulted in an increase of *Streptococcus mutans* numbers [27] and a reduction of biofilm formation was observed on hydrophobic surfaces such as dental materials in vivo [28, 29]. Bacterial attachment becomes irreversible if bacterial adhesins such as lipoteichoic acid or lectins interact with receptors of the pellicle components [22, 24, 30]. Several pellicle molecules, including some lipid components [27], are considered as bacterial binding sites. In consequence, systematic bacterial adhesion occurs, which eventually results in the development of an exceedingly organized solid biofilm [5, 22, 31].

## Conventional biofilm management

The regular and consequent removal of oral plaque, as well as the retardation of biofilm formation are the decisive targets of dental prevention methods. A significant reduction of caries incidence can be achieved by fluoride application, especially since there are no noticeable side-effects if used properly [32]. However, the persistent need for caries treatment confirms that the protective effects of fluorides nonetheless are limited and require optimization. For example, the oral sustainability of fluorides is restricted and

**Fig. 1** Conceivable mechanisms of pellicle modification by lipid components and their impact on bacterial adhesion. **a** Initial and mature pellicle under influence of lipid constituents. TEM confirmed the attachment of lipid micelles to the pellicle surface directly after rinses with vegetable oil (consult Fig. 3). The sustainable integration of these substances could hydrophobize the pellicle and therewith prevent acidic ion diffusion and enamel demineralization. Nevertheless, there is some evidence that externally added lipid constituents might also facilitate pellicle degradation by detracting relevant protective lipids from the pellicle. **b** Bacterial adhesion. Hydrophobic interactions partially influence bacterial adhesion. A hydrophobic pellicle surface might hamper irreversible bacterial attachment. In contrast, lipids incorporated into the pellicle structure could also serve as bacterial nutrients and binding sites. Yet, bacterial affinity to hydrophobic substances could be advantageous if lipid application can remove biofilm bacteria. Further research will be required to verify the existing hypotheses

although they reduce bacterial viability, an inhibiting effect on bacterial adhesion is less clear [32, 33]. Additionally, chlorhexidine is a common active substance for chemical plaque control but electron microscopic studies have shown a limited efficacy of chlorhexidine against adherent bacterial biofilms [34]. Moreover, the side effects like discolorations, a tainting effect, and a shift of the common oral flora do not qualify it as a daily supplement of oral hygiene [35, 36].

Modern concepts try to protract biofilm formation at the start. This includes a wide variety of different approaches like nanomaterials, immunization, or bacterial replacement therapy [37–41]. However, additional research clarifying the effectiveness of those expensive methods will be necessary and a negative impact on the ecology of the oral cavity cannot fully be eliminated. Simultaneously natural, biological products come more and more into focus. They are often easily obtainable worldwide, generally biocompatible, and undesirable side effects are not to be expected. Several foods, such as polyphenolic beverages, are recognized for their wholesome effect [42]. Polyphenols are regarded as strong antioxidants with potential health benefits [43–45] and they exhibit a wide range of antibacterial, antifungal, and anti-inflammatory effects which also qualify them for the prevention of caries and periodontitis [46]. Another promising approach in dental prevention methods can be to reinforce the protective pellicle properties by modifying its composition. In this context, the enrichment of lipids in the pellicle has come into focus as they might help prevent erosion, have antibacterial effects, and be efficient in the modification of pellicle's physicochemical properties to delay bacterial adhesion [47–50].

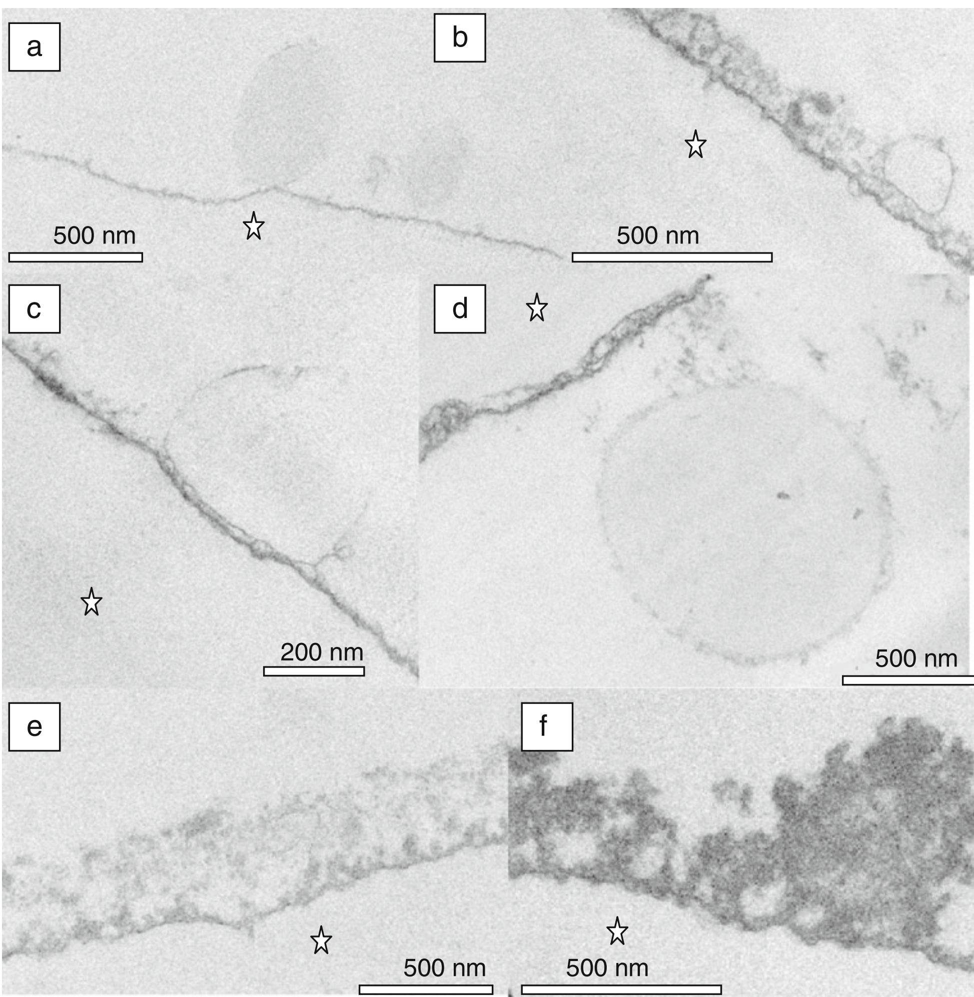



**Fig. 2** Transmission electron microscopy: effect of rinses with safflower oil on the initial pellicle layer. After formation of a 3-min pellicle in situ, the subject rinsed with the edible oils for 10 min. Directly after the rinse, oil micelles and droplets were visible, either attached to the pellicle (**a**–**c**) or near the pellicle surface (**d**). Another 110 min later, the 120 min pellicle (**e**) was of lower density than the respective control (no rinse, **f**). Please note that the enamel was removed during preparation of the samples; the former enamel side is marked with an *asterisk*. The interaction of the lipids with the pellicle requires further research. Original magnification, 30,000-fold. Please compare with Fig. 1

