Aus der Poliklinik für Zahnerhaltung und Parodontologie

Poliklinik der Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. dent. Reinhard Hickel

Antimikrobielle Peptide in Kombination mit Antibiotika als Ansatz zur Bekämpfung oralpathogener Biofilme

Dissertation

zum Erwerb des Doktorgrades der Zahnmedizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Sabina Noreen Würsching

aus München

2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. med. dent. Karin C. Huth, MME
Mitberichterstatter:	PD Dr. Anja Liebermann, M.Sc.
	Prof. Dr. Andrea Wichelhaus
	Prof. Dr. Franz-Xaver Reichl
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. med. dent. Maximilian Kollmuß
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	21.02.2022

Eidesstattliche Versicherung

Würsching, Sabina Noreen

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

„Antimikrobielle Peptide in Kombination mit Antibiotika als Ansatz zur Bekämpfung oralpathogener Biofilme“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 23.02.2022

Sabina Noreen Würsching

Inhaltsverzeichnis

1. Publikationsliste 5

2. Beitrag zu den Veröffentlichungen 6

   2.1. Veröffentlichung I 6

   2.2. Veröffentlichung II 6

3. Einleitung 8

   3.1. Problematik Biofilm-assoziierter Erkrankungen 8

   3.2. Zunahme von Antibiotikaresistenzen – eine globale Herausforderung 10

   3.3. Antimikrobielle Peptide als moderner Therapieansatz 12

   3.4. Ziele des Forschungsvorhabens 13

4. Zusammenfassung 15

5. Abstract (English) 17

6. Veröffentlichung I 19

7. Veröffentlichung II 37

8. Literaturverzeichnis zur Einleitung 64

9. Danksagung 66

10. Lebenslauf .... **Fehler! Textmarke nicht definiert.**

1. Publikationsliste

1. **Wuersching SN**, Huth KC, Hickel R, Kollmuss M. Inhibitory effect of LL-37 and human lactoferricin on growth and biofilm formation of anaerobes associated with oral diseases. Anaerobe. 2021 Feb 67:102301. doi: 10.1016/j.anaerobe.2020.102301.

2. **Wuersching SN**, Huth KC, Hickel R, Kollmuss M. Targeting antibiotic tolerance in anaerobic biofilms associated with oral diseases: Human antimicrobial peptides LL-37 and lactoferricin enhance the antibiotic efficacy of amoxicillin, clindamycin and metronidazole. Anaerobe. 2021 Aug 25;71:102439. doi: 10.1016/j.anaerobe.2021.102439.

3. Kollmuss M, Tolksdorf K, Wuersching SN, Hickel R, Huth KC. Effect of polyhexanide as antiseptic mouth rinse against oral pathogens in an in vitro biofilm model. Acta Odontol Scand. 2021 Mar 15:1-8. doi: 10.1080/00016357.2021.1899280. **(nicht Teil dieser kumulativen Dissertationsschrift)**

2. Beitrag zu den Veröffentlichungen

Im Folgenden wird der eigene Beitrag und der Beitrag der Ko-Autoren zu den beiden Publikationen, angelehnt an das CRediT-Statement, dargelegt [1]. Diese Richtlinie dient auch für wissenschaftliche Verlage als Leitfaden für die Bewertung der Anteile unterschiedlicher Autoren.

2.1. Veröffentlichung I

Inhibitory effect of LL-37 and human lactoferricin on growth and biofilm formation of anaerobes associated with oral diseases

WUERSCHING, Sabina:
Conceptualization, Methodology, Formal Analysis, Investigation, Data curation, Writing – Original Draft, Visualization

HUTH, Karin Christine:
Writing – Review & Editing

HICKEL, Reinhard:
Writing – Review & Editing

KOLLMUSS, Maximilian:
Conceptualization, Resources, Writing – Review & Editing, Supervision, Project Administration

2.2. Veröffentlichung II

Targeting antibiotic tolerance in anaerobic biofilms associated with oral diseases: Human antimicrobial peptides LL-37 and Lactoferricin enhance the antibiotic efficacy of amoxicillin, clindamycin and metronidazole

WUERSCHING, Sabina:
Conceptualization, Methodology, Formal Analysis, Investigation, Data curation, Writing – Original Draft, Visualization

HUTH, Karin Christine:
Writing – Review & Editing

HICKEL, Reinhard:
Writing – Review & Editing

KOLLMUSS, Maximilian:

Resources, Writing – Review & Editing, Supervision, Project Administration

Somit erfolgten sämtliche Schritte angefangen bei der genauen Ausgestaltung der Fragestellung, Versuchsplanung und –durchführung sowie die vollständige Auswertung der gewonnen Daten mit Erstellung aller Abbildungen durch Sabina Würsching. Ebenso verfasste sie die beiden Manuskripte selbstständig und war bei der 2. Veröffentlichung auch als corresponding author für die Kommunikation mit den Gutachern und dem Verlag verantwortlich.

3. Einleitung

3.1. Problematik Biofilm-assoziierter Erkrankungen

Die Therapie von Biofilm-assoziierten Infektionskrankheiten stellt die Medizin vor immer größer werdende Herausforderungen. Biofilme werden mit einer Vielzahl von Infektionen wie bakterieller Endokarditis, Osteomyelitis, Wundinfektionen oder chronisch rezidivierenden Pneumonien bei Mukoviszidose-Patienten in Verbindung gebracht. Auch die beiden Hauptkrankheitsbilder der Zahnheilkunde, Karies und Parodontitis, nehmen ihren Ursprung in dysbiotischen Biofilmen. Zudem sind in den menschlichen Körper eingebrachte Fremdkörper wie Katheter, Endoprothesen, künstliche Herzklappen oder Zahnimplantate Orte, an denen es vermehrt zu Infektionen durch mikrobielle Besiedelung kommen kann.

Biofilme sind oberflächen-assoziierte Gruppierungen von Mikroorganismen, welche in einer selbst produzierten Matrix aus extrazellulären polymeren Substanzen eingebettet sind [2]. Im Vergleich zu ihrer planktonischen Lebensform verändern sich die Eigenschaften der Mikroorganismen, was unter anderem eine Steigerung ihrer Pathogenität bewirkt. So werden beispielsweise obsolete, energieverbrauchende Stoffwechselmechanismen im Biofilm eingestellt, um diesen effizienter zu gestalten [3]. Insbesondere in den tiefen Schichten des Biofilms, in denen das Angebot an Nährstoffen und Sauerstoff geringer ist, kommt es zu einer Verlangsamung des Zellmetabolismus durch verminderte Expression von Proteinen, die für metabolische Prozesse benötigt werden [4]. Diese metabolisch inaktiven Zellen zeigen dadurch auch eine reduzierte Empfindlichkeit gegenüber Antibiotika, da deren Wirkmechanismus oft auf eine ausreichend hohe Stoffwechselaktivität der Ziel-Zelle angewiesen ist. Eine Besonderheit bei der Ernährung des Biofilms ist die Tatsache, dass die Mikroorganismen einer Spezies die Stoffwechselprodukte einer anderen verwerten können. Die so entstehenden Ernährungskreisläufe ermöglichen ein autarkes Überleben der Bakterien im Biofilm für einen langen Zeitraum [5]. Außerdem ist die Biofilm-Matrix in der Lage, Nährstoffe und Wasser zu speichern, sodass die nutritive Abhängigkeit der Mikroorganismen von einer stetigen exogenen Nährstoffzufuhr deutlich reduziert ist [6]. Darüber hinaus kann durch die Koexistenz verschiedener Keimspezies ein Austausch von Resistenzfaktoren und Überlebensstrategien erfolgen. Dieser erfolgt durch eine Übertragung von Genen, die für Resistenzmechanismen und veränderte Stoffwechselprozesse codieren [7]. Der Einbau von Efflux-Pumpen in die Biofilm-Matrix ist ein solcher Resistenzmechanismus, der die Mikroorganismen im Biofilm durch aktiven Heraustransport bestimmter Antibiotika vor Abtötung schützt [8]. Zudem verhindern

in der Biofilm-Matrix vorkommende Exopolysaccharide die Penetration von antimikrobiellen Substanzen in den Biofilm, sodass die Resistenz der im Biofilm lebenden Bakterien gegenüber Antibiotika im Vergleich zu planktonischen Bakterien um das 1000-1500-Fache erhöht ist [9,10].

Aus diesen Gründen ist eine alleinige Gabe von antimikrobiellen Wirkstoffen oftmals nicht ausreichend, um ein adäquates Therapieergebnis zu erzielen. Vielmehr ist es erforderlich, neben der Applikation antimikrobieller Substanzen, eine mechanische Zerstörung der Integrität des Biofilms herbeizuführen.

Dies gilt auch für die Behandlung der Parodontitis, bei der es aufgrund einer bakteriellen Besiedelung der Zahn- und Wurzeloberfläche zu einer Entzündung und einem daraus resultierenden Abbau der parodontalen Stützgewebe bis hin zum Zahnverlust kommt [11]. Das moderne Behandlungskonzept der Parodontitis basiert auf einer regelmäßigen mechanischen Reinigung der Zahn- und Wurzeloberflächen mit dem Ziel, ein langfristig entzündungsfreies Parodont zu schaffen [12]. Bei erfolgreicher Elimination des Biofilms und der darin organisierten pathogenen Bakterien kommt es zu einer Ausheilung der Entzündung und in der Folge stabilen parodontalen Verhältnissen. Bei etwa 10% der betroffenen Patienten kann allerdings ein rasch progredienter Verlauf der Parodontitis beobachtet werden [13]. Die Gründe hierfür sind unter anderem Allgemeinerkrankungen wie Diabetes mellitus oder der Konsum von Nikotin, was sich auf die Mikrozirkulation des Parodonts negativ auswirkt. Oftmals lässt sich aber kein eindeutiger Auslöser für einen solchen schweren Verlauf identifizieren, hier sind daher auch genetische Einflüsse denkbar. In diesen Fällen kann eine alleinige mechanische Reinigung der betroffenen Zahnoberflächen kein zufriedenstellendes Therapieergebnis liefern. Zudem gibt es parodontale Erkrankungen, die primär einen fulminanten, nekrotisierenden Verlauf nehmen. Diese sind in der Regel mit einem erworbenen Defekt des Immunsystems, wie einer floriden HIV-Infektion oder einer neu aufgetretenen hämatologischen Erkrankung assoziiert. In all diesen Fällen mit bestehender Gefahr eines weiteren, schnellen Knochen- und damit Zahnverlusts werden im direkten zeitlichen Zusammenhang mit einer Reinigung der Zahnoberflächen zusätzlich systemisch wirkende Antibiotika zur pharmakologischen Elimination der Bakterien eingesetzt [14]. Dies geschieht unter der Annahme, dass die pathogenen Bakterien nach einer Auflockerung der Biofilmmatrix für antimikrobielle Substanzen besser zugänglich sind [15]. Dennoch gilt es zu beachten, dass es anatomische Strukturen gibt, die grundsätzlich einer mechanischen Reinigung nicht zugänglich sind, sodass in diesen Fällen eine vollständige mechanische Elimination des Biofilms ohne chirurgische Entfernung der gesamten betroffenen Gewebe nicht möglich ist. Dies gilt insbesondere auch

für andere Biofilm-assoziierte Erkrankungen, wie chronische Mittelohrentzündungen, Pneumonien oder bakterielle Endokarditiden, bei denen eine primär chirurgische Therapie aus funktionellen Gründen nicht in Frage kommt. In solchen Fällen sind antimikrobielle Substanzen als wesentlicher Bestandteil einer konservativen Therapie nicht wegzudenken und werden dementsprechend häufig verordnet.

3.2. Zunahme von Antibiotikaresistenzen – eine globale Herausforderung

Der notwendige und häufig lebensrettende Einsatz von antimikrobiellen Substanzen in der Medizin hat in der Biologie der Krankheitserreger seine Spuren hinterlassen. Die Resistenzbildung von Mikroorganismen gegenüber Antibiotika ist eine Problematik, die seit Mitte der 1950er-Jahre enorm zugenommen hat [7]. Heute stellt die Antibiotikaresistenz eine globale Herausforderung für die Behandlung einer Vielzahl von Infektionserkrankungen dar. Laut der Weltgesundheitsorganisation (WHO) sterben jährlich fast 700.000 Menschen in Folge einer Infektion, die mit Antibiotika nicht mehr behandelbar ist. Seit einigen Jahren ist eine Zunahme von Atemwegserkrankungen, Harnwegsinfekten und sexuell übertragbaren Krankheiten mit multiresistenten Erregern zu beobachten. Der häufige und nicht immer angemessene Einsatz von Antibiotika in der Medizin ist eine entscheidende Ursache für die Entstehung von Resistenzen. Beispielsweise werden Antibiotika von vielen Haus- und Kinderärzten, entgegen aktueller Empfehlungen, zur primären Therapie von unkomplizierten Atemwegsinfekten verschrieben, obwohl diese in den meisten Fällen eine virale Ursache haben und oft auch ohne den zusätzlichen Einsatz von antimikrobiellen Substanzen selbstbegrenzend verlaufen würden [16,17]. In Hinblick auf die Prävention von Resistenzbildungen wäre es also durchaus sinnvoll, vor Verabreichung eines Antibiotikums einen gesicherten Nachweis darüber zu erzielen, ob die Infektion überhaupt durch Bakterien verursacht wurde und wenn ja, ob das gewählte Antibiotikum auch eine ausreichende Wirkung gegen diese aufweist. Daher kommt einem mikrobiologischen Erregernachweis mit Erstellung eines Antibiogramms eine entscheidende Rolle im verantwortungsbewussten Einsatz von antibiotischen Substanzen zu. Da eine vollständige mikrobiologische Diagnostik trotz zahlreicher Verbesserungen immer noch 24 bis 48 Stunden dauert, gibt es häufig Situationen, in denen auf ein solches Vorgehen verzichtet werden muss. Dies gilt insbesondere bei schweren Infektionserkrankungen, die einen unverzüglichen Beginn einer Antibiose zwingend erfordern, da sonst das Risiko für Komplikationen und potentiellen Schaden für den Patienten zu hoch wäre. Zudem gibt es klinische Bilder, die mit sehr großer Wahrscheinlichkeit einer bakteriellen Ätiologie zuzuordnen sind, wie beispielsweise Harnwegsinfekte, die in der Regel durch das

Enterobakterium *Escherichia coli* oder andere Bakterien der Darmflora ausgelöst werden [18]. Hier erfolgt die Auswahl des Antibiotikums empirisch, d.h. es wird ohne vorherigen mikrobiologischen Erregernachweis ein erfahrungsgemäß wirksames Antibiotikum eingesetzt. Dieses Vorgehen der kalkulierten Antibiose ist in der Medizin weit verbreitet und hat den Vorteil, dass die Therapie schnell begonnen werden kann. Dennoch fördert dies, auch durch das meist relativ unspezifische Wirkungsspektrum der eingesetzten Substanz, die Entstehung weiterer Antibiotikaresistenzen [19]. Auch vom Patienten eigenständig veranlasste Handlungen, wie das frühzeitige Beenden einer Antibiotikatherapie oder das Aufbewahren von Antibiotika zur meist nicht angemessenen Selbstmedikation bei späteren Erkrankungen, spielen eine entscheidende Rolle in der Ausbreitung von Resistenzen [20]. Um den bereits bestehenden Antibiotikaresistenzen entgegen zu wirken, wurden in der Vergangenheit immer wieder neue Antibiotika und Chemotherapeutika entwickelt. Allerdings stellen Infektionen mit multiresistenten Erregern immer noch eine immer größer werdende Herausforderung dar. Daher muss nicht selten auf Reserveantibiotika wie Vancomycin oder Linezolid zurückgegriffen werden. Jedoch nimmt beispielsweise auch die Prävalenz Vancomycin-resistenter Enterokokken (VRE) oder multiresistenter gram-negativer Erreger (MRGN) wie *Pseudomonas aeruginosa* bei nosokomialen Infektionen stetig zu [21].

Auch in der Zahnmedizin werden häufig Antibiotika verschrieben, schätzungsweise sind weltweit sogar 10% aller Arzneimittelverordnungen für Antibiotika auf Zahnärzte zurückzuführen [22]. Dabei handelt es sich vor allem um Breitbandantibiotika, die gegen eine Vielzahl der zu vermutenden Erreger gerichtet sind. Klassischerweise werden bei akuten odontogenen Infektionen β-Lactam-Antibiotika wie Amoxicillin, oft in Kombination mit dem β-Lactamase-Inhibitor Clavulansäure, verschrieben. Bei Vorliegen einer Penicillin-Allergie kommt das Lincosamid Clindamycin als Alternative zum Einsatz [23]. Auch zur Behandlung parodontaler Erkrankungen, insbesondere bei Vorliegen der oben beschriebenen Problematik einer unzureichenden Möglichkeit der mechanischen Biofilmentfernung, werden systemisch wirkende Antibiotika therapeutisch eingesetzt. Hierbei wird als Standardmedikation eine Kombination aus Amoxicillin und dem Nitroimidazol Metronidazol eingesetzt, deren Wirkungsspektrum alle, insbesondere auch obligat anaerobe Bakterien, umfasst, die nach dem derzeitigen Kenntnisstand eine Schlüsselfunktion in der Pathogenese parodontaler Infektionen einnehmen [24,25]. Daher kommt auch dem Fachgebiet der Zahnmedizin eine besondere Verantwortung im Umgang mit Antibiotika zu, damit diese auch in Zukunft für schwere Infektionen mit hoher Wirksamkeit zur Verfügung stehen.

Vor all diesen Hintergründen ist es von größter Wichtigkeit, alternative, neue antimikrobielle Strategien zur Bekämpfung von Biofilm-assoziierten Erkrankungen zu entwickeln, um die Häufigkeit nicht-therapierbarer Infektionen und die damit einhergehende Letalität nachhaltig zu senken.

3.3. Antimikrobielle Peptide als moderner Therapieansatz

Seit einigen Jahren stehen antimikrobielle Peptide (AMPs) zunehmend im wissenschaftlichen Fokus unterschiedlicher medizinischer Fachdisziplinen. AMPs sind natürlich vorkommende kurze, zumeist kationische Peptide mit einer Länge von 12 bis 50 Aminosäuren [26]. Sie sind Bestandteil des angeborenen Immunsystems und weisen ein breites Wirkungsspektrum gegen eine Vielzahl von Mikroorganismen auf. AMPs wurden in vielen verschiedenen Lebewesen nachgewiesen und kommen insbesondere in epithelialen Geweben und neutrophilen Granulozyten vor. In der menschlichen Mundhöhle werden AMPs in den Schleimhäuten und Speicheldrüsen exprimiert, wo sie eine wichtige Stellung in der primären Infektionsabwehr einnehmen [27].