## Lipids and hydrophobic fluids and their effects on bioadhesion

Lipids play a vital role in all organisms, not only regarding the storage of energy (e.g., neutral lipids), or as structural elements of cell membranes (e.g., phospholipids). They are also heavily involved in signal transduction processes (e.g., isoprenoides/steroids). Thus, the term “lipid” comprises a diverse range of compounds, varying in characteristics, structure, and functionality. For this reason, there is no widely accepted definition of what is considered a lipid. Traditionally, lipids are described as nonpolar compounds insoluble in water but readily soluble in organic solvents such as alcohols, ethers, hydrocarbons, and chloroform. However, a definition of this kind excludes many substances that are widely regarded as lipids and are as soluble in water as in organic solvents. Therefore, Christie [51] introduced another definition, which describes lipids as fatty acids and their derivatives, and compounds biosynthetically or functionally related to them. In the last decades, many attempts regarding a comprehensive classification system for lipids were made [52–55]. Figure 3 gives a brief overview of different lipid classes. Due to their amphiphilic and hydrophobic properties, most of these substances are of potential interest for the purpose of biofilm management from a theoretical point of view.

When solid substrates are exposed to some aqueous solution, biofilm formation naturally occurs. It is an ubiquitous phenomenon whose delaying modification challenges medicine as well as industrial technologies [28, 56, 57]. Ionic, electrostatic, and hydrophobic interactions are important determinants for the adherence of microorganisms to a variety of surfaces and materials [58, 59]. It could be postulated that making a solid surface more hydrophobic might decrease its wettability and hamper biofilm accumulation in a moist environment [22]. A slight reduction of protein adsorption and bacterial adhesion was indeed observed on glass surfaces that had been hydrophobized in vitro by plasma polymerization of a low molecular weight siloxane [60, 61]. Furthermore, less biofilm formation was also observed in vivo on hydrophobic surfaces of voice prostheses in comparison to hydrophilized ones [62]. Similarities apply to the oral cavity as comparable findings were generated by an in vivo experiment that compared plaque formation on

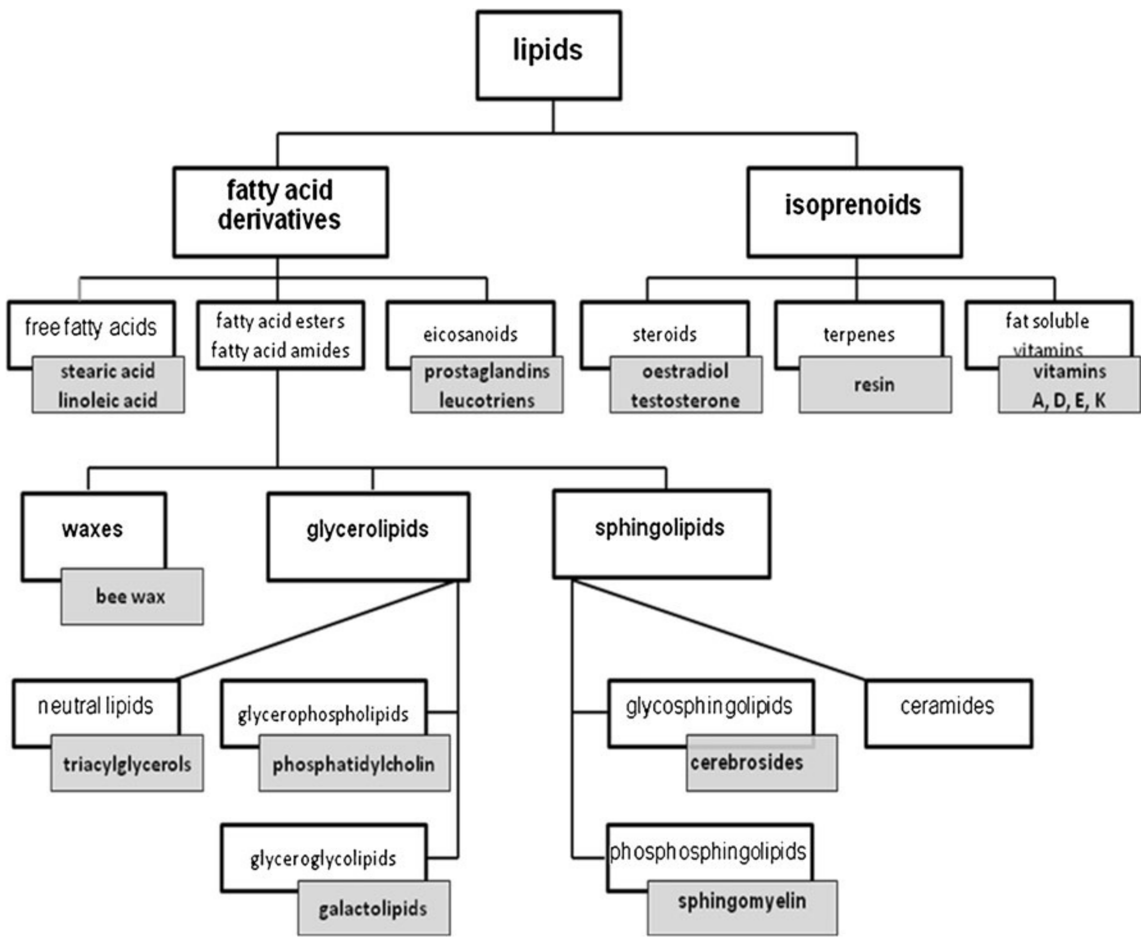



**Fig. 3** Overview of various lipid classes with examples. Figure summarizes data presented in textbooks by Lottspeich [158] and Stryer [159]

enamel and intraorally worn material strips with different surface free energy levels such as parafilm and fluorethylenepropylene (Teflon) [63]. The results of the study showed remarkably less plaque formation on surfaces with low surface-free energy which the authors explained by a decreased binding force between bacteria and substrata of low surface-free energy. According to investigations of biofilm formation on different dental restorative and implant materials, considerably less bacteria adhere to supragingival hydrophobic surfaces in the oral cavity than to hydrophilic ones [28]. The same was observed on polysiloxane pretreated crowns in vivo [60, 61].

Nevertheless, the few studies investigating the relevance of hydrophobic interactions on oral bioadhesion obtained divergent results regarding the effect of hydrophobic surfaces on bacterial colonization, as there is indications that they either reduce or enable bacterial adhesion [22, 28, 48, 64, 65].

Furthermore, the different investigations underline the distinct impact of the oral environment including saliva and in vivo pellicle formation [22, 66]. According to a study published by van der Mei et al., intraorally formed pellicles naturally tend to be more hydrophobic than salivary pellicles formed in vitro [66]. Although no analysis of the pellicles' specific lipid content was carried out, it was shown by the authors that the application of dietary lipids from salad oil in vitro increases the hydrophobicity of intraoral conditioning films. Due to these considerations, not only permanent hydrophobization of dental materials but also the application of mouth rinses for the purpose of transient surface hydrophobization might be of interest. Yet, several hypotheses concerning the effects of those expectable modifications so far remain unresolved (Fig. 1). A less recent publication by Rykke and Rölla described a notably retarded pellicle formation on enamel specimens in situ after extraoral silicon oil pretreatment [64]. Due to hydrophobization, less protein seemed to adhere to the hydroxyapatite surface and a different amino acid composition was determined for the resulting basal pellicle. The modulated pellicle structure as well as specific lipophilic components of the pellicle could either hamper but possibly also facilitate the attachment of certain microorganisms due to enforced hydrophobic interactions (Fig. 1) [28, 48, 67]. Latest information gained by transmission electron microscopy is illustrated in Fig. 1 and indicates modified, less electron-dense pellicle

structures after rinses with vegetable oils, compared with controls [48]. The effects of lipid application on the biochemical compositions of the pellicle as well as the resulting impact on bioadhesion have not been clarified entirely until now. A loosened ultrastructure could evolve optimized or weakened protective properties. Yet, the said study determined no significant impact of rinses with three different vegetable oils on the bacterial colonization of enamel over 8 h [48]. As well as having an impact on pellicle formation and its susceptibility against acids and bacterial adhesion, it is also conceivable that applied lipids extract relevant protective lipophilic components from the pellicle and provide bacterial receptors or even substrates for microorganisms. Among others, bacterial adherence depends on the attraction resulting of hydrophobic interactions [22, 68]. Cell surface hydrophobicity is derived by the bacterial membrane composition, however, distributing bacteria in aqueous liquids reduces their affinity to hydrophobic surfaces considerably and influences microbial surface thermodynamics [27, 69]. It is noteworthy that intraoral rinses with silicon oil led to the embedding of oil vesicles full of viable bacteria into the biofilm as shown in Fig. 4. This indicates that bacteria could indeed prefer a hydrophobic environment. Last but not least, publications suggest the effectiveness of specific lipids as antimicrobial agents [47, 70, 71]. For example, oleic acid and linoleic acid were tested positive on the growth inhibition of either *group-A-streptococcus beta-hemolytic-non-A-streptococcus* or *Candida* in vitro [72]. This has to be differentiated from the adoption of triglycerides. Yet, there are also indications that bacterial enzyme activities might be increased by pellicle lipids [67].

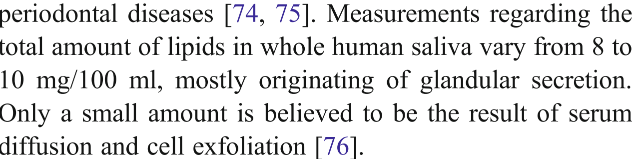
Within the context of the observations described above, lipids deserve to be investigated thoroughly for their potential role as antiplaque agents in vivo, considering the physiology of lipid in the oral environment.

## Physiology of lipids in the oral cavity

### Lipids in human saliva

Saliva is an important protective system of the oral cavity, where it serves as a lubricant and buffer; several of its components were proved to have an anti-inflammatory or antimicrobial effect and it contains a diversity of inorganic substances important for the protection and repair of hard oral tissue [73].

Despite the extensive work done on some salivary constituents, only a few attempts have been made to examine the significance and quantification of its lipid components and data predominantly refer to the scientists around Slomiany. Their findings revealed essential aspects about the correlation between the individual lipid constitution of saliva and pellicle and the susceptibility to caries and



periodontal diseases [74, 75]. Measurements regarding the total amount of lipids in whole human saliva vary from 8 to 10 mg/100 ml, mostly originating of glandular secretion. Only a small amount is believed to be the result of serum diffusion and cell exfoliation [76].

Studies based on saliva secreted by the major salivary glands identified 70–95 % of all lipids as nonpolar molecules varying from free fatty acids to cholesterol, cholesteryl esters, and mono-, di-, and triacylglycerols [77]. These neutral components are complemented by polar lipids such as glycolipids and 2–5 % phospholipids [17, 76]. Glycolipids mainly consist of glyceroglucolipids and even though they only account for 20–30 % of all salivary lipids, they appear to participate in several protective functions of human saliva [17, 78]. A summary of the major lipids contained in parotid and

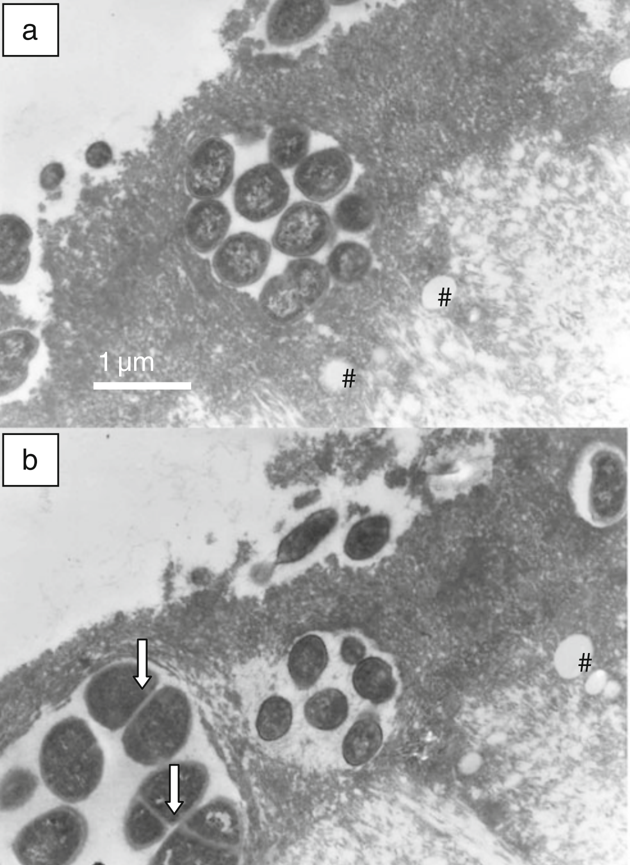



**Fig. 4** Biofilm formation in situ over 6 h on pre-etched enamel after initial application of silicone oil. Silicone oil was used as a model for a hydrophobic and inert substance. The lacunae of the etched surface were filled with protein structures, covered by a typical mature pellicle layer. Please note the included oil droplets surrounded by ordinary pellicle structures (*number sign*). The general ultrastructure of the pellicle was not altered. Interestingly, groups of bacteria were detected in silicone oil drops surrounded by pellicle structures. It seems as if the bacteria preferred the hydrophobic milieu; inside these lipophilic drops, the bacteria are intact and viable, mitosis can be seen in some cases (*arrows*). The residual enamel structures were removed during preparation of the sample. Original magnification, 10,000-fold

submandibular saliva is given in Tables 1 and 2. Labial saliva contains four to five times more lipids per milliliter of saliva and exhibits significantly higher percentages of phospholipids and glycolipids than saliva secreted from parotid and submandibular glands [17, 79]. This might serve as a lubricant protecting the lips from dehydration.