Das antimikrobielle Wirkprinzip der AMPs beruht auf einer Membraninteraktion, die durch ihre amphiphatische Struktur und geringe Molekülgröße ermöglicht wird. Der genaue Wirkmechanismus lässt sich am besten am Beispiel der Bakterienzelle veranschaulichen. Die bakterielle Zellmembran ist aus einer Doppelschicht aus Phospholipiden aufgebaut, die in wässriger Lösung so angeordnet sind, dass die hydrophoben Lipidreste nach innen und die polaren Phosphatköpfe nach außen zeigen. Elektrostatische Wechselwirkungen zwischen den negativ geladenen Phosphatköpfen und den kationischen Anteilen der AMPs ermöglichen eine Anlagerung der AMPs an die bakterielle Membran [28]. Es kommt zu einer Depolarisationsstörung und infolge dessen zu einer unwiderruflichen Zerstörung der Zellmembran. Durch den daraus resultierenden osmotischen Einstrom von Wasser lysiert die Bakterienzelle letztendlich.

Im Gegensatz zu den klassischen Antibiotika, gegen die sich üblicherweise innerhalb weniger Jahre nach deren Entwicklung Resistenzen bilden, existieren AMPs schon seit mehreren Millionen Jahren ohne merkliche Neigung zur Entstehung von Resistenzen [29]. Dies macht sie zu einem attraktiven potentiellen Therapeutikum für die Behandlung von Infektionserkrankungen. Neben der alleinigen Applikation von AMPs zu therapeutischen Zwecken ist auch eine Kombinationstherapie mit konventionellen antibiotischen Wirkstoffen denkbar, da sehr hoch dosierte AMPs durchaus auch negative Effekte im Organismus hervorrufen können. Eine solche Kombination könnte sich die Wirkungsmechanismen beider

Substanzklassen zu Nutze machen: So wäre es denkbar, dass AMPs die bakterielle Zellmembran auflockern und so einen Weg für intrazellulär wirksame Antibiotika ebnen. Dies wäre ein neuer Ansatzpunkt, vor allem in der Therapie Biofilm-assoziierter Infektionen.
Eines der am besten untersuchten AMPs ist das Cathelicidin LL-37, welches in einer Vielzahl von Zellen, unter anderem in der Mundhöhle, nachgewiesen werden konnte [30]. Neben einer breiten antimikrobiellen Wirkung zeigt LL-37 auch immunmodulatorische Eigenschaften und spielt eine entscheidende Rolle bei der Inaktivierung bakterieller Endotoxine [31,32]. Auch an der Heilung von Gewebe ist LL-37 beteiligt, was sich insbesondere bei einem Mangel an LL-37 zeigt, da es hier zu einer deutlichen Verzögerung der Reepithelialisierung kommen kann [33]. Daher ist es nicht verwunderlich, dass viele Krankheitsbilder in der Dermatologie, wie Psoriasis und Rosazea, mit der gestörten Expression von LL-37 assoziiert wird [34,35]. Ein angeborener Mangel an LL-37 kann auch in der Mundhöhle zu sichtbaren Konsequenzen führen. Am besten untersucht ist hier das Krankheitsbild Morbus Kostmann, welches mit einem genetisch bedingten Mangel an antimikrobiellen Peptiden, unter anderem LL-37, einhergeht. Die betroffenen Patienten leiden oftmals an einer schweren Form der Parodontitis, die sich durch Veränderungen in der Immunantwort durch den Mangel an LL-37 im Sulkusfluid erklären lässt [36]. Auch auf die Anzahl der durch Karies betroffenen Zähne scheint die Konzentration von LL-37 einen Einfluss zu haben, da sich eine signifikante negative Korrelation zwischen der Anzahl an kariösen Läsionen und der Konzentration von LL-37 im Speichel zeigte [37].
Ein weiterer wichtiger Vertreter der antimikrobiellen Peptide ist Lactoferricin, welches ein Derivat des multifunktionalen Enzyms Lactoferrin ist. Ähnlich zu LL-37 sind für humanes Lactoferricin (LfcinH) vielfältige antimikrobielle und immunmodulatorische Eigenschaften beschrieben worden [38,39]. So zeigte LfcinH bei *P. aeruginosa* und *Porphyromonas gingivalis* eine Hemmung des bakteriellen Wachstums, auch bei der Ausbildung von Biofilmen. Allerdings liegen zu LfcinH trotz vielversprechender erster Ergebnisse noch keine Daten für Multispezies-Biofilme mit oralpathogenen Bakterien vor.

3.4. Ziele des Forschungsvorhabens

In dieser vorliegenden Arbeit soll erstmalig anhand von zwei repräsentativen Bakterienkombinationen die Wirkung der antimikrobiellen Peptide LL-37 und LfcinH auf oralpathogene Biofilme umfassend untersucht werden. Dabei soll neben der Wirkung auf das planktonische Wachstum auch die Menge des gebildeten Biofilms quantifiziert werden. In einem zweiten Versuchsteil soll die Wirkung der AMPs auf ausgereifte Biofilme genauer

betrachtet werden. Hier werden neben der alleinigen Applikation von AMPs auch Kombinationen mit in der Zahnmedizin häufig verwendeten Antibiotika getestet. Zudem sollen Untersuchungen zur Veränderung der Stoffwechselaktivität in reifen Biofilmen in Abhängigkeit von den Wachstumsbedingungen durchgeführt werden. Ziel dieses Projekts ist es, eine belastbare Datenbasis für eine weitere, klinische Anwendung von AMPs in der Zahnmedizin zu schaffen.

4. Zusammenfassung

Im Hinblick auf eine immer mehr zunehmende Resistenzproblematik bei der antimikrobiellen Therapie von Infektionskrankheiten kommt alternativen Therapieansätzen eine immer größere Bedeutung zu. Eine vielversprechende Gruppe an alternativen Substanzen sind antimikrobielle Peptide (AMPs), die als Teil des Immunsystems vielfältige Aufgaben im Körper übernehmen. Auch ein therapeutischer Einsatz synthetisch hergestellter Peptide mit gleicher Struktur ist dabei denkbar, da sich der Ansatzpunkt der antimikrobiellen Wirksamkeit grundlegend von dem klassischer Antibiotika unterscheidet.

Das Ziel des vorliegenden Projekts war es, einen detaillierten Einblick in die Wirkung von antimikrobiellen Peptiden auf oralpathogene Bakterien zu erlangen. Es wurden die AMPs LL-37, ein Cathelicidin, und das Lactoferrin-Derivat Lactoferricin H zur Untersuchung ausgewählt. Dazu wurden zwei orale Biofilmmodelle mit einer jeweils repräsentativen Auswahl an Mikroorganismen etabliert. Zur Simulation eines supragingivalen Biofilms kamen dazu die Bakterienspezies *S. mutans*, *S. sanguinis* und *A. naeslundii* zum Einsatz, für die Nachbildung eines parodontalpathogenen, subgingivalen Biofilms wurden *V. parvula*, *P. micra* und *F. nucleatum* verwendet. Die Interaktion der AMPs mit den ausgewählten Bakterien wurde in vier unterschiedlichen Experimenten untersucht:

1. Wirkung der AMPs auf planktonisches Bakterienwachstum (Veröffentlichung I)
2. Einfluss der AMPs auf die Menge an gebildetem Biofilm (Veröffentlichung I)
3. Effektivität von AMPs bzw. ausgewählten Antibiotika gegen reife Biofilme (Veröffentlichung II)
4. Wirkung einer Kombination aus AMPs und Antibiotika auf reife Biofilme (Veröffentlichung II)

Die Versuchsergebnisse konnten zeigen, dass die AMPs LL-37 und Lactoferricin H sowohl eine Wachstumshemmung planktonischer Bakterien bewirken, als auch zu einer geringeren Bildung von Biofilmmasse führen, wenn während des Reifungsprozesses AMPs zugegeben werden. Es zeigten sich in Abhängigkeit von den untersuchten Bakterien und den AMPs unterschiedliche Schwellenkonzentrationen für eine erhöhte Reduktion des bakteriellen Wachstums. Im zweiten Teil dieses Projekts konnte gezeigt werden, dass eine Kombination aus antimikrobiellen Peptiden und klassischen Antibiotika eine synergistische antimikrobielle Wirkung zeigt, wenn diese auf ausgereifte Biofilme appliziert wird. Hierbei stellte sich, insbesondere für anaerob wachsende Biofilme, die Applikation von Amoxicillin oder Clindamycin und einem AMP als die wirksamste, über eine Addition der Einzeleffekte

hinausgehende, Kombination heraus. Eine mögliche Ursache hierfür ist, dass sich die Wirkungsmechanismen von AMPs und Antibiotika ergänzen. So könnte die durch AMPs aufgelockerte Biofilmmatrix durch antibiotische Wirkstoffe leichter passiert werden, was zu einer verbesserten Wirkung führen kann.

Im Zuge der Versuche konnten weiterhin interessante neue Erkenntnisse über das Ausmaß der metabolischen Veränderungen in verschiedenen Biofilmen gewonnen werden. So zeigten sich unterschiedliche Ausmaße der metabolischen Herabregulierung bei fakultativ und obligat anaerob wachsenden Biofilmen. Diese Erkenntnis ist von größter Wichtigkeit für die Interpretation von mikrobiologischen Studienergebnissen, da hier oft stoffwechselbasierte Assays für eine Quantifizierung von Bakterien verwendet wird.

Zusammenfassend liefern die Ergebnisse dieser Arbeit eine Datenbasis für weitere, auch klinische, Untersuchungen der Wirkung von AMPs auf oralpathogene bakterielle Biofilme. Es konnte die Wirksamkeit eines neuen Ansatzes mit einer Kombination aus verschiedenen antimikrobiellen Wirkstoffen nachgewiesen werden. Die darüber hinaus gewonnenen Erkenntnisse über metabolische Veränderungen in Biofilmen ermöglichen es, den Faktor der Stoffwechselaktivität in eine möglichst präzise Quantifizierung von Bakterienzellen einfließen zu lassen. Dies erhöht die Aussagekraft von Untersuchungen, da es zu einer geringeren Verfälschung der Bakterienquantifizierung durch metabolische Anpassungen kommt.

5. Abstract (English)

The rapid emergence of antimicrobial resistance in the past decades has driven research into the development of novel antimicrobial strategies to combat biofilm-associated infections. The antimicrobial peptides (AMPs) are a class of naturally occurring anti-infective agents of the innate immune system and they are involved in the primary defense against invading pathogens. Their mechanism of action is based on low specific interactions with microbial cell membranes, which allows the AMPs to target a broad spectrum of microorganisms. Previous studies have demonstrated promising features of the AMPs, making them attractive as alternative therapeutic agents to antibiotics for treating infectious diseases in the future.

The purpose of this research was to assess the in vitro effects of two human antimicrobial peptides (AMP), LL-37 and Lactoferricin H (LfcinH), on oral pathogens. We established two multi-species biofilm models, which aimed to represent biofilms that are responsible for the two most common dental infections, caries and periodontitis. The facultative anaerobic bacteria *S. mutans*, *S. sanguinis* and *A. naeslundii* are present in supragingival dental plaque and were therefore used to mimic a caries-inducing biofilm. The second biofilm model comprised the obligate anaerobic bacteria *V. parvula*, *P. micra* and *F. nucleatum*, which are thought to be key contributors to the formation of the subgingival biofilms in periodontal disease. These two biofilm models were used for answering four research questions:

1. Inhibitory effect of AMPs on planktonic bacterial growth (Publication I)
2. Inhibitory effect of AMPs on biofilm formation (Publication I)
3. Anti-biofilm effects of AMPs and antibiotics separately on matured polymicrobial biofilms (Publication II)
4. Anti-biofilm effects of AMPs combined with antibiotics on matured polymicrobial biofilms (Publication II)

Our results demonstrated that the planktonic growth of the facultative and obligate anaerobic strains was inhibited in the presence of LL-37 and LfcinH. Both AMPs also had an inhibitory effect on the formation of both types of biofilms. Furthermore, the AMPs exhibited a threshold concentration for the reduction of bacteria in both their planktonic state and in biofilms.

Matured biofilms exhibited an enhanced antibiotic tolerance towards amoxicillin, clindamycin and metronidazole, which are the three most frequently prescribed antibiotics in dentistry. However, the combined application of antibiotics with AMPs led to an enhanced reduction of matured biofilms. Particularly the combination of AMPs with amoxicillin or clindamycin reduced the biofilms formed by obligate anaerobic bacteria significantly compared to the

antibiotics alone. One possible explanation for this observation is that the presence of the AMPs enhances the dispersion of biofilms and thereby facilitates the antibiotics' accessibility to the specific target site of the microbial cell.

For examining the possible mechanisms of antibiotic tolerance in biofilms, we conducted investigations on the metabolic state of the bacteria residing within. Our results revealed different levels of metabolic downshifts in sessile facultative and obligate anaerobic bacteria compared to their planktonic state. This finding is valuable for any future work dealing with in vitro biofilm models, because cell metabolism-based assays are often used for quantifying bacteria in biofilms. However, knowing that sessile bacteria exhibit a downregulated metabolism, these assays may not be adequate tools for determining the number of bacteria in biofilms.

In conclusion, this work lays the groundwork for further studies examining the therapeutic application of AMPs for the treatment of biofilm-related diseases. Our results give new insights into the efficacy of combined antimicrobial strategies involving human AMPs as well as into the mechanisms of antibiotic tolerance in biofilms. In addition, this work provides further details on metabolic particularities of biofilms which can help to improve the methodology in future biofilm research.

6. Veröffentlichung I

Inhibitory effect of LL-37 and human lactoferricin on growth and biofilm formation of anaerobes associated with oral diseases

Sabina Noreen **Wuersching**, Karin Christine **Huth**, Reinhard **Hickel**, Maximilian **Kollmuss**

Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Goethestraße 70, 80336 Munich, Germany

Journal: ***Anaerobe*** (Impact Factor 2020: 3,331)

Wuersching SN, Huth KC, Hickel R, Kollmuss M. Inhibitory effect of LL-37 and human lactoferricin on growth and biofilm formation of anaerobes associated with oral diseases. Anaerobe. 2021 Feb 67:102301. doi: 10.1016/j.anaerobe.2020.102301.

Received 3 November 2020
Received in revised form 20 November 2020
Accepted 23 November 2020
Available online 26 November 2020

Abstract

This study was conducted to evaluate the antimicrobial potential of the antimicrobial peptides (AMP) LL-37 and human Lactoferricin (LfcinH) on the planktonic growth and biofilm formation of oral pathogenic anaerobes related to caries and periodontitis. Multi-species bacterial suspensions of either facultative anaerobic bacteria (FAB: *Streptococcus mutans*, *Streptococcus sanguinis*, *Actinomyces naeslundii*) or obligate anaerobic bacteria (OAB: *Veillonella parvula*, *Parvimonas micra*, *Fusobacterium nucleatum*) were incubated with different concentrations of AMP solutions for 8 h. Planktonic growth was registered with an ATP-based cell viability assay for FAB and via plate counting for OAB. Biofilms were grown on ZrO_2 discs for 4 days in a mixture of the multi-species bacterial suspensions and AMP solutions. Biofilm mass was quantified using a microtiter plate biofilm assay with crystal violet staining. An overall planktonic growth inhibition and biofilm mass reduction of FAB and OAB was registered for LL-37 and LfcinH. Significant inhibitory threshold concentrations of LL-37 were observed in all experiments ($p < 0.0001$). No significant threshold was observed for LfcinH. Biofilm mass of OAB was barely reduced by LfcinH. The complete mechanisms of the AMPs are not fully understood yet. While LL-37 shows promising features as potential therapeutic for biofilm-associated oral diseases, LfcinH seems unsuitable for this particular indication. For clinical AMP use, further investigations will be necessary.

Keywords

Anaerobes, biofilm, antimicrobial peptides, LL-37, human Lactoferricin

Abbreviations

AMP = antimicrobial peptide; *LF* = Lactoferrin; *LfcinH* = human Lactoferricin; *FAB* = facultative anaerobic bacteria; *OAB* = obligate anaerobic bacteria

1. Introduction

Dental caries and severe periodontal diseases, being the two most common oral health conditions, are the main contributors for the loss of teeth. The Global Burden of Diseases, Injuries, and Risk Factors Study 2017 (GBD 2017) estimated dental caries of permanent teeth to be prevalent in 2.3 billion people and inflammatory periodontal diseases to affect nearly 800 million people worldwide [1]. Both conditions have been proven to have an infectious etiology and are subject to the development of bacterial biofilms, which are known to be involved in several other diseases as well, such as urinary catheter infections, infections of joint prostheses and endocarditis [2]. Biofilms can be perceived as a sessile community of microorganisms that are irreversibly attached to a surface and embedded in an autogenously produced matrix of extracellular polymeric substances. The physiological attributes of such a microbial community grant a drastically enhanced resistance against all kinds of antimicrobial agents, including a wide range of antibiotics. Several mechanisms have been identified for this feature, such as delayed penetration of antimicrobials through the biofilm matrix, physiological changes in the sessile microorganisms and exchange of mobile genetic elements encoding resistance determinants [3]. Bearing these mechanisms in mind, it is evident that the treatment of oral conditions associated with biofilms cannot be solely based on the application of antimicrobials and thus requires a mechanical disruption of the biofilm on the surfaces of dental tissues. This approach has been advocated by a large number of randomized clinical trials that have shown an increased therapeutic efficacy in the treatment of rapidly progressing periodontitis when adjunctive systemic antibiotics were administered immediately after subgingival debridement [4]. Prompt application of systemic antibiotics is based on the premise that specific pathogenic bacteria residing in periodontal pockets can be eradicated prior to recolonization and biofilm formation on dental tissues [5].

Regarding the use of antibiotics in the field of dentistry, it should be noted that dental practitioners routinely prescribe antibiotics for a number of prophylactic and therapeutic indications aside from adjunctive treatment of periodontal disease. While antibiotic prophylaxis prior to dental procedures is mainly indicated for preventive management of endocarditis, the therapeutic administration of antibiotics is most frequent in the treatment of odontogenic infections, especially in cases where the infection cannot be controlled by local debridement. As cultures from the patients' pus or exudate are not commonly grown, the microorganisms causing the infection are lacking certainty and thus antibiotic agents are often prescribed on a presumptive basis. This empirical approach for the administration of antibiotics has led to an over- and misuse of broad-spectrum agents in both dentistry and medicine and

has propelled the development of antibiotic drug resistance enormously in the recent years [6]. The urge to reduce excessive antibiotic use has been driving research into the development of novel agents with antimicrobial and anti-biofilm properties. The antimicrobial peptides (AMPs) are a new generation of antimicrobials with a broad activity spectrum against bacteria, fungi, viruses and even parasites and have therefore been receiving extensive attention over the past decades [7]. As essential components of innate defense mechanisms, antimicrobial peptides are gene-encoded molecules and are found in basically every living organism, specifically on mucosal surfaces, on the body surface and within the granules of phagocytes. They are derivatives of larger precursor peptides and are released by posttranslational modifications such as proteolytic processing. Antimicrobial peptides are small molecules composed of usually no more than 50 – predominantly basic – amino acids that contain a net positive charge, yet the cationic structure is altered by sections of hydrophobic residues, providing the molecule with an amphipathic design in its folded state [8]. These properties allow the antimicrobial peptides to target membranes that display an overall negative charge on their outermost portion. Unlike eukaryotic cells, whose lipids with negatively charged headgroups are directed towards the cytoplasm, bacterial membranes are equipped with a considerable amount of negatively charged phospholipids on the outer section of the bilayer [9]. In most cases, the antimicrobial peptides' mode of action is based on permeabilization of the lipid membranes via peptide-membrane interaction, which is facilitated by the opposite net charge of the peptides and lipids [10].