The dynamic interaction between lipids, salivary proteins, and glycoproteins is regarded as determinant for many physical, chemical, and functional properties of saliva [17]. It was confirmed in several studies carried out by Slomiany that elevated levels of lipids in saliva are often associated with a higher incidence of caries and periodontal diseases [17, 74, 75, 80]. These observations might be derived by a facilitated bacterial adherence involving hydrophobic bonds or high levels of lysophosphatidylcholine might enhance glucosyltransferase activity promoting glucan formation and bacterial adhesion [67]. Glucoglycerolipids as well as phospholipids interact with proteins, particularly low and high molecular weight glycoproteins, either by hydrophobic forces or covalent linkage [17]. The function of those lipid–protein complexes is not fully clarified yet, but they seem to influence the viscoelastic and hydrophobic properties of the secretions. Additionally, salivary mucins have an important impact on the pellicle formation and epithelial integration of mucous tissue, interact with inorganic ions but might also serve as bacterial receptors [16, 81–83]. Compositional differences in lipid distribution referring to caries activity were observed in different studies and are summarized in Table 1.

The saliva of caries-resistant individuals contains smaller amounts of total lipids with a significantly lower percentage of neutral lipids and phospholipids whereas only minor differences occurred in the content of glyceroglucolipids. Although caries susceptibility does not directly refer to a certain group of associated fatty acids, Table 2 reveals a tendentially frequent appearance of elevated levels of stearic acid and docosanoic acid in salivary lipids of caries-susceptible individuals [74].

Interestingly, saliva of caries-susceptible individuals exhibits more lipids associated with mucous glycoproteins and although the mucins of both groups show similar protein and carbohydrate content, several differences were detected for their lipid distribution. Neutral lipids derived from mucus glycoproteins of saliva of caries-resistant individuals contain about 50 % more cholesterol, 38 % more cholestryl ester, and 32 % less triacylglycerols than the associated neutral lipids of salivary mucous glycoproteins of caries-susceptible individuals [17]. The phospholipids associated with glycoproteins of caries-resistant individuals have a higher content of phosphatidylethanolamine while those associated with mucus glycoproteins of caries-susceptible individuals are rich in sphingomyelin and phosphatidylcholine [17]. And also, the glycoproteins of caries-susceptible individuals contain significantly more bound fatty acids [84, 85] which make them less prone to peptic degradation and exhibit a lower buoyant density and more viscosity than those of caries-resistant individuals [16, 17]. Besides, it was reported that the saliva of heavy calculus formers contained about 50 % more lipids, larger amounts of free fatty acids in general, more cholesterol esters and glyceroglucolipids, whereas light calculus formers exhibit higher levels of triglycerides and free cholesterol [75].

In summary, it can be stated that elevated levels of specific lipids in saliva are associated with a higher incidence of caries and periodontal disease. As emphasized earlier, bacterial adherence is, among others, initiated by hydrophobic interactions [24], which might be facilitated by a lipid-rich environment (Fig. 1).

## Lipids in the pellicle

There are considerable distinctions between the lipid composition of saliva and the pellicle [74, 86]. First of all, the amount of lipids incorporated in the pellicle is significantly higher. Although they account for 22–23 % of the pellicle's

**Table 1** Average content and standard deviation of proteins and lipids in the parotid and submandibular saliva of caries-resistant (CR) and caries-susceptible (CS) individuals based on data provided by Slomiany et al. [160]

| Constituent (mg/100 ml of saliva) | Parotid | | Submandibular | |
|---|---|---|---|---|
| | CR | CS | CR | CS |
| Proteins | 180.36±60.20 | 221.30±78.92 | 102.92±49.32 | 132.36±44.35 |
| Total lipids | 4.81±0.28 | 7.63±0.57 | 5.20±0.37 | 8.01±0.32 |
| Neutral lipids | 2.89±0.34 | 5.35±0.58 | 3.23±0.40 | 5.64±0.52 |
| Free fatty acids | 1.32±0.22 | 2.33±0.41 | 1.39±0.11 | 2.34±0.30 |
| Mono- and diglycerides | 0.09±0.02 | 0.11±0.03 | 0.12±0.03 | 0.19±0.04 |
| Tricglycerides | 0.58±0.11 | 0.98±0.14 | 0.60±0.15 | 1.34±0.19 |
| Cholesterol | 0.44±0.07 | 0.51±0.15 | 0.50±0.10 | 0.51±0.14 |
| Cholesterol esters | 0.46±0.08 | 1.42±0.35 | 0.62±0.13 | 1.26±0.37 |
| Glycolipids | 1.27±0.08 | 1.21±0.13 | 1.46±0.23 | 1.56±0.29 |
| Phospholipids | 0.09±0.02 | 0.12±0.03 | 0.10±0.02 | 0.15±0.03 |

**Table 2** Fatty acid composition of parotid saliva lipids from caries-resistant (CR) and caries-susceptible (CS) subjects as measured by Slomiany et al. [160]

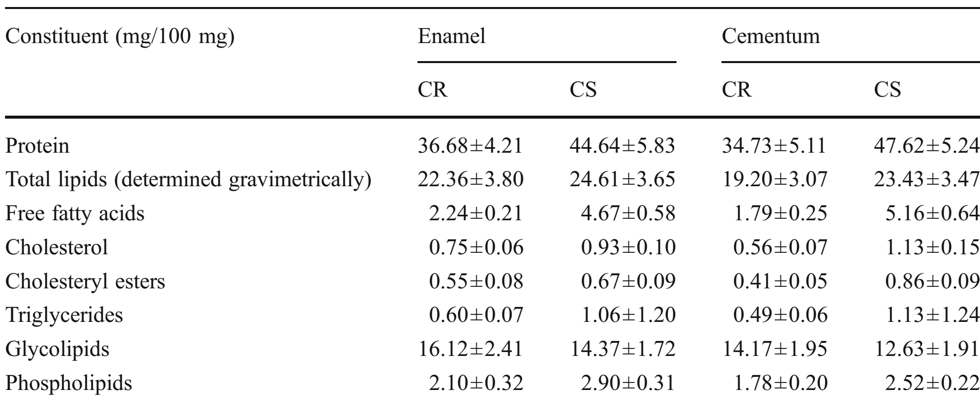
| Fatty acid | Neutral lipids | | Glycolipids | | Phospholipids | |
|---|---|---|---|---|---|---|
| | CR | CS | CR | CS | CR | CS |
| Percent of total lipids | | | | | | |
| 16:0 | 23.1 | 12.0 | 9.8 | 14.9 | 29.1 | 13.8 |
| 16:1 | 3.3 | 3.8 | 1.2 | 3.3 | 2.5 | 2.3 |
| 18:0 | 26.9 | 27.2 | 20.5 | 36.5 | 23.3 | 32.3 |
| 18:1 | 23.2 | 9.9 | 15.9 | 14.9 | 19.3 | 16.8 |
| 18:0α-OH | 2.7 | 2.7 | 2.2 | 2.6 | 1.3 | 2.8 |
| 20:0 | 1.9 | – | 9.2 | 11.4 | 5.5 | 5.7 |
| 20:1 | – | – | 3.8 | – | – | – |
| 22:0 | 8.0 | 36.8 | 4.9 | 11.1 | 10.7 | 16.4 |
| 24:0 | 4.1 | 2.4 | – | – | – | 4.1 |
| 24:1 | 3.6 | 2.7 | 4.7 | 3.0 | 4.8 | 4.0 |
| 24:0α-OH | 1.7 | – | – | – | – | – |
| 26:0 | – | – | 10.9 | – | – | – |
| 26:1 | – | – | 12.3 | – | – | – |
| Unidentified | 1.5 | 2.5 | 4.6 | 2.3 | 3.5 | 1.8 |

dry weight little is known about their individual distribution and precise function [24]. The presence of phospholipids is suggested to have an important impact on the tenacity of the pellicle [87]. Therefore, compositional differences and the extent of interactions with proteins and glycoproteins of the pellicle could modulate bacterial adhesion [88]. Table 3 summarizes the distribution of different pellicle lipids. As shown, glycolipids are the major lipid fraction of the pellicle and might be a significant source of pellicle glucose [16, 88]. Very isolated studies indicate that inter-individual differences in the lipid composition of the pellicle might reflect differences in the caries activity of the individuals [87, 88]. In comparison to caries-susceptible subjects, the pellicle of caries-resistant subjects contained 42 % less neutral lipids and 31 % less phospholipids but had a higher proportion of cholesterol, cholesterol esters, and sphingomyelin, which might have a caries-protective effect [87]. Once more, advances concerning the value of pellicle lipids for the protection of the tooth tissue refer to the working group around Slomiany. They conducted an in vitro study which proved a considerable reduction of lactic acid retardation after extracting lipids out of the experimental pellicles of caries-resistant and caries-susceptible individuals by chloroform methanol followed by thin layer chromatography with hexane-diethyl ether acid [87]. In conclusion, lipid removal caused 50–52 % less lactic acid retardation capacity for the pellicles of caries-resistant subjects and 30–32 % less for the caries-susceptible ones [87]. Accordingly, a generally protective effect of the hydrophobic pellicle constituents against acidic noxae could be hypothesized. Yet, first experiments suggest that although rinses with safflower oil modulate the ultrastructural pellicle composition by a loose attachment of lipid micelles, they have a rather negative effect on the susceptibility of the pellicle against acids [49]. Electronmicroscopic investigations showed that the modified pellicle is of lower density than in controls and exposing these pellicle samples to hydrochloric acid with low pH values led to a remarkable pellicle degradation. But since all indications regarding the protective function of lipids in human saliva as well as in the formation of the acquired pellicle only refer to a few studies, additional research based on modern analytical techniques is required. This applies for the physiological lipid composition of the pellicle as well as for the impact of edible oils on the initial oral biofilm.

## Methods for quantification of lipids in the saliva and in the oral biofilm

Analytical methods for determination of lipids are as various as the lipid classes themselves. Besides recently published books dealing with this topic [51, 89], there are also several journal reviews available [90–94]. Therefore, only a rough survey of the most important techniques for lipid analysis is given in Table 4 providing widely accepted references.

**Table 3** Average lipid content and standard deviation of pellicles formed on enamel and cementum in vitro from saliva of caries-resistant (CR) and caries-susceptible (CS) subjects, based on data provided by Slomiany et al. [161]



| Constituent (mg/100 mg) | Enamel | | Cementum | |
|---|---|---|---|---|
| | CR | CS | CR | CS |
| Protein | 36.68±4.21 | 44.64±5.83 | 34.73±5.11 | 47.62±5.24 |
| Total lipids (determined gravimetrically) | 22.36±3.80 | 24.61±3.65 | 19.20±3.07 | 23.43±3.47 |
| Free fatty acids | 2.24±0.21 | 4.67±0.58 | 1.79±0.25 | 5.16±0.64 |
| Cholesterol | 0.75±0.06 | 0.93±0.10 | 0.56±0.07 | 1.13±0.15 |
| Cholesteryl esters | 0.55±0.08 | 0.67±0.09 | 0.41±0.05 | 0.86±0.09 |
| Triglycerides | 0.60±0.07 | 1.06±1.20 | 0.49±0.06 | 1.13±1.24 |
| Glycolipids | 16.12±2.41 | 14.37±1.72 | 14.17±1.95 | 12.63±1.91 |
| Phospholipids | 2.10±0.32 | 2.90±0.31 | 1.78±0.20 | 2.52±0.22 |

**Table 4** General survey of common, well-established analytical methods for lipid analysis. Journal reviews and books are listed as references

| Analytical Method | Principle | Analytes | Reference |
|---|---|---|---|
| Gas chromatography (GC; GC/MS) | Separation by partition between a solid/liquid stationary phase and a gaseous mobile phase; detection most commonly with a FID or coupled with MS | Volatile compounds, determination of fatty acids after derivatisation | [51, 162, 163] |
| High-performance liquid chromatography (HPLC) | Separation between a solid stationary phase and a liquid mobile phase under high pressure; various detection possibilities including MS, UV, RI | Depending on the broad range of lipid classes depending on the detector | [91] |
| Thin-layer chromatography (TLC) | Separation between a solid stationary phase and a liquid mobile phase; detection most commonly via staining | Quick and inexpensive separation, identification of individual lipid classes | [92] |
| Mass spectrometry (MS)<br>Soft ionization methods<br>MALDI and ESI | Mass spectrometry converts molecules to ions. Resulting ions and their characteristic fragments are separated by their mass to charge ratio (*m/z*); ESI often coupled with HPLC | Direct analyte detection; multiple lipid classes; screening of biological tissue | [93, 100, 164, 165] |
| Nuclear magnetic resonance (NMR) | Nondestructive spectroscopic technique that gives detailed structure information | Basically all lipid classes; location of the position of double bonds; isomeric lipid analysis | [94, 166] |

Independent of the specific analytical method, the first and crucial step of lipid analysis is the extraction of lipids from the matrix combined with cleanup by removing any nonlipid contaminants from the extract (e.g., proteins and polysaccharides). The importance of sample preparation is often underestimated and therefore carried out hurriedly and incorrectly [89]. It should be kept in mind that, in case of errors occurring during the extraction procedure, even the best analytics are worthless. A comprehensive summary of this sophisticated topic can be found in [95]. Several extraction procedures are found in scientific literature. The best described and most commonly used procedures were introduced by Folch et al. [96] and Bligh and Dyer [97]. As lipids are naturally occurring substances, there is a high risk of cross-contamination. Thus, various precautions concerning sample handling, solvents, and glassware have to be taken [89]. This is especially important for lipid analysis of pellicle samples, which represents a multistep procedure of minute quantities from specimen generation to the final chemical analytical determination.