One of the best studied antimicrobial peptides is the human cathelicidin LL-37, which is found in neutrophils, epithelial cells, saliva and gingival crevicular fluid, hence contributing to the broad-spectrum defense of the oral cavity [11,12]. In a lipophilic environment, such as in bacterial membranes, LL-37 exhibits an amphipathic alpha-helical secondary structure, which is responsible for the peptides' antimicrobial properties. LL-37 accesses the periplasmic space of the bacterial cell by binding to the outer membrane and its lipopolysaccharides, followed by translocation across the outer membrane. The bactericidal effect is proposed to be induced by transmembrane pore-forming mechanisms such as toroidal pore formation, which eventually leads to lysis of the microbial cell [13,14]. Susceptibilities of periodontal and non-periodontal oral pathogens to LL-37 have been reported in previous studies, making it a major subject of interest to further oral research [15,16].

Lactoferrin (LF) is a 703-amino acid iron-binding glycoprotein and is considered a member of the transferrin family [17]. Originally identified in bovine, and later in human milk, lactoferrin is one of the major proteins in all human exocrine secretions, such as colostrum, tears, saliva,

seminal and gastrointestinal fluids, nasal and bronchial mucosa, and plasma [18]. A broad spectrum of functional properties have been ascribed to lactoferrin, including antiviral, antimicrobial, antifungal, anti-parasitic, immunomodulatory and antioxidant activity [19]. A fair amount of antimicrobial peptides have been identified that are derived from LF by the action of proteolytic enzymes, the three most studied being LF1-11, Lactoferrampin and Lactoferricin [20]. Particularly human Lactoferricin (LfcinH), a 25-residue peptide, is of great interest to oral research, as growth inhibitory effects and anti-biofilm activity of LfcinH and other LF-related agents against periodontopathic bacteria have been described in previous studies [21]. Much like several other antimicrobial peptides, the mode of action of LfcinH is presumably based on membrane perturbation of the bacterial membrane [22]. Moreover, clinical trials assessing LF-concentrations in patients with periodontal disease have determined higher levels of salivary LF in patients with moderately and rapidly progressing periodontitis compared to healthy patients, suggesting a stimulated secretion of LF from the salivary glands in sites with periodontal disease [23].

Both LL-37 and LfcinH show numerous promising features and are potential antimicrobial agents for the prevention and treatment of periodontal diseases and other oral pathologies. Among the studies evaluating the bactericidal effect of these antimicrobial peptides, their effect on a more authentic microbial in-vitro model of the oral cavity, namely with multi-species cultures, has not been investigated yet. Therefore, this study was conducted to examine the effectiveness of LL-37 and LfcinH on the planktonic growth of oral pathogens as well as on the formation of the oral biofilm. All of the experiments were performed in multi-species bacterial suspensions with selected facultative anaerobic and obligate anaerobic bacteria that were specifically chosen as microbial representatives for caries and periodontal disease.

2. Materials and Methods

Bacterial strains and growth media

Bacterial strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). For all of the experiments, the bacteria were divided into two groups: (1) facultative anaerobic bacteria (FAB) *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguinis* (ATCC 10556) and *Actinomyces naeslundii* (ATCC 19039) and (2) obligate anaerobic bacteria (OAB) *Veillonella parvula* (ATCC 17745), *Parvimonas micra* (ATCC 33270) and *Fusobacterium nucleatum* (ATCC 49256). All strains were grown and maintained on Schaedler agar plates supplemented with Vitamin K_1 and 5% Sheep Blood (Becton Dickinson, Franklin Lakes, NJ, USA). For growth in liquid media, all of the bacteria were cultured in Brain-Heart-Infusion broth (BHI, Becton Dickinson) supplemented with Hemin (5 µg/ml) and Vitamin K_1 (1 µg/ml). For an oxygen-free growth media that was required by the OAB, liquid media was boiled and cooled under CO_2 and autoclaved under N_2. In all of the performed experiments, bacterial suspensions in liquid media were incubated with the equal volume of antimicrobial agents (or water for the control group). In order to keep the ratio of nutrients to liquid unaffected when combined with another liquid, the BHI broth was prepared containing double the amount of nutrients and supplements (2X BHI).

Facultative anaerobic bacteria were grown at a temperature of 37°C and a humidity level of 60% in a CO_2 enriched atmosphere with 5.8% CO_2. Obligate anaerobic bacteria were grown at 37°C in an anaerobic gas atmosphere (GasPak EZ container system, Becton Dickinson).

Antimicrobial peptides (AMPs)

For this study, two different antimicrobial peptides, LL-37 (Innovagen AB, Lund, Sweden) and LfcinH (AnaSpec Inc., Fremont, CA, USA), were used. Amino acid sequences are shown in ***Table 1***. Both AMPs were prepared in 0.001% acetic acid and tested at five different concentrations: 250, 100, 25, 10, 2.5 µg/ml. Since equal parts of the AMP solution and the bacterial suspensions were combined during incubation for the experiments, stock solutions were prepared in double concentration of the selected test concentrations.

AMP	Amino acid sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
LfcinH	TKCFQWQRNMRKVR-G-PPVSCIKRDS

Table 1: Amino acid sequences of LL-37 and LfcinH

Discs for biofilm growth

Zirconium dioxide discs (ZrO_2, DEGOS Dental GmbH, Regenstauf, Germany) for developing biofilms were manufactured by cutting 6 mm diameter ZrO_2 rods into 1 mm thick segments, followed by a sintering process according to the manufacturer's instructions. Prior to use the discs were sandblasted with Al_2O_3 (120 µm, 1.5 bar), cleaned in an ultrasonic bath and autoclaved.

Inhibitory effect of AMPs on the growth of planktonic bacteria

Bacterial colonies of each strain were inoculated individually in 5 ml of 2X BHI broth and incubated overnight under the required growth conditions for FAB and OAB as mentioned above. The bacterial suspensions were diluted until an optical density of 0.1 at 620 nm was reached (Varioskan Microplate Reader, Thermo Fisher Scientific, Waltham, MA, USA). For a multi-species bacterial suspension, equal parts of the three strains were combined and briefly mixed for each group. Stock solutions of AMPs were prepared and equal amounts of both diluted bacteria and AMP stock solutions were added to the wells of a 96-well-plate (Corning Inc., Corning, NY, USA). For the control group, the same amount of sterile water was added to the multi-species bacterial suspension.

Growth of FAB was monitored using an ATP-based cell viability assay (BacTiter-Glo Microbial Cell Viability Assay; Promega, Madison, WI, USA). Luminescence was measured after 4 h by combining equal amounts of the BacTiterReagent and the bacterial suspension/AMP solution in a 96 half-area well plate (Corning Inc., Corning, NY, USA) and incubating the contents on an orbital shaker for 5 min at room temperature. Relative Light Units (RLUs) were registered in a Luminometer (GloMax Navigator System; Promega, WI, USA).

For the OAB, the number of viable cells after incubation with the antimicrobial agents was quantified by counting the number of colony-forming units (CFUs). After 8 h of incubation, 10-fold serial dilutions of the bacterial suspension/AMP solution were prepared in 0.9% sodium chloride and plated on agar plates using a spread plate method. After three days of incubation of the plates, CFUs were counted following FDA guidelines (only plates with 25 to 250 colonies were considered).

In both assays the bacterial growth of the test groups was compared to that of the control group, whose growth was defined as 100%. For both facultative and obligate anaerobic multi-species bacterial suspensions, all experiments were performed in duplicate wells for each condition and repeated at least three times.

Inhibitory effect of AMPs on the biofilm formation

Bacterial suspensions from overnight cultures in 2X BHI broth were diluted to an OD_{620nm} of 0.1 as mentioned above. Equal amounts of the three strains used for growing a multi-species biofilm were combined and mixed. Stock solutions of AMPs were prepared. ZrO_2 discs were placed in the wells of a 96-well-plate with a flat bottom (Corning Inc., Corning, NY, USA). Equal amounts of both diluted bacteria and AMP solutions were added to the wells, making sure that the discs were covered entirely in liquid. To prevent evaporation of the liquid during incubation, the well plates were covered with a lid. The discs were incubated at 37°C for four days in compliance with the required growth conditions for either group.

For quantifying the amount of biofilm grown on the ZrO_2 discs, a microtiter plate biofilm assay was performed by modifying a previously reported protocol [24]. The discs were rinsed in 0.9% sodium chloride and moved to clean wells. The discs were stained by adding a 0.1% aqueous crystal violet solution to the wells and incubating them for 10 min at room temperature. After removing the discs from the wells, any excess staining solution was discharged by drying the discs on a clean paper towel. For solubilizing the dye, the stained discs were placed in clean wells and 250 µl of 30% acetic acid were added to each well. After incubation on an orbital shaker at 50 rpm for 10 min at room temperature, the contents of each well were briefly mixed by pipetting. For every sample, two replicates containing each 100 µl of the crystal violet solution/acetic acid solution were transferred to separate wells in an optically clear flat-bottom 96-well plate. The optical density (OD) was used as a parameter to define the amount of biofilm and was measured at 600 nm (OD_{600}) in a microplate reader. Mean values of the two replicates were calculated for every disc and compared to the control group. The OD of the control group was defined as 100%.

For both facultative and obligate anaerobically grown multi-species biofilms, all experiments were performed in duplicate for each condition and repeated at least three times.

Statistics

All statistical analyses were implemented in Python 3.7.6 using *scipy* and *scikit* for the statistical tests and *matplotlib* for creating the plots [25]. Data were tested for normality with Shapiro-Wilk test. Homoscedasticity was assessed with Levene's test. Differences among group means were evaluated with a Kruskal-Wallis test for non-parametric data. Post hoc analysis was conducted by means of a Dunn's test with a non-negative two stage false discovery rate correction. P-values < 0.05 were considered significant.

3. Results

Effects of LL-37 and LfcinH on the planktonic growth of multi-species cultures

Both AMPs suppressed the planktonic growth of FAB. The median growth compared to the control group ranged from 43% to 77% and from 49% to 63% when incubated with the chosen concentrations of LL-37 and LfcinH, respectively (***Table 2***).

For visualizing the inhibitory effect of each AMP concentration, the measured values were plotted as the percentage of the control group in box-and-whisker-plots as shown in ***Figure 1***.

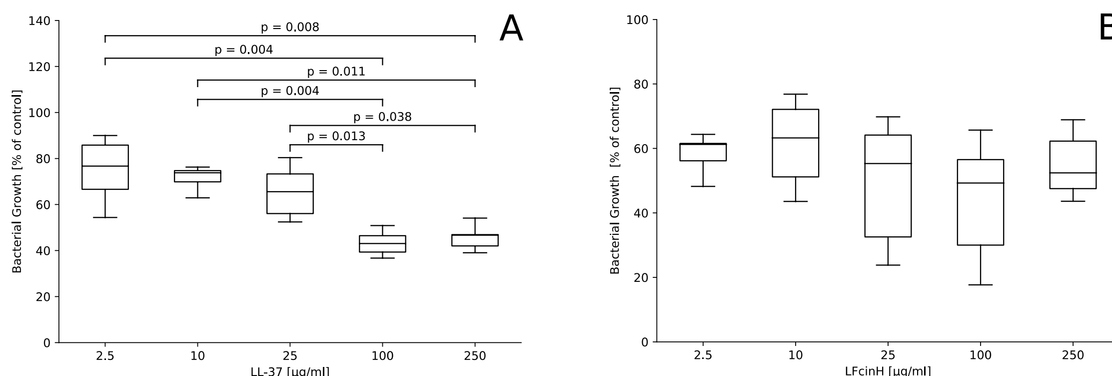

Figure 1: Growth inhibition of facultative anaerobic bacteria in a multi-species suspension by LL-37 (**A**) and LfcinH (**B**). Bacterial growth shown as the percentage of the control group for five selected test concentrations. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ. P-values determined by Dunn's test with a non-negative two stage false discovery rate correction.

The suppression of the planktonic growth by LL-37 showed a concentration dependence: at 100 µg/ml a sharp drop in bacterial growth was observed causing statistical significances (Kruskal-Wallis test, $p < 0.0001$). Although the planktonic bacterial growth was overall inhibited by LfcinH, it did not show a significant concentration dependence. A minimal increase in growth at 250 µg/ml was observed for both AMPs.

For the OAB, both AMPs showed an inhibitory effect on bacterial growth during incubation. The median growth was reduced to a percentage ranging from 11% to 65% for LL-37 and from 52% to 64% for LfcinH (***Table 2***). The median values indicate that the growth inhibition by LL-37 was greater for the OAB than for the FAB.

c (AMP) [µg/ml]		**2.5**	**10**	**25**	**100**	**250**
FAB	LL-37	*76.71% (19.21)*	*73.82% (4.87)*	*65.56% (17.22)*	*43.06% (7.12)*	*46.67% (4.90)*
	LfcinH	*61.25% (5.38)*	*63.27% (20.93)*	*55.30% (31.59)*	*49.27% (26.49)*	*52.42% (14.74)*
OAB	LL-37	*50.55% (13.56)*	*64.94% (12.80)*	*51.85% (12.29)*	*10.75% (10.38)*	*29.25% (23.76)*
	LfcinH	*52.24% (17.74)*	*54.49% (23.70)*	*58.99% (32.39)*	*53.07% (14.73)*	*64.24% (13.82)*

Table 2: Median growth of planktonic facultative (FAB) and obligate anaerobic (OAB) multi-species cultures after incubation with the AMPs LL-37 and LfcinH in different concentrations. Data presented as the percentage of the control group with the Interquartile Range (IQ).

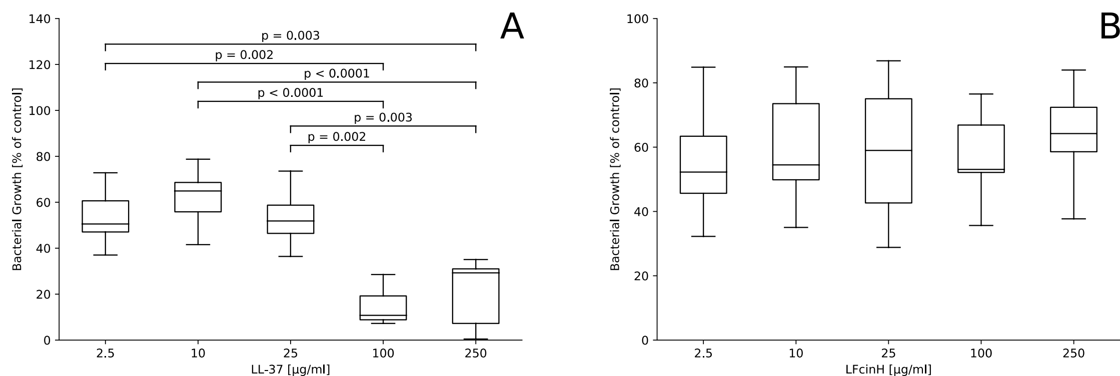

Figure 2: Growth inhibition of obligate anaerobic bacteria in a multi-species suspension by LL-37 (**A**) and LfcinH (**B**). Bacterial growth shown as the percentage of the control group for five selected test concentrations. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ. P-values determined by Dunn's test with a non-negative two stage false discovery rate correction.

Plots for the bacterial counts are shown in ***Figure 2***. For LL-37, significant differences between the concentrations were found (Kruskal-Wallis test, $p < 0.0001$). As previously observed for the FAB, the bacterial growth showed a sharp drop at a LL-37 concentration of 100 µg/ml, followed by a slight increase in growth at 250 µg/ml. On the other hand, LfcinH did not show any significant differences between the selected concentrations. Once again, the median growth of the bacteria seemed to be slightly promoted at 250 µg/ml.

Effects of LL-37 and LfcinH on the inhibition of biofilm growth in a multi-species biofilm

For both AMPs an inhibitory effect on the biofilm grown with the FAB was observed. Biofilm growth compared to the control group ranged from 48% to 73% and from 53% to 78% after incubation with LL-37 and LfcinH, respectively (***Table 3***).

Descriptive statistical analyses of produced biofilm mass by FAB are shown in ***Figure 3*** for each AMP. The in-vitro biofilm growth was influenced by the AMPs in a concentration dependent manner: while the mass of produced biofilms after incubation with LL-37 was reduced at higher concentrations, LFcinH led to the opposite effect. Both AMPs caused statistical significances (Kruskal-Wallis test, $p < 0.0001$).

c (AMP) [µg/ml]		**2.5**	**10**	**25**	**100**	**250**
FAB	LL-37	*71.66% (18.28)*	*72.60% (10.50)*	*62.40% (25.92)*	*51.29% (8.98)*	*48.29% (5.48)*
	LfcinH	*73.19% (21.00)*	*52.71% (15.87)*	*55.06% (26.20)*	*76.39% (25.37)*	*78.44% (18.65)*
OAB	LL-37	*70.06% (18.41)*	*62.49% (15.27)*	*75.59% (26.38)*	*69.28% (19.98)*	*43.92% (12.48)*
	LfcinH	*77.95% (22.33)*	*64.70% (16.66)*	*67.30% (30.02)*	*69.51% (15.59)*	*62.81% (15.54)*

Table 3: Median biofilm mass in a multi-species, facultative anaerobic (FAB) and obligate anaerobic (OAB) biofilm influenced by LL-37 and LfcinH during incubation. Data shown as the percentage of the control group for five selected AMP concentrations.

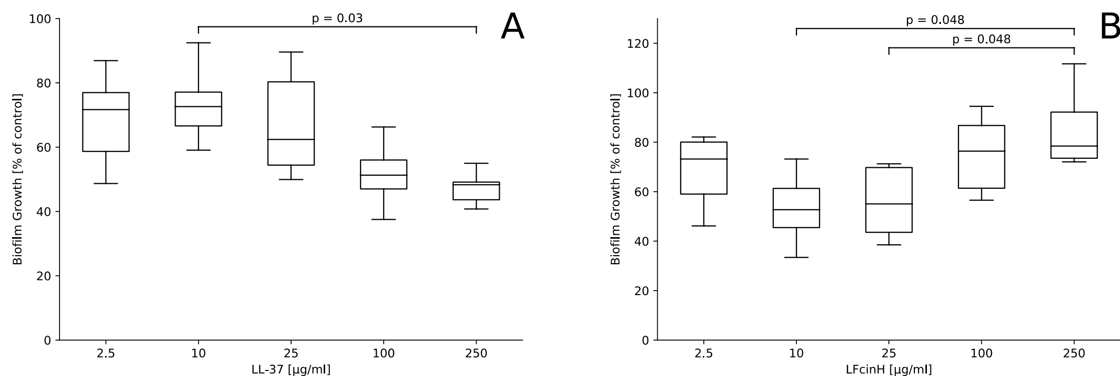

Figure 3: Inhibition of biofilm formation by LL-37 (**A**) and LfcinH (**B**). Multi-species biofilm formed by facultative anaerobic bacteria. Optical density shown as the percentage of the control group for five different test concentrations. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ. P-values determined by Dunn's test with a non-negative two stage false discovery rate correction.

For the OAB, the measured OD compared to the control group showed a range of 44% to 76% and 63% to 78% for LL-37 and LfcinH, respectively. Median values for the OD are shown in ***Table 3*** as the percentage of the control group.

Box-and-whisker-plots showing the biofilm mass in an obligate anaerobically grown biofilm are presented in ***Figure 4***. For LL-37 a sharp drop in biofilm production was observed at an AMP concentration of 250 µg/ml, causing a statistical significance (Kruskal-Wallis test, $p < 0.0001$). Despite the median values for LfcinH, which suggest an inhibitory activity, the box-and-whisker-plot reveals barely any inhibition.

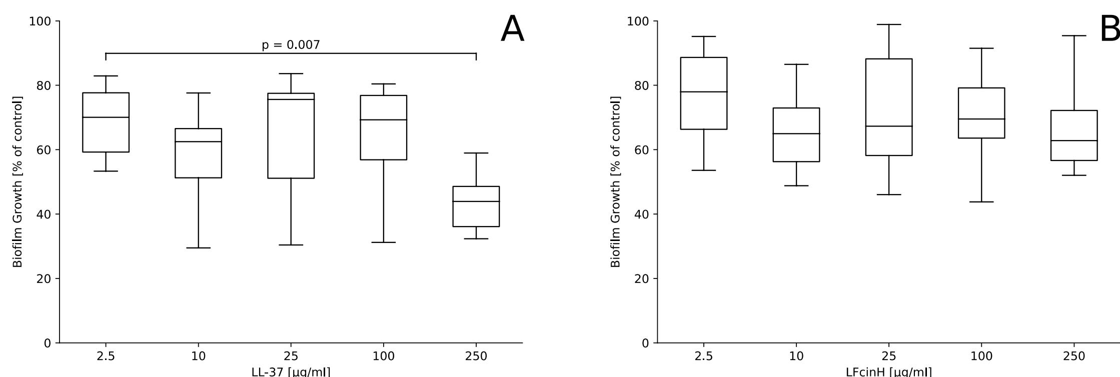

Figure 4: Inhibition of biofilm formation by LL-37 (**A**) and LfcinH (**B**). Multi-species biofilm formed by obligate anaerobic bacteria. Optical density shown as the percentage of the control group for five different test concentrations. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ. P-values determined by Dunn's test with a non-negative two stage false discovery rate correction.

4. Discussion

In this study, the antimicrobial potential of two human AMPs was tested on planktonic growth and biofilm formation of either FAB or OAB that are involved in caries and periodontitis. The bacterial species as well as the grouping were primarily determined based on their relevance to the oral diseases, caries and periodontitis. *S. mutans*, *S. sanguinis* and *A. naeslundii* are predominant species in supragingival dental plaque and were therefore used to mimic caries-inducing biofilms. This type of facultative anaerobic three-strain biofilm model has also been used in previous studies as a modification of the Zürich biofilm model [26,27]. While *S. sanguinis* and *A. naeslundii* are among the first bacteria to colonize the dental surfaces and are known to interact with each other via coaggregation mechanisms during plaque formation, *S. mutans* is considered the key microorganism for the development of dental caries. In contrast to the predominantly facultative anaerobic species in caries-inducing biofilms, plaque formation in periodontitis is characterized by increasing proportions of motile, gram-negative and obligate anaerobic species. Therefore, *V. parvula*, *P. micra* and *F. nucleatum* were chosen as obligate anaerobic representatives for subgingival biofilms. Particularly *F. nucleatum* is numerically predominant in subgingival plaque, presumably due to metabolic communication and coaggregation properties with other bacteria. For instance, both of the other species chosen for the obligate anaerobic three-strain biofilm model, *V. parvula* and *P. micra,* are known to coaggregate with *F. nucleatum,* as previous studies have discovered [28,29].

For testing the susceptibility of these bacterial species to antimicrobial substances, we opted for AMPs that are components of the human innate immune system. This requirement was fulfilled by LL-37 and LfcinH, which are both found in human serum as well as in human saliva. Salivary levels reported for LL-37 range from 2.2-30.5 ng/mL [30,31] and for LFcinH from 1-8 µg/mL [32,33]. In this study, the AMPs were prepared in alternating 2.5- and 4-fold dilutions, starting at 250 µg/ml. We deliberately selected concentrations that were higher than the physiological AMP levels in order to generate data for potential therapeutic usage.

During incubation with LL-37, the planktonic growth of multi-species FAB and OAB was affected in a concentration dependent manner. The inhibition progressed in two stages: primary reduction of planktonic bacterial growth was registered at the lower AMP concentrations, followed by a second significant reduction of growth at 100 µg/ml for both groups of bacteria. These findings suggest that LL-37 has a “threshold concentration” which seems to be between 25 µg/ml and 100 µg/ml for both FAB and OAB. Furthermore, our results indicate that the planktonic multi-species OAB are more susceptible to LL-37 than the FAB, especially after reaching the presumed threshold concentration. We propose that the availability of oxygen

might affect the antimicrobial activity of LL-37. A previous study that compared the antimicrobial action of LL-37 under aerobic and anaerobic conditions revealed that certain facultative anaerobic bacteria showed an increased susceptibility towards LL-37 under anaerobiosis [34]. For further research it would be therefore interesting to alter the experimental setup by testing the LL-37 susceptibility of the FAB we used in our study under oxygen deprivation and to subsequently draw a comparison to their susceptibility tested under aerobic conditions. Taking into account that we are aiming to represent a caries-inducing biofilm with these bacteria, this indeed appears to be a reasonable approach, since the availability of oxygen especially in the depth of matured biofilms is restricted and the proposed method would therefore mimic these conditions. As opposed to LL-37, the planktonic growth inhibition of FAB and OAB by LfcinH did not show a concentration dependency. Moreover, no threshold concentration was seen within the range of the chosen concentrations. As streptococci are known to produce a wide range of enzymes, proteolytic degradation of LfcinH may be a possible explanation for the observed results. Unlike LL-37, whose antimicrobial action seemed to be affected by the availability of oxygen, LfcinH did not inhibit the OAB more than the FAB, as the median values showed a very similar range. Thus, LfcinH's antimicrobial activity seems to function independently of oxygen availability.

Throughout all of the experiments performed on planktonic bacteria, we observed that the parameters used for growth measurement appeared to increase at the highest AMP concentrations. We suggest that this seemingly promoted growth is merely an artifact and probably results from reduced antimicrobial activity of the AMPs caused by protein precipitation at extremely high concentrations. In accordance with this proposition, the precipitation was perceivable as turbidity of the AMP solution/bacterial suspension. As the physiological concentrations of the AMPs in biological fluids are considerably lower than the ones tested in-vitro and it is therefore unlikely for AMP precipitation to occur in-vivo.

The amount of biofilm mass was affected by adding AMP solutions to the bacterial suspensions during biofilm formation, however the overall inhibition was less pronounced in comparison to the planktonic growth inhibition. Moreover, the threshold concentration for LL-37 appeared at 250 µg/ml instead of at 100 µg/ml as for the planktonic bacteria. It is a well-known fact that bacteria exhibit an enhanced antimicrobial resistance in biofilms, thus it is not surprising that the overall inhibitory effect of the AMPs on the biofilm formation was weaker at the given concentrations. The reduced antimicrobial potency of the AMPs in biofilms is most likely attributable to the physiological properties of microbial communities. Bacterial DNA and polymers of the extracellular matrix create a hydrated and charged environment surrounding

the bacterial surface which can delay the access, penetration and translocation of the cationic AMPs. Moreover, it is important to bear in mind that the peptides' tertiary structure is dependent on their environmental features, such as temperature, pH, electrolytic surroundings and hydrophilicity of the solvent. Previous studies have shown that LfcinH exhibits a partially folded, ribbon-like structure with a coiled backbone in aqueous solution, whereas a membrane mimetic solution induces a more pronounced helical conformation [35]. The latter may be therefore more apt to penetrate lipid membranes as the helix bears stronger amphipathic properties than the ribbon structure. In this study, acetic acid was used as solvent for the AMPs and BHI was used as growth medium for the bacteria. Since both liquids are aqueous solutions, the partially folded conformation of LfcinH undoubtedly predominated in the bacterial suspension/AMP solution used to submerge the ZrO_2 discs for biofilm growth. However, the biofilm surface does not primarily exhibit bacterial cells, instead the outermost layer contains a substantial amount of exopolysaccharides and other components of the extracellular matrix. The bacterial membranes in biofilms are therefore not markedly exposed to their surroundings and chances for LfcinH to interact with the membranes and to resultantly adapt a helical conformation are certainly lower. It is reasonable to say that the full antimicrobial potential of LfcinH cannot be utilized in our biofilm model, which may explain the weaker inhibitory effect it had on the biofilm formation compared to the planktonic growth of the same multi-species bacterial suspensions. Nevertheless, membrane interaction and perturbation do not seem to be the only mechanisms of action of the AMPs, or else no anti-biofilm effect would have been assessed at all. Previous studies have shown that anti-biofilm effects of AMPs are likely to stem from inhibition of bacterial adhesion on solid surfaces or interference with quorum sensing signals which lead to a downregulation of the two most important quorum sensing systems Las and Rh1 [36]. The components of the extracellular matrix and metabolic products used for opportunistic growth in biofilms are further possible target areas for AMPs.

There are numerous immunomodulatory strategies that AMPs use for host defence during inflammatory processes. For example, AMPs mediate the inflammatory response with impact on epithelial and inflammatory cells, resulting in cytokine release, cell proliferation, angiogenesis, wound healing and chemotaxis [37].

Despite promising features of AMPs for potential therapeutic use, many challenges remain for future development. For example, when ingested after systemic application, peptides tend to be degraded or bound and inactivated by protein binding in the blood stream [38]. Moreover, high concentrations of ions, proteins and polysaccharides are in-vivo conditions in biological fluids and may alter the antimicrobial potency of the AMPs [37]. Since the activity of AMPs

is not always clearly defined, toxicity against eukaryotic cells is a relevant matter and must be considered especially for AMPs which are not of human origin. Ultimately, if AMPs are meant to be routinely prescribed, the pharmaceutical production cost is an important factor for creating large quantities. As the synthesis of peptides requires a complex purification step to isolate the desired peptide from its contaminant, the cost for producing AMPs is generally much higher compared to conventional antibiotics.

5. Conclusion

Our results demonstrated that two AMPs of human origin affect planktonic and biofilm growth of the FAB and OAB tested in this study. The inhibition of planktonic growth and biofilm formation by LL-37 progressed depending on the AMP concentration in two stages: subsequent to a primary reduction at lower concentrations, LL-37 seems to have an inhibitory threshold concentration for a second significant reduction of planktonic growth and biofilm mass at higher concentrations, which varied depending on the experimental setup. Furthermore, the antimicrobial activity of LL-37 may be affected by the availability of oxygen, as the OAB were more susceptible to LL-37 than the FAB. Contrarily, LfcinH inhibited the planktonic growth of FAB and OAB in a similar manner regardless of the oxygen presence. However, no inhibitory threshold concentration was observed for either bacterial group within the range of the tested LfcinH concentrations. Biofilm formation was inhibited by low LfcinH concentrations, whereas the amount of biofilm mass produced by OAB was barely reduced by LfcinH.

While our results reveal promising features for LL-37 as a potential therapeutic for biofilm-associated oral diseases, LfcinH appears to be unsuitable for this very indication in spite of its well-known antimicrobial properties. Examining the effect of these AMPs on matured biofilms and investigating combined strategies of antimicrobials, for example with conventional antibiotics, are some future considerations. Nevertheless, many more investigations are necessary for eventually applying AMPs as therapeutic antimicrobial in the case of biofilm-associated oral diseases.

Acknowledgements

Our special thanks go to the dental laboratory *Dentaltechnik Kollmuß GmbH* and the master dental technician Robert Kollmuß for kindly providing us with the ZrO_2 discs used in this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] S.L. James, D. Abate, K.H. Abate, S.M. Abay, C. Abbafati, N. Abbasi et al., Global, regional, and national incidence, prevalence, and years lived with disability for 354 Diseases and Injuries for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Study 2017, Lancet. 392 (2018) 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7.

[2] M. di Luca, G. Maccari, R. Nifosí, Treatment of microbial biofilms in the post-antibiotic era: Prophylactic and therapeutic use of antimicrobial peptides and their design by bioinformatics tools, Pathog. Dis. 70 (2014) 257–270. https://doi.org/10.1111/2049-632X.12151.

[3] L. Grassi, G. Maisetta, S. Esin, G. Batoni, Combination Strategies to Enhance the Efficacy of Antimicrobial Peptides against Bacterial Biofilms., Front. Microbiol. 8 (2017) 2409. https://doi.org/10.3389/fmicb.2017.02409.

[4] A. Mombelli, A. Almaghlouth, N. Cionca, D.S. Courvoisier, C. Giannopoulou, Differential benefits of amoxicillin-metronidazole in different phases of periodontal therapy in a randomized controlled crossover clinical trial., J. Periodontol. 86 (2015) 367–375. https://doi.org/10.1902/jop.2014.140478.

[5] A.J. van Winkelhoff, T.E. Rams, J. Slots, Systemic antibiotic therapy in periodontics., Periodontol. 2000. 10 (1996) 45–78. https://doi.org/10.1111/j.1600-0757.1996.tb00068.x.

[6] S.S. Oberoi, C. Dhingra, G. Sharma, D. Sardana, Antibiotics in dental practice: how justified are we., Int. Dent. J. 65 (2015) 4–10. https://doi.org/10.1111/idj.12146.

[7] B.A. Dale, L.P. Fredericks, Antimicrobial peptides in the oral environment: expression and function in health and disease., Curr. Issues Mol. Biol. 7 (2005) 119–133. https://doi.org/10.1093/jac/dki103.

[8] R.E.W. Hancock, H.-G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies., Nat. Biotechnol. 24 (2006) 1551–1557. https://doi.org/10.1038/nbt1267.

[9] M. Zasloff, Antimicrobial peptides of multicellular organisms., Nature. 415 (2002) 389–395. https://doi.org/10.1038/415389a.

[10] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides., Biochim. Biophys. Acta. 1462 (1999) 55–70. https://doi.org/10.1016/s0005-2736(99)00200-x.

[11] M. Murakami, T. Ohtake, R.A. Dorschner, R.L. Gallo, Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva., J. Dent. Res. 81 (2002) 845–850. https://doi.org/10.1177/154405910208101210.

[12] M. Puklo, A. Guentsch, P.S. Hiemstra, S. Eick, J. Potempa, Analysis of neutrophil-derived antimicrobial peptides in gingival crevicular fluid suggests importance of cathelicidin LL-37 in the innate immune response against periodontogenic bacteria., Oral Microbiol. Immunol. 23 (2008) 328–335. https://doi.org/10.1111/j.1399-302X.2008.00433.x.

[13] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238–250. https://doi.org/10.1038/nrmicro1098.

[14] A.J. Duplantier, M.L. van Hoek, The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds, Front. Immunol. 4 (2013) 1–14. https://doi.org/10.3389/fimmu.2013.00143.

[15] S. Ji, J. Hyun, E. Park, B.-L. Lee, K.-K. Kim, Y. Choi, Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils., J. Periodontal Res. 42 (2007) 410–419. https://doi.org/10.1111/j.1600-0765.2006.00962.x.

[16] J.E. Goeke, S. Kist, S. Schubert, R. Hickel, K.C. Huth, M. Kollmuss, Sensitivity of caries pathogens to antimicrobial peptides related to caries risk., Clin. Oral Investig. 22 (2018) 2519–2525. https://doi.org/10.1007/s00784-018-2348-7.

[17] M.H. Metz-Boutigue, J. Jollès, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, P. Jollès, Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins., Eur. J. Biochem. 145 (1984) 659–676. https://doi.org/10.1111/j.1432-1033.1984.tb08607.x.

[18] N. Bruni, M.T. Capucchio, E. Biasibetti, E. Pessione, S. Cirrincione, L. Giraudo, A. Corona, F. Dosio, Antimicrobial Activity of Lactoferrin-Related Peptides and Applications in Human and Veterinary Medicine., Molecules. 21 (2016). https://doi.org/10.3390/molecules21060752.

[19] F. Berlutti, F. Pantanella, T. Natalizi, A. Frioni, R. Paesano, A. Polimeni, P. Valenti, Antiviral properties of lactoferrin--a natural immunity molecule., Molecules. 16 (2011) 6992–7018. https://doi.org/10.3390/molecules16086992.

[20] M. Sinha, S. Kaushik, P. Kaur, S. Sharma, T.P. Singh, Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein., Int. J. Pept. 2013 (2013) 390230. https://doi.org/10.1155/2013/390230.

[21] H. Wakabayashi, K. Yamauchi, T. Kobayashi, T. Yaeshima, K. Iwatsuki, H. Yoshie, Inhibitory effects of lactoferrin on growth and biofilm formation of Porphyromonas gingivalis and Prevotella intermedia., Antimicrob. Agents Chemother. 53 (2009) 3308–3316. https://doi.org/10.1128/AAC.01688-08.

[22] H. Wakabayashi, M. Takase, M. Tomita, Lactoferricin derived from milk protein lactoferrin., Curr. Pharm. Des. 9 (2003) 1277–1287. https://doi.org/10.2174/1381612033454829.

[23] P. Glimvall, C. Wickström, H. Jansson, Elevated levels of salivary lactoferrin, a marker for chronic periodontitis?, J. Periodontal Res. 47 (2012) 655–660. https://doi.org/10.1111/j.1600-0765.2012.01479.x.

[24] J.H. Merritt, D.E. Kadouri, G.A. O'Toole, Growing and analyzing static biofilms., Curr. Protoc. Microbiol. Chapter 1 (2005) Unit 1B.1. https://doi.org/10.1002/9780471729259.mc01b01s00.