Although salivary analysis has been considered important in terms of oral health for a long time [98], and the fact that there are notable indications that lipids play a significant role in this context, there is still a paucity of data on the nature and amounts of lipids in human saliva. Analytical methods predominantly used for this task are thin layer chromatography and gas chromatography coupled with flame ionization detector (GC-FID). Most of the data derive from studies carried out in the 1980s and refer almost exclusively to the workgroup around Slomiany [17, 75, 80, 86].

The analytical procedures used in all these salivary studies are basically the same. Samples are extracted with chloroform/methanol (2:1, *v* /*v*) and filtered to remove insoluble protein residue. The lipids dissolved in the extract are fractionated on silica columns into neutral lipids, phospholipids, and glycolipids, which are separated into their individual lipids by TLC, respectively. For identification, authentic standards were used. Fatty acid composition is determined by GC-FID after acidic methanolysis.

Regarding the nature, function, and composition of lipids in the acquired pellicle, the current state of research gives only preliminary information. Studies on pellicle composition are hampered by the fact that only limited amounts of pellicle material can be harvested and recovered from human teeth in vivo for analytical investigation [24]. In vitro pellicle samples on the other hand give only limited insight in the process of bioadhesion in the oral cavity. Accordingly, in situ approaches are most convenient. They are usually based on enamel slabs exposed to the oral fluids with aid of individual splints [48, 49]. Due to the high tenacity of the pellicle, extensive desorption procedures based on EDTA, sodium hypochloride, and ultrasonication are required to harvest the complete pellicle for purpose of analysis [99]. The existing knowledge of the lipids' protective properties for the pellicle is solely based on two publications [87, 88]. The methodology used for lipid determination is similar to that described above for salivary samples. Therefore, additional research with current analytical methods that are more specific and more sensitive such as gas chromatography coupled with mass spectrometry (GC/MS) are necessary to provide a more thorough understanding of the lipids involved in pellicle formation [100].

## Vegetable oils—a natural source of lipids

The following considerations regarding the value of lipids in preventive dentistry will deliberately be focused on vegetable oils, as they are a natural, biocompatible, inexpensive, and worldwide accessible source of these substances [101].

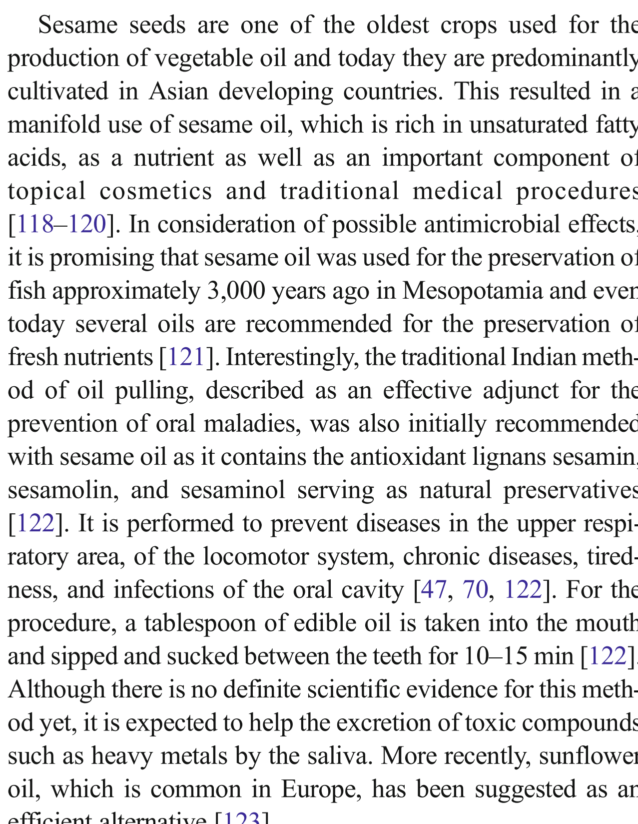
Although health-promoting effects were also described for marine oils or essential oils their ubiquitous application is naturally restricted by the elaborate extraction. Last but not least, there are also concerns about the medical use of animal fats questioning their biocompatibility and religious as well as cultural aspects.

Vegetable oils are extracts obtained from oil plants and seeds, which have been diversely used in many cultures for centuries. Not only foods, but cosmetics, medical products, and technologies cannot be imagined without their incorporation.

In general, vegetable oils are composed of triglycerides and their fatty acids consist of 8–24 carbon atoms with saturation levels determined by the amount of double bonds [101]. Valuable oil is rich in mono- and polyunsaturated fatty acids, which are considered to have a health-promoting effect [102, 103]. Further important components of vegetable oils are liposoluble vitamins, lecithin, phytosterols, minerals, and trace elements [104]. The stability of vegetable oils against oxidation processes not only depends on the amount of unsaturated fatty acids but is also determined by the presence of antioxidants like phenols, chelating agents, and oxygen quenchers [105]. Different types of vitamin E, tocopherol α, -β, -γ, -δ, can be distinguished, acting as radical quenchers, preventing oxidation and stabilizing cell membranes of the human body [106, 107]. Additionally, polyphenols as secondary plant products are believed to have anti-inflammatory and antimicrobial effects [108–110]. Despite the similar basic elements, there are specific differences concerning the chemical composition of various vegetable oils.

## Characteristics of typical vegetable oils and their relevance in preventive medicine

Over the last years, further knowledge has been gained about the impact of lipid components on human health. Especially the noticeable linkage between the dietary intake of certain lipids and coronary heart diseases as well as chronic degenerative diseases has repeatedly been examined and might offer potential prophylactic measures [111, 112]. There is decisive evidence that vegetable oils rich in unsaturated fatty acids have a health promoting effect: an enhanced consumption of unsaturated fatty acids can lower blood cholesterol levels and reduces the risk of atherosclerosis [113]. Omega-3 and omega-6 fatty acids are essential fatty acids. Besides their protective effect on the cardiovascular system, omega-3 fatty acids might also prevent dementia and macular degeneration [114–116]. Other publications discuss a possible protection against certain cancer types such as prostate, colorectal, or lung cancer, but relating epidemiological studies achieved inhomogeneous results [117].



Sesame seeds are one of the oldest crops used for the production of vegetable oil and today they are predominantly cultivated in Asian developing countries. This resulted in a manifold use of sesame oil, which is rich in unsaturated fatty acids, as a nutrient as well as an important component of topical cosmetics and traditional medical procedures [118–120]. In consideration of possible antimicrobial effects, it is promising that sesame oil was used for the preservation of fish approximately 3,000 years ago in Mesopotamia and even today several oils are recommended for the preservation of fresh nutrients [121]. Interestingly, the traditional Indian method of oil pulling, described as an effective adjunct for the prevention of oral maladies, was also initially recommended with sesame oil as it contains the antioxidant lignans sesamin, sesamolin, and sesaminol serving as natural preservatives [122]. It is performed to prevent diseases in the upper respiratory area, of the locomotor system, chronic diseases, tiredness, and infections of the oral cavity [47, 70, 122]. For the procedure, a tablespoon of edible oil is taken into the mouth and sipped and sucked between the teeth for 10–15 min [122]. Although there is no definite scientific evidence for this method yet, it is expected to help the excretion of toxic compounds such as heavy metals by the saliva. More recently, sunflower oil, which is common in Europe, has been suggested as an efficient alternative [123].