[25] G. van Rossum, F. Drake, Python 3 Reference Manual, (2019). https://www.python.org.

[26] A.M. Marttinen, P. Ruas-Madiedo, C. Hidalgo-Cantabrana, M.A. Saari, R.A. Ihalin, E.M. Söderling, Effects of xylitol on xylitol-sensitive versus xylitol-resistant Streptococcus mutans strains in a three-species in vitro biofilm., Curr. Microbiol. 65 (2012) 237–243. https://doi.org/10.1007/s00284-012-0151-2.

[27] B. Guggenheim, M. Guggenheim, R. Gmür, E. Giertsen, T. Thurnheer, Application of the Zürich biofilm model to problems of cariology., Caries Res. 38 (2004) 212–222. https://doi.org/10.1159/000077757.

[28] P. Kolenbrander, R. Andersen, L. Moore, Coaggregation of Fusobacterium nucleatum, Selenomonas flueggei, Selenomonas infelix, Selenomonas noxia, and Selenomonas sputigena with strains from 11 genera of oral bacteria., Infect. Immun. 57 (1989) 3194–3203.

[29] A. Horiuchi, E. Kokubu, T. Warita, K. Ishihara, Synergistic biofilm formation by Parvimonas micra and Fusobacterium nucleatum., Anaerobe. 62 (2020) 102100. https://doi.org/10.1016/j.anaerobe.2019.102100.

[30] S. Davidopoulou, E. Diza, D. Sakellari, G. Menexes, S. Kalfas, Salivary concentration of free LL-37 in edentulism, chronic periodontitis and healthy periodontium., Arch. Oral Biol. 58 (2013) 930–934. https://doi.org/10.1016/j.archoralbio.2013.01.003.

[31] C.W. Cheah, A.R. Al-Maleki, J. Vadivelu, M. Danaee, S. Sockalingam, N.A. Baharuddin, R.D. Vaithilingam, Salivary and serum cathelicidin LL-37 levels in subjects with rheumatoid arthritis and chronic periodontitis., Int. J. Rheum. Dis. (2020). https://doi.org/10.1111/1756-185X.13919.

[32] D.H. Fine, Lactoferrin: A Roadmap to the Borderland between Caries and Periodontal Disease., J. Dent. Res. 94 (2015) 768–776. https://doi.org/10.1177/0022034515577413.

[33] L. Rosa, A. Cutone, M.S. Lepanto, R. Paesano, P. Valenti, Lactoferrin: A Natural Glycoprotein Involved in Iron and Inflammatory Homeostasis., Int. J. Mol. Sci. 18 (2017). https://doi.org/10.3390/ijms18091985.

[34] A. Eini, A. Sol, S. Coppenhagen-Glazer, Y. Skvirsky, A. Zini, G. Bachrach, Oxygen deprivation affects the antimicrobial action of LL-37 as determined by microplate real-time kinetic measurements under anaerobic conditions., Anaerobe. 22 (2013) 20–24. https://doi.org/10.1016/j.anaerobe.2013.04.014.

[35] H.N. Hunter, A.R. Demcoe, H. Jenssen, T.J. Gutteberg, H.J. Vogel, Human lactoferricin is partially folded in aqueous solution and is better stabilized in a membrane mimetic solvent, Antimicrob. Agents Chemother. 49 (2005) 3387—3395. https://doi.org/10.1128/aac.49.8.3387-3395.2005.

[36] J. Overhage, A. Campisano, M. Bains, E.C.W. Torfs, B.H.A. Rehm, R.E.W. Hancock, Human host defense peptide LL-37 prevents bacterial biofilm formation., Infect. Immun. 76 (2008) 4176–4182. https://doi.org/10.1128/IAI.00318-08.

[37] P.Y. Chung, R. Khanum, Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria., J. Microbiol. Immunol. Infect. 50 (2017) 405–410. https://doi.org/10.1016/j.jmii.2016.12.005.

[38] J. Bradshaw, Cationic antimicrobial peptides : issues for potential clinical use., BioDrugs. 17 (2003) 233–240. https://doi.org/10.2165/00063030-200317040-00002.

7. Veröffentlichung II

Targeting antibiotic tolerance in anaerobic biofilms associated with oral diseases: Human antimicrobial peptides LL-37 and Lactoferricin enhance the antibiotic efficacy of amoxicillin, clindamycin and metronidazole

Sabina Noreen **Wuersching**, Karin Christine **Huth**, Reinhard **Hickel**, Maximilian **Kollmuss**

Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Goethestraße 70, 80336 Munich, Germany

Journal: ***Anaerobe*** (Impact Factor 2020: 3,331)

Wuersching SN, Huth KC, Hickel R, Kollmuss M. Targeting antibiotic tolerance in anaerobic biofilms associated with oral diseases: Human antimicrobial peptides LL-37 and lactoferricin enhance the antibiotic efficacy of amoxicillin, clindamycin and metronidazole. Anaerobe. 2021 Aug 25;71:102439. doi: 10.1016/j.anaerobe.2021.102439.

Received 2 June 2021
Received in revised form 27 July 2021
Accepted 22 August 2021
Available online 25 August 2021

Abstract

Antimicrobial peptides are receiving increasing attention as potential therapeutic agents for treating biofilm-related infections of the oral cavity. Many bacteria residing in biofilms exhibit an enhanced antibiotic tolerance, which grants intrinsically susceptible microorganisms to survive lethal concentrations of antibiotics. In this study, we examined the effects of two endogenous human antimicrobial peptides, LL-37 and human Lactoferricin, on the antibiotic drug efficacy of amoxicillin, clindamycin and metronidazole in two types of polymicrobial biofilms, which aimed to represent frequent oral diseases: (1) facultative anaerobic (*Streptococcus mutans*, *Streptococcus sanguinis*, *Actinomyces naeslundii*) and (2) obligate anaerobic biofilms (*Veillonella parvula*, *Parvimonas micra*, *Fusobacterium nucleatum*). LL-37 and Lactoferricin enhanced the anti-biofilm effect of amoxicillin and clindamycin in facultative anaerobic biofilms. Metronidazole alone was ineffective against facultative anaerobic biofilms, but the presence of LL-37 and Lactoferricin led to a greater biofilm reduction. Obligate anaerobic biofilms showed an increased drug tolerance to amoxicillin and clindamycin, presumably due to metabolic downshifts of the bacteria residing within the biofilm. However, when combined with LL-37 or Lactoferricin, the reduction of obligate anaerobic biofilms was markedly enhanced for all antibiotics, even for amoxicillin and clindamycin. Furthermore, our results suggest that antimicrobial peptides enhance the dispersion of matured biofilms, which may be one of their mechanisms for targeting biofilms. In summary, our study proves that antimicrobial peptides can serve as an auxiliary treatment strategy for combatting enhanced antibiotic tolerance in bacterial biofilms.

Keywords

biofilm, drug tolerance, cationic antimicrobial peptides, LL-37, human lactoferricin, anaerobes

Abbreviations

AMP = antimicrobial peptide; *LfcinH* = human Lactoferricin; *FAB* = facultative anaerobic bacteria; *OAB* = obligate anaerobic bacteria; *AMX* = amoxicillin; *CLI* = clindamycin; *MDZ* = metronidazole

1. Introduction

Biofilm-related infections have emerged as one of the biggest challenges facing medicine today. The discovery of biofilms dates back to the 17th century, when the scientist Antonie van Leeuwenhoek described a vast accumulation of microbes he had observed through his microscope on scrapings of dental plaque from his teeth [1]. Since the early 1980s, bacterial biofilms have been identified as the culprit of various tissue infections, including osteomyelitis, endocarditis, dental caries and periodontitis. Furthermore, they are frequent causes of infections originating from implanted devices, such as joint prostheses, catheters, prosthetic heart valves and dental implants [2]. Particularly the oral cavity is at an increased risk for bacterial infections due to its unique interface between the internal and external environment on the alveolar process, the thickened bone ridge of the jaw forming the tooth sockets. It is the only region of the human body where hard tissues break through the epithelial surface, making it more vulnerable and susceptible to infections, especially in view of its constant exposure to pathogenic microorganisms in the oral cavity [3]. Oral biofilms are often composed of a consortium of interacting microorganisms rather than just one bacterial species. The development of such polymicrobial biofilms involves sophisticated mechanisms of inter-species communication, and once in a matured state, the biofilm benefits the microorganisms by offering a protective environment against the host's defense system [4]. One of the reasons for the biofilms' inherent antibiotic resistance (= tolerance) is the biofilm matrix, which is composed of polysaccharides, DNA and proteins, and serves as a barrier against the penetration of antimicrobial agents [5]. Particularly antibiotics that are designed to target a specific domain of the microbial cell fail to reach the target site of the sessile bacteria at a sufficient concentration. Aside from the fact that increasing the antibiotic concentration does not necessarily ensure a penetration of the biofilm matrix, the administration of larger antibiotic doses is also often limited in clinical practice due to the higher risk of toxic side effects [6].
In view of the antibiotics' limitations for the antimicrobial treatment of biofilms, there is a great demand for the development of alternative strategies to combat biofilm-related infections, especially in cases where the complete mechanical removal of the infected tissues is not possible. The antimicrobial peptides (AMPs) are a part of the innate immune system and a class of naturally occurring agents with immunomodulatory properties and a wide range of antimicrobial activity against bacteria, fungi, viruses and even parasites. Unlike antibiotics, which have been subject to the development of resistances ever since their discovery, AMPs have surprisingly not developed wide-spread resistance despite having co-evolved with bacteria over millions of years [7]. AMPs are epithelial-cell-derived, cationic peptides which

have been identified on various epithelial surfaces as a part of the primary defense line against invading pathogens. In the oral cavity, AMPs are to be found within the granules of neutrophils as well as in epithelial cells of the oral mucosa and the salivary glands [3]. Though being relatively small in size (10-50 amino acids), AMPs exhibit an amphipathic structure with a net positive charge, enabling them to gain access to the periplasmic space of the microbial cell via mechanisms such as transmembrane pore formation, which eventually cause cell lysis [8]. Their wide antimicrobial spectrum, their low tendency to induce resistance and their prevalence in the oral cavity make them attractive as potential therapeutic agents for the antimicrobial treatment of biofilm-related oral infections. One of the best studied AMPs is LL-37, a member of the cathelicidin family, which is known to contribute to the broad-spectrum defense in the oral cavity by modulating inflammatory and immune responses, accelerating the angiogenesis, promoting wound healing, and neutralizing lipopolysaccharides [3]. Changes in LL-37 levels are associated with chronic inflammatory diseases, including psoriasis, lupus erythematosus, gingivitis and periodontal disease [9,10]. A further, naturally occurring protein with antimicrobial activity is lactoferrin, an iron-binding glycoprotein of the transferrin family, which is frequently found in human exocrine secretions, including saliva, gingival crevicular fluid, tears and milk. Lactoferricin is a functional antimicrobial peptide derived from lactoferrin through pepsin-mediated proteolysis and is thought to be a potent antibacterial and anti-fungal agent. A previous study demonstrated anti-biofilm activity of bovine lactoferricin against matured mono-species biofilms formed by periodontal pathogens, suggesting a potential therapeutic application for the treatment and prevention of periodontal disease [11]. However, to our knowledge there are only few studies examining the anti-biofilm effects of human Lactoferricin (LfcinH) in association with oral diseases.

In our previous work, we demonstrated that LL-37 and LfcinH interfere with the planktonic growth and biofilm formation of facultative and obligate anaerobic oral pathogens that are responsible for dental caries and periodontitis [12]. Unlike many antibiotics which are dependent on an active cell metabolism for a sufficient effect, AMPs, having a low specificity of their molecular target, are able to even target metabolically dormant cells, which are to be found in substantial regions of matured biofilms [13]. This prompts the question whether these AMPs are able to enhance the antimicrobial potency of antibiotics in preformed polymicrobial biofilms, for example by virtue of facilitating the antibiotics' accessibility to the microorganisms within the biofilm. The objectives of this study were to examine synergistic effects of human AMPs, such as LL-37 and LfcinH, and common antibiotics used to treat biofilm-related infections in the oral cavity. For a more realistic approach, we opted for multi-

species biofilms and included two different microbial compositions which are frequently found in common oral conditions.

2. Materials and Methods

Antimicrobials

The term "antibiotic" describes any class of organic molecule with bacteriostatic or bactericidal activity and not a specific class of pharmaceuticals. For the sake of clarity, "antibiotic" will be used solely for pharmaceuticals and not for the antimicrobial peptides in this study. All antibiotics were obtained from Merck (Merck KGaA, Darmstadt, Germany). Amoxicillin (AMX), clindamycin (CLI), metronidazole (MDZ) and combined amoxicillin and metronidazole (AMX/MDZ) were tested at five different concentrations, which included each agent's reported serum (SER) and gingival crevicular fluid (GCF) concentrations after oral application [14–17]. The AMPs LL-37 (Innovagen AB, Lund, Sweden) and LfcinH (AnaSpec Inc., Fremont, CA, USA) were tested at five different concentrations in alternating 2.5- and 4-fold dilutions. Their amino acid sequences are shown in ***Table 1***. Antimicrobial combinations were tested by adding two different AMP concentrations to each antibiotic SER and GCF concentration. Final concentrations of all antimicrobial substances are shown in ***Table 2*** along with their required solvents.

AMP	Amino acid sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
LfcinH	TKCFQWQRNMRKVR-G-PPVSCIKRDS

Table 1: Amino acid sequences of the antimicrobial peptides LL-37 and human Lactoferricin (LfcinH)

Bacterial strains and cultivation

Two types of bacteria were examined in this study: (1) facultative anaerobic bacteria (FAB) *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguinis* (ATCC 10556) and *Actinomyces naeslundii* (ATCC 19039) and (2) obligate anaerobic bacteria (OAB) *Veillonella parvula* (ATCC 17745), *Parvimonas micra* (ATCC 33270) and *Fusobacterium nucleatum* (ATCC 49256). All strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were grown on Schaedler agar plates supplemented with Vitamin K1 and 5% Sheep Blood (Becton Dickinson, Franklin Lakes, NJ, USA). Planktonic cultivation of all bacteria was performed in Brain-Heart-Infusion broth (BHI, Becton Dickinson) supplemented with Hemin (5 µg/mL) and Vitamin K1 (1 µg/mL). The BHI medium for the OAB was prepared under an anaerobic atmosphere. FAB were cultivated at a temperature of 37°C and a humidity level of 60% in a 5.8% CO2 enriched atmosphere. All steps involving the OAB were performed in an anaerobic chamber (Sheldon Manufacturing Inc, Cornelius, OR, USA). OAB were grown at a temperature of 37°C in a 5% H_2, 10% CO_2,

85% N_2 atmosphere. The described media and incubation conditions for the FAB and OAB apply to all experiments unless otherwise stated.

Antimicrobial	Solvent	Test concentrations [µg/mL]				
AMX	1M NH_4OH	1	3.8^{SER}	10	14^{GCF}	20
CLI	$(CH_3)_2SO$ *(= DMSO)*	0.5	1	2$^{SER/GCF}$	5.0	10
MDZ	H_2O	0.5	1	3.6^{GCF}	6.1^{SER}	10
AMX/MDZ		1/1	3.8/6.1^{SER}	10/6.1	14/3.6^{GCF}	20/10
LL-37 LfcinH	0.0001% CH_3COOH	2.5	10*	25	100*	250

Table 2: Final test concentrations of the antimicrobials amoxicillin (AMX), clindamycin (CLI), metronidazole (MDZ), combined amoxicillin and metronidazole (AMX/MDZ), LL-37 and human Lactoferricin (LfcinH), shown with the solvents required to prepare each agent. SER: serum concentration; GCF: gingival crevicular fluid concentration; *, AMP concentrations selected for testing antimicrobial combinations with SER and GCF concentrations

Antimicrobial susceptibility testing

Prior to performing any susceptibility tests on multi-species biofilms, each individual strain was tested for intrinsic resistances against the antibiotics used in this study. Minimal inhibitory concentrations (MIC) of AMX, CLI and MDZ were determined for each strain using the ETEST method (bioMèrieux SA, Marcy-l'Étoile, France). Colonies were inoculated in supplemented BHI and turbidity was adjusted to a McFarland standard of 0.5 (FAB) or 1 (OAB). Bacterial suspensions were plated on Schaedler agar plates and incubated with the ETEST strips for 20-24 h (FAB) or 48-72 h (OAB) under the required growth conditions. MICs were interpreted according to the breakpoints reported in the guidelines from the Clinical and Laboratory Standards Institute (CLSI) [18].

Biofilm development

FAB and OAB biofilms were each developed on zirconium dioxide (ZrO_2) discs (6 mm diameter, 1 mm thickness) in independent experiments. The following steps were performed for both types of biofilms. The bacterial strains used for forming a three-species FAB or OAB biofilm were grown overnight in BHI broth medium under their required growth conditions. The cultures were diluted in fresh medium until an optical density of 0.1 at 600 nm was reached (Varioskan Microplate Reader, Thermo Fisher Scientific, Waltham, MA, USA). Equal volumes of the three diluted strains were combined in a sterile reaction tube and vortexed briefly. ZrO_2 discs were placed in the wells of a 96-well-plate with a flat bottom and the diluted three-species cell suspension was added to the wells, making sure that the discs were covered entirely in liquid. The well plates were incubated for four days under the required growth conditions. Biofilm growth was verified on an extra set of ZrO_2 discs along with each experiment using a modified crystal violet staining protocol as previously described [12].

Exposure of the matured biofilms to the antimicrobials and biofilm extraction

The selected concentrations of the antibiotic solutions were added to a volume of 10% to fresh medium (BHI) in the wells of a 96-well-plate. AMPs were added to a volume of 50%. Discs with four-day grown biofilms were removed from the bacterial suspension and washed twice in 0.9% sodium chloride solution in order to remove planktonic cells from the surface. Subsequently, the discs were submerged in the prepared antimicrobial solutions and incubated for four hours. The discs were then removed from the wells and washed twice in sodium chloride. The discs were stored in reaction tubes containing sodium chloride for one hour. Biofilm extraction was performed in three steps by modification of a previously reported protocol [19]:

1. Tubes were vortexed at full speed for 60 s.
2. A probe-based sonication was performed at 8 W for 60 s. Reaction tubes were cooled on ice during sonication to prevent heat damage to the cells.
3. Tubes were vortexed for another 60 s at full speed.

Quantification of viable cells

Ten-fold serial dilutions of the sonicates were prepared in sodium chloride and 100 µL of each dilution were plated on Schaedler agar plates using a spread plate method. The agar plates were incubated for 48 h (FAB) and 72 h (OAB) under the required growth conditions mentioned above. The CFU were counted following FDA guidelines (only plates with 25 to 250 colonies were considered).

Relative quantification of planktonic bacteria released from matured biofilms

Planktonic cells released from biofilms after incubation with the AMPs were quantified relatively by means of the BacTiter-Glo™ microbial cell viability assay (Promega, Madison, WI, USA), a luminescence-based viability kit which lyses cells to release intracellular ATP. The luminescent signal is directly proportional to the amount of ATP present, which again is directly proportional to the number of cells. After four hours of incubation with the biofilms, the medium containing the antimicrobial solutions was combined with the sodium chloride solution used for washing each disc after antimicrobial exposure. Equal amounts of this mixture and the BacTiterReagent were combined in the wells of an opaque 96-well plate and incubated for 5 min at room temperature. The same procedure was performed with the sonicates containing the cells extracted from the biofilms. Relative light units (RLUs) were recorded in a Luminometer (GloMax Navigator System, Promega, WI, USA).

Evaluation of metabolic adaptations in biofilms

In addition to performing the antimicrobial susceptibility tests on matured biofilms, metabolic adaptations of the bacteria residing in biofilms were examined. ATP-levels of the bacteria in matured, untreated FAB and OAB biofilms were compared to those of the planktonic bacteria dispersed from biofilms. Discs with four-day grown biofilms were removed from the bacterial suspension and washed twice in sodium chloride solution. Subsequently, the discs were submerged in fresh medium and incubated for four hours without any antimicrobial agent. The discs were washed twice and moved to reaction tubes containing sodium chloride. Sessile bacteria were extracted from the biofilms in the same manner as described above. The medium containing the planktonic cells dispersed from biofilms during the four-hour incubation was combined with the sodium chloride solution used for washing the biofilms after incubation. Since ATP is an indicator of the bacteria's cell metabolism, we used the BacTiter-Glo™ microbial cell viability assay's feature to measure the amount of ATP in terms of RLUs in the sonicates as well as in the medium/sodium chloride mixture. Additionally, ten-fold serial dilutions of both solutions were plated on agar plates to determine the CFU/mL. The CFU/RLU ratio was calculated for the sessile bacteria and for the planktonic bacteria dispersed from biofilms.

Statistics

All experiments were performed in duplicate for each condition and repeated five times. Statistical analyses were performed in Python 3.7.6 using scipy and scikit for inferential statistics and matplotlib for the descriptive analysis [20]. Homoscedasticity was assessed with Levene's test and data were tested for normality based on the Shapiro-Wilk test. As the data showed a non-parametric distribution, comparisons between groups were evaluated with a Kruskal-Wallis test. Post hoc analysis was conducted by means of a Dunn's test with a two-stage false discovery rate correction. P-values < 0.05 were considered statistically significant. Effect sizes were estimated by computation of Hedges' g with a 95% confidence interval.

3. Results

Antibiotic susceptibilities

MICs of AMX, CLI and MDZ for each strain are summarized in ***Table 3***. All strains were susceptible to AMX and CLI. The facultative anaerobes were resistant to MDZ.

Strain	Antibiotic	Range (µg/mL)	MIC (µg/mL)	Interpretation
Facultative anaerobic bacteria				
A. naeslundii	Amoxicillin	0.016 - 256	**0.023**	S
(ATCC 19039)	Clindamycin	0.016 - 256	**0.125**	S
	Metronidazole	0.016 - 256	> 256	R
S. mutans	Amoxicillin	0.016 - 256	**0.064**	S
(ATCC 25175)	Clindamycin	0.016 - 256	**0.094**	S
	Metronidazole	0.016 - 256	> 256	R
S. sanguinis	Amoxicillin	0.016 - 256	**0.25**	S
(ATCC 10556)	Clindamycin	0.016 - 256	**0.094**	S
	Metronidazole	0.016 - 256	> 256	R
Obligate anaerobic bacteria				
F. nucleatum	Amoxicillin	0.016 - 256	**0.064**	S
(ATCC 49256)	Clindamycin	0.016 - 256	**0.016**	S
	Metronidazole	0.016 - 256	**0.016**	S
P. micra	Amoxicillin	0.016 - 256	**2.0**	S
(ATCC 33270)	Clindamycin	0.016 - 256	**0.094**	S
	Metronidazole	0.016 - 256	**0.064**	S
V. parvula	Amoxicillin	0.016 - 256	**1.5**	S
(ATCC 17745)	Clindamycin	0.016 - 256	**0.94**	S
	Metronidazole	0.016 - 256	**6.0**	S

Table 3: Antimicrobial susceptibilities of the facultative and obligate anaerobic bacteria used in this study. Minimal inhibitory concentrations (MIC) determined with the ETEST method in the shown ranges. MICs interpreted according to guidelines form the Clinical and Laboratory Standards Institute (CLSI). R, resistant; S, susceptible.

Effects of the antibiotics alone

The effects of the antibiotics AMX, CLI, MDZ and combined AMX/MDZ on matured FAB and OAB biofilms are shown in ***Figure 1***. Within the range of the tested concentrations, FAB biofilms were mainly affected by AMX and CLI, whereas OAB biofilms were only reduced by MDZ and combined AMX/MDZ. The presence of AMX or CLI in FAB biofilms resulted in a significant reduction of CFU/mL at all tested concentrations, including the SER and GCF concentrations of both antibiotics. The median bacterial counts in FAB biofilms dropped by one log-scale with AMX and by one to two log-scales with CLI compared to the control group. FAB biofilms were barely affected by MDZ. A significant reduction of bacterial counts was registered only at 10 µg/mL (highest MDZ test concentration). However, the combination of AMX and MDZ reduced FAB biofilms significantly at the GCF concentration as well as at the

highest AMX/MDZ concentration. OAB biofilms were neither affected by AMX nor by CLI within the range of the tested concentrations. As opposed to the FAB biofilms, OAB biofilms were significantly reduced by MDZ, even at the SER and GCF concentration. Furthermore, MDZ showed an explicit concentration dependency and caused a 5-log drop for the median CFU/mL at the highest concentration. The presence of AMX combined with MDZ enhanced the biofilm reduction, even at both SER and GCF concentrations.

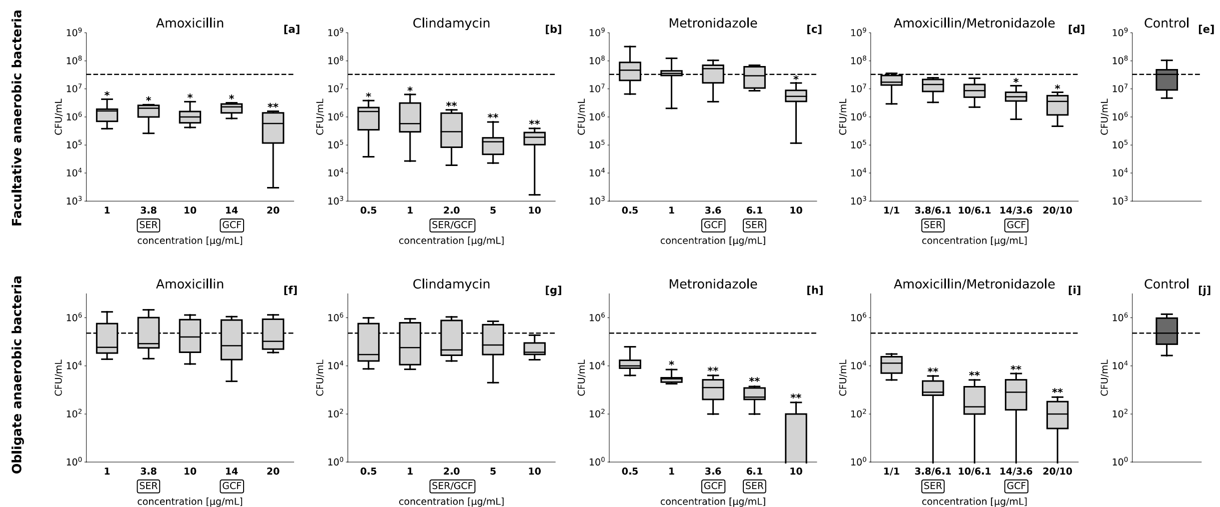

Figure 1: Bacterial counts (CFU/mL) in matured biofilms formed by facultative **(a-e)** and obligate anaerobic **(f-j)** bacteria after 4 h of incubation with different concentrations [µg/mL] of amoxicillin, clindamycin, metronidazole and combined amoxicillin/metronidazole. Test concentrations include the serum (SER) and gingival crevicular fluid (GCF) concentration for each antibiotic agent. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ on a base-10 logarithmic scale. P-values determined by Dunn's test with a non-negative false discovery rate correction. *, $p < 0.05$ compared with the control (no test agent); **, $p < 0.001$ compared with the control (no test agent).

Effects of the AMPs

The effects of the AMPs LL-37 and LfcinH on the bacteria residing in matured FAB and OAB biofilms are shown in ***Figure 2***. Both biofilms were affected by LL-37 and LfcinH and showed similar susceptibilities to the AMPs: the CFU/mL were significantly reduced by both AMPs and showed a concentration dependency. Maximum reduction of bacterial counts was achieved at the highest test concentration for both AMPs (250 µg/mL), which led to a drop in CFU/mL by more than one log-scale. The BacTiter-Glo™ microbial cell viability assay provided similar results regarding biofilm reduction by the AMPs (***Figures 3c and 3d***).

Furthermore, the BacTiter-Glo™ microbial cell viability assay was used for relative quantification of planktonic bacteria released from FAB and OAB biofilms after incubation with the AMPs (***Figures 3a and 3b***). The RLU-measurements for the planktonic bacteria varied depending on the AMP type and the AMP concentration. The control group served as a reference for the test groups, since the biofilms in the control group were incubated in the absence of any antimicrobials. LL-37 reduced mean RLUs of released planktonic bacteria from

FAB biofilms at all concentrations with the exception of 10 µg/mL, where the mean RLUs were higher compared to the control group. On the contrary, LfcinH increased the number of planktonic facultative anaerobic bacteria at all concentrations. Although the RLUs in released facultative anaerobes were generally higher for LfcinH than for LL-37, both AMPs showed a similar concentration dependency with a smooth rise and fall in RLUs. Maximum RLUs in bacteria released from FAB biofilms were registered at a LL-37 concentration of 10 µg/mL and at LfcinH concentrations of 25 and 100 µg/mL. As opposed to the FAB biofilms, RLUs in planktonic bacteria released from OAB biofilms by the agency of both AMPs were lower compared to the control group at all tested concentrations. While LfcinH showed no explicit concentration dependency and reduced the mean RLUs to approximately 70% of the control group, LL-37 led to a sharp drop in RLUs at 10 µg/mL, reducing the planktonic bacteria to a percentage of 20%. Subsequently, the mean RLUs slightly increased up to a concentration of 100 µg/mL and began to decrease at 250 µg/mL.

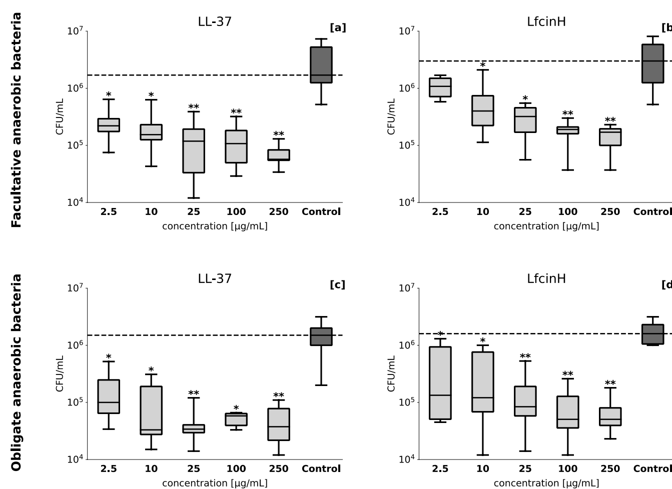

Figure 2: Bacterial counts (CFU/mL) in matured biofilms formed by facultative **(a-b)** and obligate anaerobic **(c-d)** bacteria after 4 h of incubation with different concentrations [µg/mL] of LL-37 and human Lactoferricin (LfcinH). Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ on a base-10 logarithmic scale. P-values determined by Dunn's test with a non-negative false discovery rate correction. *, $p < 0.05$ compared with the control (no test agent); **, $p < 0.001$ compared with the control (no test agent).

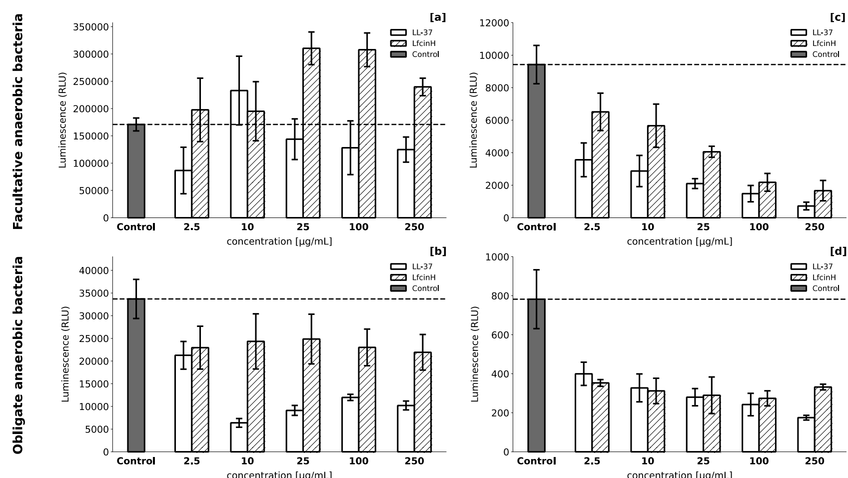

Figure 3: Influence of different concentrations [µg/mL] of LL-37 and human Lactoferricin (LfcinH) on planktonic bacteria dispersed from biofilms **(a-b)** and on sessile bacteria residing within biofilms **(c-d)** formed by facultative anaerobic and obligate anaerobic bacteria. Bacteria quantified by means of a luminescence-based cell viability assay. Data are shown as relative light units (RLUs).

Effects of antibiotics combined with AMPs

The anti-biofilm effects of AMX, CLI, MDZ and AMX/MDZ combined with LL-37 and LfcinH are shown in terms of CFU/mL in ***Figures 4 and 5***. For the purpose of comparing the level of reduction achieved by the antibiotics and the AMPs alone with the biofilm reduction achieved by the antimicrobial combinations, the effect size of each experimental condition was determined and visualized in a forest plot (***Figure 6***).

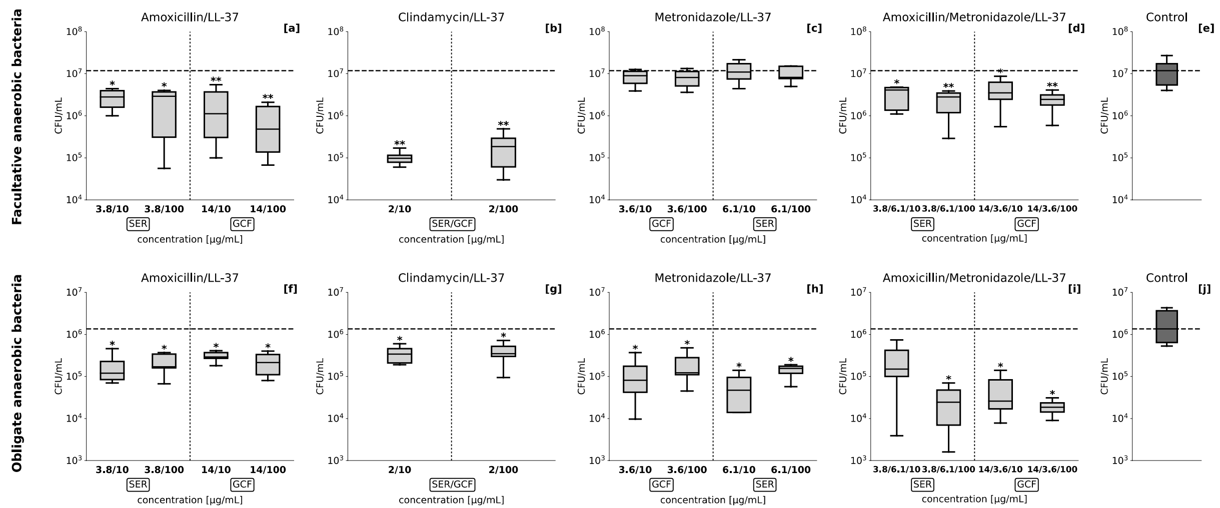

Figure 4: Bacterial counts (CFU/mL) in matured biofilms formed by facultative **(a-e)** and obligate anaerobic **(f-j)** bacteria after 4 h of incubation with antibiotic solutions (amoxicillin, clindamycin, metronidazole, amoxicillin/metronidazole) combined with LL-37. For each antibiotic agent, the serum (SER) and the gingival crevicular fluid (GCF) concentration [µg/mL] was combined with a lower (10 µg/mL) and a higher (100 µg/mL) LL-37 concentration. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ on a base-10 logarithmic scale. P-values determined by Dunn's test with a non-negative false discovery rate correction. *, $p < 0.05$ compared with the control (no test agent); **, $p < 0.001$ compared with the control (no test agent).

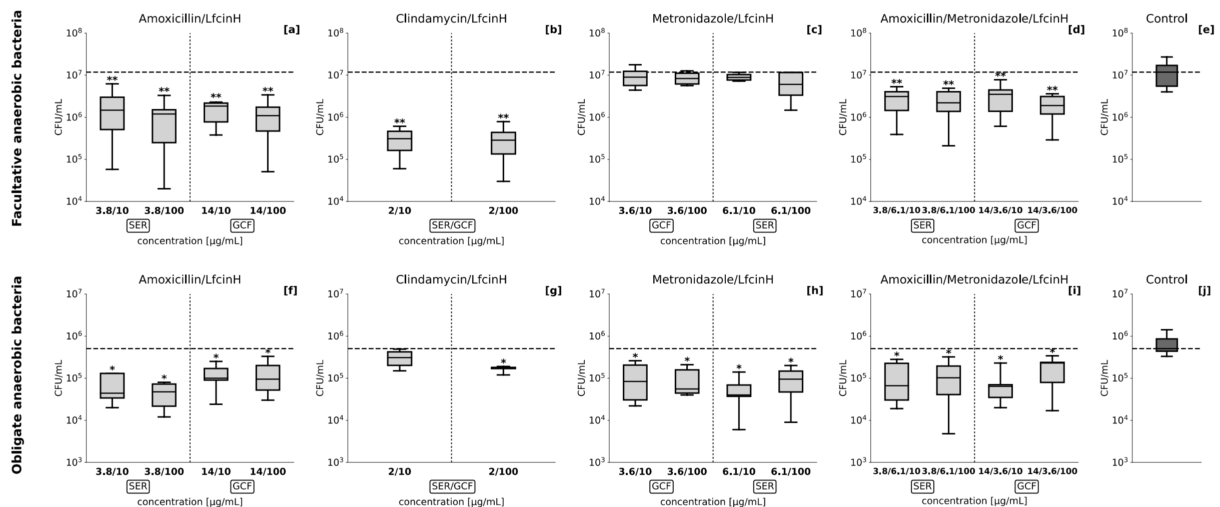

Figure 5: Bacterial counts (CFU/mL) in matured biofilms formed by facultative **(a-e)** and obligate anaerobic **(f-j)** bacteria after 4 h of incubation with antibiotic solutions (amoxicillin, clindamycin, metronidazole, amoxicillin/metronidazole) combined with human Lactoferricin (LfcinH). For each antibiotic agent, the serum (SER) and the gingival crevicular fluid (GCF) concentration [µg/mL] was combined with a lower (10 µg/mL) and a higher (100 µg/mL) LfcinH concentration. Data are presented as medians and interquartile ranges (IQ) with whiskers extending to a maximum of 1.5 IQ on a base-10 logarithmic scale. P-values determined by Dunn's test with a non-negative false discovery rate correction. *, $p < 0.05$ compared with the control (no test agent); **, $p < 0.001$ compared with the control (no test agent).