There is extensive literature referring to health-promoting benefits of olive oil as a dietary ingredient, commonly described as the Mediterranean diet [124, 125]. About 70 % of the olive oil's fatty acids are mono-unsaturated fatty acids, with oleic acid as the major compound [104, 126]. Additionally, olive oil contains a diversity of secondary plant products such as phenolic components; squalen; vitamins A, E, and K; and phytosterols [104, 110]. These substances are suggested to have an antioxidative, immunomodulatory, and antimicrobial effect. Therefore, olive oil is presumed to prevent coronary heart diseases, neural degeneration, cell damage, and even oral mal-odor [125, 127, 128].

Sunflower oil and rapeseed oil are very standard cooking oils whereas linseed oil is known to have a fishy taste and low stability against oxidation. Nevertheless, the particularly high amount of linolenic acid makes linseed oil valuable for the treatment of high serum cholesterol and triglycerides and hypertonia [129]. Furthermore, linseed oil was promoted to have an antibacterial effect on *Staphylococcus aureus* and dermatologic poultices seem to facilitate cell regeneration [130].

Safflower oil is considered as particularly valuable and healthy for the human consumption (Fig. 2). Two types of the oil have to be distinguished: one which is rich in monounsaturated oleic acid and another rich in linoleic acid [104]. The high content of unsaturated fatty acids, especially linoleic acid qualifies the oil for the reduction of cholesterol levels and might even have a positive impact on diabetes [131, 132]. Due to the dominance of essential ω-6-fatty

acids, safflower oil was also widely spread as a traditional component of parenteral nutrition solutions [133]. However, uncertainty has emerged if an excess supply of linoleic acid could increase the production of proinflammatory lipid mediators [134].

## Efficacy of vegetable oils and their derivatives for the prevention of oral diseases

Besides the positive effects on the general health constitution, traditional oil pulling is also suggested to prevent tooth decay, bleeding gums, and halitosis [47, 50, 70, 135–137]. Patients repeatedly described a positive impact on their oral tissues and although there is no clear scientific explanation to this phenomenon yet, several popular scientific sources promote the procedure as a daily supplement of oral hygiene. It was to be expected that there are numerous research findings on the effects of edible oils in preventive dentistry but there are only a few scientific studies. Some mouth care preparations, predominantly in the field of natural cosmetics, contain vegetable oils as ingredients, varying from "mouth-oil" to toothpaste or saliva substitutes [138]. It is important to note that their distribution is rather based on an empirical application, as previous findings and literature regarding the efficacy of vegetable oils are controversial and require further investigation. Nevertheless, a few studies attempt to scientifically prove a potential of vegetable oils to inhibit oral biofilm accumulation and to have a protective impact on the oral hard and soft tissue [48, 49, 70, 135]. The following will point out beneficial results that were obtained in vitro as well as in vivo and are derived by lipid components; however, further scientific data is needed to comprehend molecular interactions. For instance, a positive correlation between dietary fats and the fatty acid composition of the few enamel lipids was noticed in vitro [139, 140]. Certain fatty acids were shown to disturb bacterial adhesion as an in vitro study revealed for *S. aureus* under the influence of oleic acid [141].

### Caries

If similarities apply for cariogenic bacteria, mouth rinses containing lipids could delay biofilm maturation and prevent carious lesions. The adhesion and total count of *S. mutans* were inhibited repeatedly by vegetable oil-based products in vitro [142, 143]. For example, in order to determine an inhibiting effect of olive oil on bacterial growth, *S. mutans* solutions were incubated with olive oil overnight and total viable counts conducted afterwards. Additionally, oil pretreated microscope slides were likewise incubated with *S. mutans* and again total viable counts were determined. Although none of the experiments were conducted under clinical conditions, they still arouse interest owing to their promising *S. mutans*-reducing effects [143]. Furthermore, Green and Hartles examined the impact of dietary lipids on caries prevalence and reported that the addition of 5 % groundnut oil to a cariogenic diet of rats could reduce the incidence of caries significantly [144]. A few attempts were undertaken to investigate the effects of rinses with edible oils under in vivo conditions [48, 70, 135, 143]. According to their knowledge about oil pulling as a traditional folk remedy, Asokan et al. conducted studies to investigate its effect on the prevention of caries as well as gingival inflammation. Their findings suggest that vegetable oils as a supplement for oral hygiene reduce the concentration of *S. mutans* in human saliva and certain fatty acids (e.g., linoleic and oleic) might inhibit bacterial regrowth [70]. Similarities were reported in vivo for almond oil, which additionally had a positive impact on the buffer capacity of human saliva [145]. Asokan et al. also proposed the process of "saponification", owing to the alkali hydrolysis of fat, as one explanation for the cleansing effect of vegetable oils [70]. However, it is not clear which lipids or other components of the oils are relevant for these partially observed effects, the interactions with the pellicle and with the initial oral biofilm have not been investigated sufficiently until now. However, as pointed out earlier, recent experiments including modern analytical techniques and transmission electron microscopic investigations did not confirm any positive impact of rinses with different edible oils on the bacterial colonization of enamel samples in situ [48].

### Erosions

Research concerning demineralization derived by erosive processes indicated in vitro that vegetable oil mouth rinses might form a protective coating against acids as illustrated in Fig. 1. Since the extraction of lipids out of tooth tissue resulted in a faster progression of caries lesions in vitro, it was concluded that hydrophobic lipids act as a diffusion barrier [146]. Similarities were described earlier according to Slomiany et al. for the effects of the acquired pellicle [87]. Furthermore, vegetable oils might increase the lipid content in the outermost layer of hard tooth tissue or more likely in the pellicle [48, 140] (Fig. 2). In vitro studies, which questioned exactly those ideas, investigated the effect of olive oil-based emulsions on dentin demineralization [147, 148]. Once, bovine dentin samples were subjected to three demineralization cycles, each one lasting 8 h in a demineralization solution with an initial pH of 5.0 and the lesion depth as well as mineral loss were determined daily up to 9 days by microradiographs. In between, the samples were stored and pretreated in different olive oil emulsions (50 or 5 % olive oil), distilled water, or a 1,500 ppm fluoride solution. In summary, a small but significant decrease of mineral loss

was observed for the samples pretreated by olive oil emulsions whereby the higher concentration showed a stronger protecting effect [148]. Comparable findings were presented for dentin and enamel samples by another in vitro study [147]: during 10 cycles, several concentrations of olive oil-containing preparations were applied to enamel and dentin samples before transfer to artificial saliva and 1 % citric acid exposure for 3 min. Afterwards, the samples were stored in artificial saliva for 30 min to enable remineralization and enamel and dentin loss were quantified using profilometric measurements. In conclusion, a significant reduction of mineral loss was noticed if samples were pretreated with 2 % olive oil. In turn, pure olive oil (100 %) was less effective which possibly relates to its low polarity and therefore less affinity to the tooth surface [147]. In contrast, first clear doubts regarding the protective effect of applied edible oils against erosive noxae were latest revealed [49]. For this purpose, enamel slabs were carried in the oral cavity for pellicle formation in situ and rinses with safflower oil were performed. Afterwards, the specimens were exposed to hydrochloric acid in vitro and the erosive mineral loss was determined by measurement of calcium and phosphate release. An even slightly increased loss of the respective minerals was recorded for the samples after application of the edible oil in situ as compared with unrinsed pellicles indicating diminished protective effects. The pellicles modified by rinsing with safflower oil were degraded faster in the acidic milieu [49]. It was postulated that the edible oil could have detracted relevant lipophilic pellicle components.