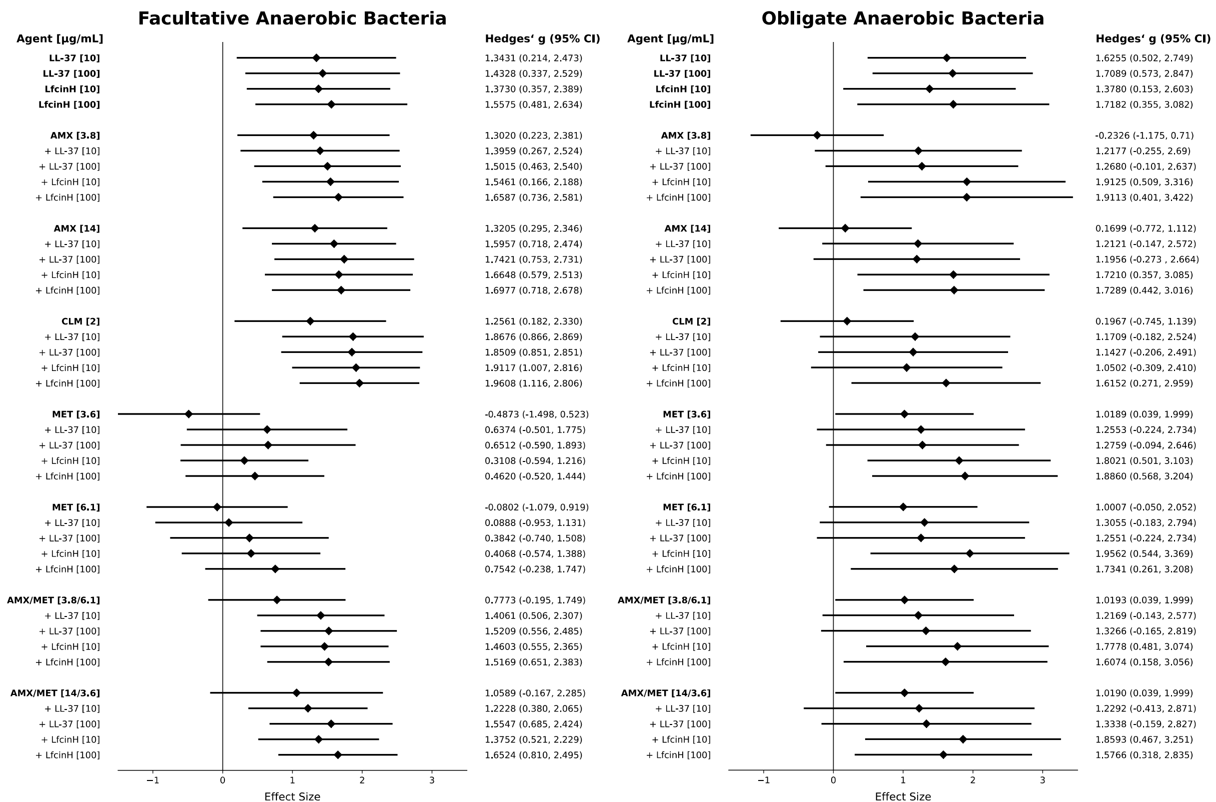

Figure 6: Forest plots showing effect sizes of different antimicrobial combinations on matured biofilms formed by facultative and obligate anaerobic bacteria. The serum (SER) and gingival crevicular fluid (GCF) concentrations [µg/mL] of the antibiotics amoxicillin (AMX), clindamycin (CLI), metronidazole (MDZ) and combined amoxicillin/metronidazole (AMX/MDZ) as well as a low (10 µg/mL) and a high concentration (100 µg/mL) of the antimicrobial peptides LL-37 and human Lactoferricin (LfcinH) are shown in bold print. For the antimicrobial combinations, SER and GCF concentrations of each antibiotic were combined with both LL-37 and LfcinH concentrations. Effect sizes were estimated by computation of Hedges' g and 95% confidence intervals (CI).

The anti-biofilm effect of AMX against FAB biofilms was only slightly improved in the presence of the AMPs. OAB biofilms were not affected by AMX alone, however a noticeable biofilm reduction was achieved at the SER and GCF concentration by the agency of LL-37 or LfcinH. The effect estimation implies that AMX/LfcinH was more effective than AMX/LL-37 against OAB biofilms. Furthermore, the antimicrobial activity of CLI in FAB biofilms was enhanced in the presence of LL-37 or LfcinH. CLI alone had no effect on OAB biofilms, but CLI combined with LL-37 or LfcinH led to a significant reduction of OAB biofilms. According to the effect estimation, MDZ/LL-37 and MDZ/LfcinH in FAB biofilms were more effective than MDZ alone, for which no anti-biofilm effect was registered at the SER and GCF concentrations. However, the improved antimicrobial activity of MDZ by the AMPs was very moderate, as no significant reduction of CFU/mL compared to the control group was observed for these combinations. The level of reduction achieved by MDZ at the SER and GCF concentration in OAB biofilms was higher when AMPs were added to the antibiotic solution. As previously observed for AMX, the effect of MDZ/LfcinH was larger than that of MDZ/LL-37. Moreover, the reduction of FAB biofilms by AMX/MDZ was increased by the

agency of LL-37 and LfcinH at the SER and GCF concentration. The anti-biofilm activity of the SER and GCF concentrations of AMX/MDZ against OAB biofilms was also enhanced in the presence of LL-37 or LfcinH. Again, the extent to which LfcinH improved the effect of AMX/MDZ was greater compared to that of LL-37.
All of the results for these antibiotic/AMP combinations did not show any significant difference among the two chosen AMP concentrations.

Metabolic adaptations in matured biofilms

The metabolic state of sessile bacteria residing in matured FAB and OAB biofilms was judged by the amount of ATP present in the sonicates containing the extracted biofilms. ATP-levels were compared to those in the solutions containing the planktonic bacteria dispersed from matured biofilms. The RLUs as well as the CFUs were determined for each solution, and the CFU/RLU ratio was calculated. Ratios for both planktonic and sessile bacteria in FAB and OAB biofilms are shown in ***Figure*** *7*.

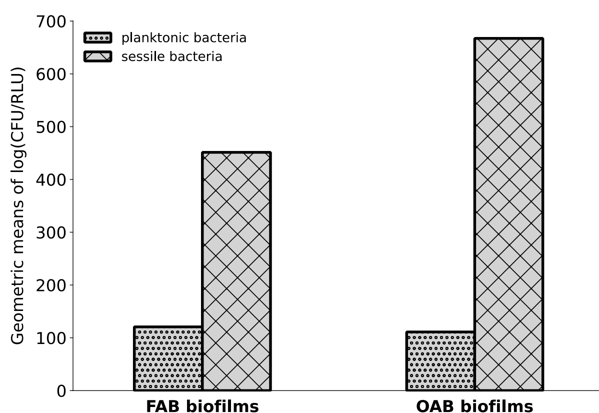

Figure 7: Metabolic activity of planktonic bacteria and sessile bacteria residing in biofilms formed by facultative (FAB) and obligate anaerobic bacteria (OAB). ATP-levels (expressed in terms of RLUs) were used as a parameter for the bacteria's cell metabolism. Data shown as geometric means of log-transformed CFU/RLU ratios.

Among the planktonic bacteria, there was no noteworthy difference in the CFU/RLU ratios, which indicates that the ATP-levels of planktonic facultative anaerobes and obligate anaerobes were very similar. However, in both biofilms the CFU/RLU ratios of sessile bacteria were larger compared to the respective planktonic bacteria, which implies that in a given amount of CFUs the ATP-levels were comparatively lower. This discrepancy was larger in OAB biofilms, as the CFU/RLU ratio in the sessile obligate anaerobes was the highest among all groups.

4. Discussion

This study investigated the anti-biofilm effects of the antimicrobial peptides LL-37 and LfcinH combined with antibiotics on in vitro biofilm models which aimed to represent common causes for oral conditions. Our FAB biofilm comprises three typical species of the supragingival dental plaque, among which *S. mutans* is recognized as a key player in the development of dental caries. *S. sanguinis* and *A. naeslundii* are commensal bacteria in the human oral cavity and pioneering colonizers of tooth surfaces and the oral mucosa. However, they can act as pathogens, for example in infections of endodontic origin by forming biofilms on foreign bodies such as filling materials used for root canal treatments, or by colonizing the periapical region of endodontically infected teeth [21,22]. Moreover, bacteremia induced by dental procedures may allow viridans group streptococci like *S. mutans* and *S. sanguinis* to incite infections in other areas besides the oral cavity, such as infective endocarditis caused by accumulation of bacteria in the heart lining [23]. This three-strain FAB biofilm model has also been used in previous studies as a modification of the Zürich biofilm model [24,25]. The OAB biofilm model aims to represent a subgingival dental biofilm, which is considered the source of periodontal diseases. It is characterized by predominantly obligate anaerobic species, including *V. parvula*, an early colonizer in oral biofilm formation, *P. micra*, a late colonizer, and *F. nucleatum*, which acts as a coaggregation bridge between early and late colonizers [26]. Recent findings have shown that some periodontal bacteria act pathogenic in tissues beyond those of the oral cavity, such as *F. nucleatum*, which is involved in the carcinogenesis of colorectal cancer by stimulating tumor growth via immunomodulatory mechanisms and virulence factors [27].

The antibiotics used in this study were selected based on their common fields of application in dentistry. Amoxicillin is the antibiotic of choice for the treatment of most odontogenic infections (usually combined with a β-lactamase inhibitor) and it is routinely prescribed as antibiotic prophylaxis in patients with high endocarditis risk before invasive dental procedures. For patients hypersensitive to penicillin, clindamycin can be used as an alternative for these two indications [28,29]. While metronidazole exhibits an excellent activity against most obligate anaerobes, it is ineffective against aerobes and facultative anaerobes. Therefore, metronidazole must be administered in combination with an agent effective against these bacteria when treating polymicrobial infections such as periodontitis, for which the antibiotic treatment involves the combined administration of metronidazole and amoxicillin [30]. For achieving an adequate antimicrobial effect, it is essential for the antibiotic to gain access to the intended target site at a sufficient concentration. Hence, the antibiotic concentrations tested in

this study were determined based on their reported serum and gingival crevicular fluid concentrations after oral application.

Despite each facultative and each obligate anaerobic strain being intrinsically sensitive to amoxicillin and clindamycin, only FAB biofilms were susceptible to both antibiotics, while their drug efficacy was reduced in OAB biofilms. Increased antibiotic tolerance in biofilms can be induced by metabolic downshifts of the sessile cells within biofilm. Many antibiotics, including amoxicillin and clindamycin, function best in metabolically active cells: amoxicillin acts bactericidal by specifically inhibiting the transpeptidase needed for the synthesis of the peptidoglycan layer in the bacterial cell wall and clindamycin, a bacteriostatic antibiotic, binds to the peptidyl transferase on the 50S subunit of bacterial ribosomes and thereby interferes with the synthesis of proteins. Both mechanisms of action target energy-consuming processes, which, however, are repressed in substantial regions of high-density biofilms, where the residing cells are deprived of the nutrients required to generate a sufficient energy charge. This applies particularly to bacteria which are located toward the inside of the biofilm, because there is a steep gradient of nutritional availability from the biofilms' periphery to its center [31]. This induced state of metabolic dormancy in large portions of the biofilm reduces the antibiotics' ability to display their full effect at concentrations which would be sufficient under normal circumstances. For judging the bacteria's metabolic activity, we compared the amount of ATP present in a given number of planktonic bacteria and sessile bacteria extracted from matured FAB and OAB biofilms which were not exposed to any antimicrobials. For this purpose, we used the RLUs obtained from the BacTiter-Glo™ microbial cell viability assay solely to determine the ATP-levels, but not to estimate the absolute number of cells, because quantification methods based on cell viability depend on the bacteria's metabolic activity and therefore would underestimate the number of metabolically dormant bacteria in this particular case. Under the premise that the solutions containing the sessile bacteria exhibit a lower ATP-production, it was essential to additionally perform a culture method which determines the CFUs, as this is the only reliable method for quantifying the number of viable bacteria independently of their metabolic state. The calculation of the CFU/RLU ratio ultimately allows us to judge the metabolic activity and to draw comparisons between sessile and planktonic bacteria. Our results demonstrate that facultative and obligate anaerobic bacteria produce a similar amount of ATP in their planktonic state, whereas sessile bacteria residing in FAB and OAB biofilms generally exhibit lower ATP-levels. These results are consistent with other investigations comparing the expression of proteins involved in metabolic processes in biofilms vs. planktonic bacteria. For example, recent proteomic analyses of *Staphylococcus*

aureus strains have shown that several proteins needed for energy acquisition, including ATP synthases, are absent in *S. aureus* biofilm cells, while being abundant in planktonic cells [32]. Furthermore, a study investigating the protein expression in *Aggregatibacter actinomycetemcomitans*, a pathogen frequently found in patients with severe periodontitis, also revealed that 37 from a total of 50 examined proteins were downregulated in cells growing in biofilms [33]. Our observations combined with the results of previous studies validate our postulation on metabolic downshifts being a mechanism of enhanced antibiotic tolerance in biofilms. Furthermore, OAB biofilms in particular seem to have a greater downregulation of ATP-production compared to FAB biofilms, since the CFU/RLU ratio we determined was even higher for sessile obligate anaerobes. Perhaps this explains why OAB biofilms were inherently resistant to amoxicillin and clindamycin as opposed to FAB biofilms, whose metabolic activity probably sufficed for the antibiotics to display their effect. Indeed, recent investigations indicate that several proteins involved in metabolic processes are repressed in biofilms of *F. nucleatum*, one of the three obligate anaerobes also forming our OAB biofilm model [34].
A further explanation for the OAB biofilms' higher antibiotic tolerance involves the properties of the biofilm matrix, which may have markedly affected the antibiotics' drug efficacy. As most antibiotics act intracellularly, it is a pivotal step for them to penetrate the biofilm matrix and pass the bacterial cell membrane in order to reach the concentration required within the cell. Possibly, structural differences within the biofilm matrices of the obligate anaerobes are responsible for creating a harsher barrier against the antibiotics. Since the components forming the matrix are autogenously produced by the bacteria and the nutrients provided by the culture media were identical for FAB and OAB biofilms, it is likely that the metabolized components produced by the obligate anaerobes form thicker or denser matrices which hinder the antibiotics from penetration. Aside from creating a mechanical barrier, the properties of the biofilm matrix may also influence the metabolic state of the bacteria. As opposed to the bacteria residing in the very center of the biofilm, cells located on the outside of the biofilm are less deprived of nutrients and therefore display a more active cell metabolism, making them more susceptible to the antibiotics [5]. If, however, the outer cells are embedded in a much thicker matrix, as we believe this is the case for the OAB biofilms, nutritional availability in the outer layers is also more limited, which again induces metabolic downshifts in the bacteria residing in this part of the biofilm. Further possible reasons for the reduced drug efficacy of amoxicillin and clindamycin in OAB biofilms are pH changes, variations in hydrophobicity within the biofilm as well as changes in the redox potential under anaerobiosis, which may delay the activity of the antibiotics.

Since the ETEST revealed that each of the obligate anaerobes was susceptible to metronidazole, it is not surprising that the OAB biofilms were also reduced by metronidazole. On the other hand, each facultative anaerobic strain was intrinsically resistant to metronidazole, and therefore the FAB biofilms were also barely affected. Indeed, this is a reasonable observation considering metronidazole's mechanism of action: despite its ability to enter both anaerobic and aerobic microorganisms by diffusing across cell membranes, metronidazole's toxicity, under the proper redox environment, is limited selectively to anaerobes. Metronidazole is a pro-drug nitroimidazole, which first has to be activated by reduction of its nitro group. This step requires an effector with a sufficiently low redox potential, such as the pyruvate:ferredoxin oxidoreductase, an enzyme only found in anaerobic bacteria for catalyzing the oxidative decarboxylation of pyruvate to form acetyl-coenzyme A. In the process of the reductive activation of metronidazole, a series of intermediate nitroso metabolites is generated, causing damage to the microbial DNA due to inhibition of the nucleid acid synthesis [35]. However, the presence of oxygen interferes with the activation of metronidazole, because oxygen, having a higher affinity for electrons, competes with metronidazole for capturing the electrons needed for its nitroreduction [36]. Consequently, the cytotoxic products are less efficiently formed, and the metronidazole concentrations needed for an adequate effect are much higher in facultative anaerobes than in obligate anaerobes. Given that metronidazole was mostly ineffective against FAB biofilms on account of its mechanism of action, the enhanced reduction of FAB biofilms after exposure to combined amoxicillin and metronidazole is most likely ascribed to the presence of amoxicillin. It may also be assumed that metronidazole's bactericidal effect in OAB biofilms initiated the dispersion of the residing cells within the biofilm, enabling amoxicillin to target bacteria that were not accessible to amoxicillin alone.