### Periodontal diseases

In contrast to the dental hard tissues, the periodontal structures and especially the gingiva underlie a considerable turnover, lipids, and other components of edible oil can directly influence cellular structures, cell membranes, etc. Accordingly, the possible effects of oils have to be analyzed from a completely different point of view. In consequence of general microorganism removal, the use of vegetable oil as a mouth rinse might reduce gingival inflammation and therefore prevent tissue degradation [47]. Particularly for both *n*-3 and *n*-6 polyunsaturated fatty acids anti-inflammatory effects have been demonstrated in different in vitro models. According to their anti-inflammatory properties, the enrichment of certain fatty acids in oral care products might be useful [149] as clinical studies also observed benefits on periodontal diseases [150, 151]. In contrast, another study stated a less obvious impact on periodontitis and further data will be required [152]. Referring to an in vivo study, a daily mouth wash with sesame oil could decrease the plaque and gingival index and decline the total amount of aerobic microorganisms in the plaque within 10 days [47]. A gingivitis-reducing effect was also reported for other oils like sunflower oil, herbal oils, or essential oils [153]. Nevertheless, those findings currently lack a detailed scientific analysis as they primarily refer to the empirical observations of a folk remedy.

### Mucosal infections

The increased utilization of plant cooking oils like perilla oil and soybean oil was reported to inhibit the incidence of minor recurrent aphthous stomatitis in vivo [154]. These promising findings mentioned above are of special interest for patients with xerostomia and pronounced oral inflammation, as such occur due to oral radiation therapy. Their mucosa appears exceedingly sensitive so that further irritation by sharp or burning mouth rinses should strictly be avoided [155]. In turn, microorganism removal is indispensible to prevent oral infections. An in situ study performed on irradiated rats examined the effect of sunflower oil on the manifestation of mucositis [156]. Although the side effect could obviously not be prevented it still appeared to emerge slightly retarded.

## Discussion

Rinses with edible oils are a well-known practice in traditional folk medicine as well as in modern alternative medicine for the prevention and therapy of oral diseases. However, scientific evidence for the efficacy of these strategies is sparse and the physicochemical background of recorded effects has not been investigated adequately. This is surprising as in vivo or in situ studies with the safe/nonhazardous edible oils can be carried out easily [48, 49]. Despite modern ultrastructural investigations, most explanations for the effects of lipophilic substances in oral prophylaxis are rather theoretical than evidence based and unfortunately the few recent studies tend to vary a lot regarding their observations.

Typical examples are the suspected interactions with the tooth surface and the postulated hydrophobization, respectively, after rinses with vegetable oils. In vitro studies suggest the protection especially against acidic noxes, though the interactions with the ubiquitous pellicle layer have not been investigated completely until now [48, 49, 143, 147]. This applies especially for the lipid composition. Yet, latest in situ studies did not confirm the expected positive impact of oil rinses on oral diseases, especially with respect to the protection of dental hard tissues [48, 49]. There is no evidence that the lipids are bound tenaciously to the pellicle following rinses with oils. If so, it is uncertain whether they are really integrated into the ultrastructure of the pellicles or whether oil micelles only stick to them without providing any preventive effect. Figure 2 summarizes data gained by transmission electron microscopy that confirm the lipid

accumulation at the pellicle's surface directly after rinses with vegetable oils [48]. Nevertheless, as pellicle formation is a highly selective process, tenacious and sustainable integration of relevant lipids in the pellicle seems doubtful and requires further research. It might also be postulated that rinses with oils could detract relevant lipophilic components from the pellicle or modulate the adsorption of other pellicle components as outlined in Fig. 2 [48, 64]. Until now, there are only two rather aged studies on the physiological lipid composition of the pellicle despite the potential relevance of this substance group [87, 88]. Modern analytical methods such as (GC; GC/MS) offer the opportunity to identify and to qualify the relevant lipid fractions in the pellicle layer though lipid analytics are still a considerable challenge [100]. Furthermore, it is not easy to deal with the small sample sizes which require distinct methodical approaches. Nevertheless, the effect of these interactions on erosion or bacterial adhesion also requires further research as potentially occurring lipid accumulation in the pellicle does not necessarily mean an improvement of protective properties [48, 49].

In situ studies based on enamel slabs exposed to the oral fluids are almost ideal to elucidate all these scientific questions. This allows harvesting of pellicle material for lipid analysis as well as fluorescence microscopic visualization and quantification of adherent bacteria to monitor the process of bioadhesion. Lipid accumulation at the pellicle's surface is not necessarily accompanied by a reduction of microbial adhesion as lipids could also promote hydrophobic interactions or even serve as substrates for certain bacteria [22]. Figures 3 and 4 show the discrepancy between lipid accumulation and their integration into the pellicle. Eventually, studies on lipids in the pellicle offer new insights into the process of bioadhesion in general.

Also the effects on the oral soft tissues and on the periodontal structures require further research based on prospective controlled clinical studies including adequate placebos. If protective effects are observed, it is necessary to identify the fractions of the edible oils relevant for the effects. Edible oils and foodstuffs rich in lipids contain a vast number of chemical substances with certainly different effects. Thereby, the phospholipids for example contained in soya milk are of special interest due to their interactions with mucins potentially making the pellicle more tenacious in contrast to triglycerides [87]. The identification of relevant groups of elements would allow the development of tailored biomimetic and biological oral healthcare preparations of high biocompatibility. Incidentally, comparable approaches are reported for dairy components that are thought to promote the anticariogenic properties of milk [41, 157]. All in all, only a few evidence based conclusions can be drawn with respect to the relevance and the efficacy of edible oils and lipids in general for preventive dentistry.

## Conclusions

Lipophilic substances could be promising supplements to conventional dental prophylaxis for the prevention of gingivitis, periodontitis, and caries but the data so far available suggest very limited evidence. The modification of the pellicle structure and of bioadhesion processes seems to be the main target of rinses with edible oils. In any case, further research is required to understand the effect of oils and lipids on the composition and characteristics of the pellicle layer and on bioadhesion in the oral cavity.

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