The AMPs LL-37 and LfcinH were deemed suitable for this study, as they are components of the human innate immune system and are present in human serum, saliva and the gingival crevicular fluid. They have shown promising antimicrobial and anti-biofilm features against oral pathogens, including bacteria causing dental caries and periodontal disease [10,11]. In our study, LL-37 and LfcinH reduced the CFU/mL of matured FAB and OAB biofilms and showed a concentration dependency. Similar to the CFUs, the RLUs decreased continuously in both biofilms depending on the AMP concentration, and therefore confirmed our results (***Figures 3c and d***). According to the effect estimation, LL-37 was slightly more effective in OAB biofilms than in FAB biofilms, whereas LfcinH showed a similar effect size in FAB and OAB biofilms. As opposed to the antibiotics, the AMPs' anti-biofilm effect seems to be less sensitive to the properties of the biofilms. While our previous findings give us reason to believe that the

antibiotics' drug efficacy is altered by matrix properties of the biofilm and by metabolic adaptations of the bacteria residing within, the AMPs seem to easily penetrate the biofilm matrices and act bactericidal even in metabolically dormant cells. In fact, our results allow us to identify different mechanisms which the AMPs may have used to achieve the observed anti-biofilm effect. The bactericidal effect of AMPs on a single microbial cell is well studied and is known to arise from interactions with the microbial cell membrane by virtue of the AMPs' amphipathic structure, causing cell death by cytoplasmic shedding [37]. This mechanism is independent of an active metabolism, and therefore the AMPs were able to act bactericidal on the sessile bacteria residing within the biofilm. However, these bacteria are embedded in a thick biofilm matrix which seemingly hindered some antibiotics from accessing the microbial cell membrane, but not the AMPs. There is evidence that the AMPs loosen the biofilm matrix by interacting with its components. Perhaps they covalently bind to the negatively charged polysaccharides in the matrix or alter the tertiary structure of proteins, thereby reducing the cohesive forces within the biofilm matrix. This causes the biofilm to gradually disintegrate and release sessile bacteria, making them more accessible to the antimicrobials within their reach. To verify this theory, we measured the number of viable cells released from the matured biofilms on the ZrO_2 discs during incubation. We combined the incubation solution containing the AMPs and the sodium chloride solution used for washing the biofilms, considering that washing the ZrO_2 discs releases cells that are only loosely attached to the biofilm after antimicrobial exposure. In this particular case, we used the RLUs to estimate and compare the number of viable cells in these solutions. Regardless of the AMPs' presence, a certain level of planktonic bacteria was to be found in the culture medium due to the biofilms' nature to disperse after maturation, allowing bacteria in select areas to escape from the biofilm and return to their planktonic state [38]. Nutrients and other osmotically active solutes in the culture medium surrounding the discs are a driving force for biofilm dispersion, which justifies why the RLUs were elevated even in the absence of an antimicrobial stimulus. Ensuing the evacuation of bacteria from the biofilm, the presence of fresh medium grants a sudden nutritional availability, which activates the metabolism of the dispersed planktonic cells and promotes further growth during the subsequent four-hour incubation. This leads to an increase in the number of viable cells in the culture medium, if no antimicrobial agent is present to quench the bacteria's planktonic growth, which was the case in the control group. However, the planktonic bacteria in the culture medium containing the AMP solutions were not continuously reduced at increasing concentrations, as opposed to the matured FAB and OAB biofilms, where the AMPs caused a gradual decrease in the number of sessile bacteria extracted

from the biofilms. In the case of the facultative anaerobes (***Figure 3a***), an imaginary line connecting the mean values of each AMP concentration shows a distinct pattern with a smooth rise and fall. Given that the mean values are a representation of an equilibrium between biofilm dispersion and the AMPs' bactericidal action, the maximum RLUs display the least favorable ratio, having an enhanced biofilm dispersion and thus a net increase in planktonic bacteria. In facultative anaerobes, this ratio is at a LL-37 concentration of 10 µg/mL, which is the only LL-37 concentration exceeding the average number of planktonic bacteria. LfcinH on the other hand, shows a quantitative shift in two directions for the facultative anaerobes: first, the absolute RLU values were higher for all LfcinH concentrations and secondly, the maximum RLUs were also registered at higher concentrations. For the planktonic obligate anaerobes (***Figure 3b***), both AMPs resulted in lower RLUs compared to the control group at all tested concentrations. This implies that the AMPs' continuous bactericidal effect outweighs any additional release of bacteria from the biofilms. From this we conclude that the AMPs are more effective in planktonic obligate anaerobic organisms than in facultative anaerobes. This proposition is also consistent with the results of our previous work, where we revealed that the inhibitory effect of AMPs on planktonic bacterial growth was also larger with obligate anaerobes than with facultative anaerobes [12]. Furthermore, the RLUs in planktonic obligate anaerobes barely differed between the LfcinH concentrations, which implies that the concentration-dependent increase in AMP-driven biofilm dispersion in OAB biofilms is proportionate to the AMP's simultaneous bactericidal action.

For the antibiotic/AMP combinations, we selected one of the lower (10 µg/mL) and one of the higher (100 µg/mL) AMP concentration and paired each with the SER and GCF concentration of the antibiotics. The effect estimation implies that all antibiotic/AMP combinations resulted in an enhanced reduction of FAB and OAB biofilms. The largest increase in biofilm reduction was registered in OAB biofilms after adding AMPs to amoxicillin and clindamycin. There are several possible explanations for the synergism we observed between the antibiotics and the AMPs. Considering that the AMPs had a disintegrating effect on the biofilm matrix as previously described, we may assume that this feature also allowed the antibiotics to affect any bacteria that they were unable to approach on their own by promoting dispersion of the cells within the biofilm. Furthermore, given that the AMPs induce pore formation in the microbial membrane, it is reasonable to hypothesize that the AMPs could have mediated the antibiotic uptake into the bacteria and thereby facilitated the antibiotics' accessibility to their designated target site. However, we do not know if these proposed mechanisms really took place or if the biofilm reduction was achieved solely by the bactericidal action of the AMPs, whilst the

antibiotics remained ineffective against the sessile bacteria, especially in the cases where the antibiotics alone were unable to reduce the biofilms. This is probably true for the antimicrobial combination of MDZ with LL-37 or LfcinH in FAB biofilms, knowing that metronidazole's mechanism of action is dependent on conditions that are not provided by the facultative anaerobes. Even though LfcinH enhanced the biofilm reduction of the antibiotics slightly more than LL-37, no overall significant difference was observed between the two AMPs. This implies that the increased antimicrobial biofilm reduction achieved by the AMPs may be ascribed to rather basic mechanisms which seem to be prevalent in different types of AMPs. Furthermore, it did not seem to play a role, if the AMPs were administered at a concentration of 10 or 100 µg/mL in combination with the antibiotics, suggesting that there may be a saturation for the AMP-driven enhancement of the antibiotics' drug efficacy. This result is of great value in consideration of a potential therapeutic application, since higher AMP concentrations are known to exert cytotoxic effects on host cells. In a previous study, LL-37 was shown to be slightly toxic to human gingival fibroblasts at concentrations of more than 20 µg/mL, while being non-toxic up to a concentration of 10 µg/mL [39].

5. Conclusions

In summary, our work provides promising findings regarding the possible therapeutic use of the AMPs LL-37 and LfcinH. We have obtained comprehensive results demonstrating that these AMPs act in synergism with antibiotics against facultative and obligate anaerobic biofilms. Our results highlight that LL-37 and LfcinH exhibit a broad therapeutic spectrum for the treatment of various polymicrobial biofilm-related infections of the oral cavity, which is a considerable advantage over many antibiotics, since they often fail to sufficiently reduce such multi-species biofilms. Furthermore, we proved that the AMPs' antimicrobial efficacy is less sensitive to metabolic adaptations of the bacteria residing within biofilms. We also showed that there is an equilibrium between AMP-driven biofilm dispersion and the AMPs' bactericidal action on the planktonic bacteria released from the biofilms. However, some limitations of our research should be mentioned. Since the development of all in vitro biofilms was performed under static conditions, the nutrient supply for the cultures was limited during biofilm formation. This may have affected certain properties of the mature biofilm, such as thickness, mass of the biofilm matrix and the distribution of the three different species within the biofilm. Our biofilm model also does not consider certain biological factors of the human saliva, such as the presence of ions, proteins, enzymes and polysaccharides as well as other AMPs, which may interact with the AMPs we tested in this study. Furthermore, the strains we used for our biofilm models were reference strains which showed no intrinsic resistance against the antibiotics with the ETEST method. Perhaps it would be useful to examine the antimicrobial efficacy of AMPs and antibiotics in clinical isolates of oral anaerobic pathogens, since they are more likely to display intrinsic or acquired antimicrobial resistances. In clinical practice, AMPs are not yet a replacement for conventional antibiotics, however their supplementary application may reduce the required antibiotic dosage and the duration of administration, thereby lowering the risk of side effects from the antibiotics. AMPs may also serve as additional auxiliary agents next to antibiotics of last resort for treating infections with highly resistant bacteria. Future studies should focus on an in vivo application of combined antimicrobial strategies with AMPs to verify their many promising features in clinical practice.

Acknowledgements

We would like to thank the dental laboratory Dentaltechnik Kollmuß GmbH and the master dental technician Robert Kollmuß for kindly supplying us with the ZrO_2 discs used in this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] N. Høiby, A short history of microbial biofilms and biofilm infections, APMIS. 125 (2017) 272–275. https://doi.org/10.1111/apm.12686.

[2] M. di Luca, G. Maccari, R. Nifosí, Treatment of microbial biofilms in the post-antibiotic era: Prophylactic and therapeutic use of antimicrobial peptides and their design by bioinformatics tools, Pathog. Dis. 70 (2014) 257–270. https://doi.org/10.1111/2049-632X.12151.

[3] B.A. Dale, L.P. Fredericks, Antimicrobial peptides in the oral environment: expression and function in health and disease., Curr. Issues Mol. Biol. 7 (2005) 119–133. https://doi.org/10.1093/jac/dki103.

[4] D.G. Davies, M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, E.P. Greenberg, The involvement of cell-to-cell signals in the development of a bacterial biofilm., Science. 280 (1998) 295–298. https://doi.org/10.1126/science.280.5361.295.

[5] N. Høiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Antibiotic resistance of bacterial biofilms, Int. J. Antimicrob. Agents. 35 (2010) 322–332. https://doi.org/10.1016/j.ijantimicag.2009.12.011.

[6] A. Hernández Ceruelos, L.C. Romero-Quezada, J.C. Ruvalcaba Ledezma, L. López Contreras, Therapeutic uses of metronidazole and its side effects: An update, Eur. Rev. Med. Pharmacol. Sci. 23 (2019) 397–401. https://doi.org/10.26355/eurrev_201901_16788.

[7] A.J. Duplantier, M.L. van Hoek, The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds, Front. Immunol. 4 (2013) 1–14. https://doi.org/10.3389/fimmu.2013.00143.

[8] R.E.W. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies., Nat. Biotechnol. 24 (2006) 1551–1557. https://doi.org/10.1038/nbt1267.

[9] B. Pahar, S. Madonna, A. Das, C. Albanesi, G. Girolomoni, Immunomodulatory Role of the Antimicrobial LL-37 Peptide in Autoimmune Diseases and Viral Infections., Vaccines (Basel). 8 (2020) 517. https://doi.org/10.3390/vaccines8030517.

[10] O. Turkoglu, G. Emingil, G. Eren, H. Atmaca, N. Kutukculer, G. Atilla, Levels of ll-37 antimicrobial peptide in the gingival crevicular fluid of young and middle-aged subjects with or without gingivitis., J. Istanbul Univ. Fac. Dent. 51 (2017) 15–21. https://doi.org/10.17096/jiufd.42354.

[11] H. Wakabayashi, K. Yamauchi, T. Kobayashi, T. Yaeshima, K. Iwatsuki, H. Yoshie, Inhibitory effects of lactoferrin on growth and biofilm formation of Porphyromonas gingivalis and Prevotella intermedia., Antimicrob. Agents Chemother. 53 (2009) 3308–3316. https://doi.org/10.1128/AAC.01688-08.

[12] S.N. Wuersching, K.C. Huth, R. Hickel, M. Kollmuss, Inhibitory effect of LL-37 and human lactoferricin on growth and biofilm formation of anaerobes associated with oral diseases, Anaerobe. 67 (2021) 102301. https://doi.org/10.1016/j.anaerobe.2020.102301.

[13] L. Grassi, G. Maisetta, S. Esin, G. Batoni, Combination Strategies to Enhance the Efficacy of Antimicrobial Peptides against Bacterial Biofilms., Front. Microbiol. 8 (2017) 2409. https://doi.org/10.3389/fmicb.2017.02409.

[14] H. Tenenbaum, Amoxicillin and clavulanic acid concentrations in gingival crevicular fluid, J. Clin. Periodontol. 24 (1997) 804–807. https://doi.org/10.1111/j.1600-051X.1997.tb01193.x.

[15] P.B. Dinis, M.C. Monteiro, M.L. Martins, N. Silva, A. Gomes, Sinus tissue pharmacokinetics after oral administration of amoxicillin/clavulanic acid., Laryngoscope. 110 (2000) 1050–1055. https://doi.org/10.1097/00005537-200006000-00030.

[16] C.B. Walker, J.M. Gordon, H.A. Cornwall, J.C. Murphy, S.S. Socransky, Gingival crevicular fluid levels of clindamycin compared with its minimal inhibitory concentrations for periodontal bacteria., Antimicrob. Agents Chemother. 19 (1981) 867–871. https://doi.org/10.1128/aac.19.5.867.

[17] M.R. Britt, D.J. Pohlod, Serum and crevicular fluid concentrations after a single oral dose of metronidazole., J. Periodontol. 57 (1986) 104–107. https://doi.org/10.1902/jop.1986.57.2.104.

[18] CLSI, Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute, (2020).

[19] K.D. Mandakhalikar, J.N. Rahmat, E. Chiong, K.G. Neoh, L. Shen, P.A. Tambyah, Extraction and quantification of biofilm bacteria: Method optimized for urinary catheters, Sci. Rep. 8 (2018) 8069.

https://doi.org/10.1038/s41598-018-26342-3.

[20] G. van Rossum, F. Drake, Python 3 Reference Manual, (2019).

[21] T. Xia, J.C. Baumgartner, Occurrence of Actinomyces in infections of endodontic origin., J. Endod. 29 (2003) 549–552. https://doi.org/10.1097/00004770-200309000-00001.

[22] K. Jhajharia, A. Parolia, K.V. Shetty, L.K. Mehta, Biofilm in endodontics: A review., J. Int. Soc. Prev. Community Dent. 5 (2015) 1–12. https://doi.org/10.4103/2231-0762.151956.

[23] I. Šutej, K. Peroš, V. Trkulja, I. Rudež, D. Barić, I. Alajbeg, H. Pintarić, R. Stevanović, D. Lepur, The epidemiological and clinical features of odontogenic infective endocarditis, Eur. J. Clin. Microbiol. Infect. Dis. 39 (2020) 637–645. https://doi.org/10.1007/s10096-019-03766-x.

[24] B. Guggenheim, M. Guggenheim, R. Gmür, E. Giertsen, T. Thurnheer, Application of the Zürich biofilm model to problems of cariology., Caries Res. 38 (2004) 212–222. https://doi.org/10.1159/000077757.

[25] A.M. Marttinen, P. Ruas-Madiedo, C. Hidalgo-Cantabrana, M.A. Saari, R.A. Ihalin, E.M. Söderling, Effects of xylitol on xylitol-sensitive versus xylitol-resistant Streptococcus mutans strains in a three-species in vitro biofilm., Curr. Microbiol. 65 (2012) 237–243. https://doi.org/10.1007/s00284-012-0151-2.

[26] R.T. Mendes, D. Nguyen, D. Stephens, F. Pamuk, D. Fernandes, T.E. Van Dyke, A. Kantarci, Endothelial cell response to Fusobacterium nucleatum, Infect. Immun. 84 (2016) 2141–2148. https://doi.org/10.1128/IAI.01305-15.

[27] N. Hashemi Goradel, S. Heidarzadeh, S. Jahangiri, B. Farhood, K. Mortezaee, N. Khanlarkhani, B. Negahdari, Fusobacterium nucleatum and colorectal cancer: A mechanistic overview, J. Cell. Physiol. 234 (2019) 2337–2344. https://doi.org/10.1002/jcp.27250.

[28] W. Wilson, K.A. Taubert, M. Gewitz, P.B. Lockhart, L.M. Baddour, M. Levison, A. Bolger, C.H. Cabell, M. Takahashi, R.S. Baltimore, J.W. Newburger, B.L. Strom, L.Y. Tani, M. Gerber, R.O. Bonow, T. Pallasch, S.T. Shulman, A.H. Rowley, J.C. Burns, P. Ferrieri, T. Gardner, D. Goff, D.T. Durack, Prevention of infective endocarditis: Guidelines from the American Heart Association, Circulation. 116 (2007) 1736–1754. https://doi.org/10.1161/CIRCULATIONAHA.106.183095.

[29] A. Sebastian, P.G. Antony, M. Jose, A. Babu, J. Sebastian, A. Kunnilathu, Institutional microbial analysis of odontogenic infections and their empirical antibiotic sensitivity, J. Oral Biol. Craniofacial Res. 9 (2019) 133–138. https://doi.org/10.1016/j.jobcr.2019.02.003.

[30] I. Brook, H.M. Wexler, E.J.C. Goldstein, Antianaerobic antimicrobials: Spectrum and susceptibility testing, Clin. Microbiol. Rev. 26 (2013) 526–546. https://doi.org/10.1128/CMR.00086-12.

[31] J.M. Stokes, A.J. Lopatkin, M.A. Lobritz, J.J. Collins, Bacterial Metabolism and Antibiotic Efficacy, Cell Metab. 30 (2019) 251–259. https://doi.org/10.1016/j.cmet.2019.06.009.

[32] S. Martínez-García, H. Peralta, G. Betanzos-Cabrera, L. Chavez-Galan, S. Rodríguez-Martínez, M.E. Cancino-Diaz, J.C. Cancino-Diaz, Proteomic comparison of biofilm vs. planktonic Staphylococcus epidermidis cells suggests key metabolic differences between these conditions, Res. Microbiol. 172 (2021) 103796. https://doi.org/10.1016/j.resmic.2020.103796.

[33] A. Llama-Palacios, O. Potupa, M.C. Sánchez, E. Figuero, D. Herrera, M. Sanz, Aggregatibacter actinomycetemcomitans Growth in Biofilm versus Planktonic State: Differential Expression of Proteins, J. Proteome Res. 16 (2017) 3158–3167. https://doi.org/10.1021/acs.jproteome.7b00127.

[34] A. Llama-Palacios, O. Potupa, M.C. Sánchez, E. Figuero, D. Herrera, M. Sanz, Proteomic analysis of Fusobacterium nucleatum growth in biofilm versus planktonic state, Mol. Oral Microbiol. 35 (2020) 168–180. https://doi.org/10.1111/omi.12303.

[35] S.A. Dingsdag, N. Hunter, Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms, J. Antimicrob. Chemother. 73 (2018) 265–279. https://doi.org/10.1093/jac/dkx351.

[36] M. Müller, Reductive activation of nitroimidazoles in anaerobic microorganisms, Biochem. Pharmacol. 35 (1986) 37–41. https://doi.org/10.1016/0006-2952(86)90552-6.

[37] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238–250. https://doi.org/10.1038/nrmicro1098.

[38] K.P. Rumbaugh, K. Sauer, Biofilm dispersion, Nat. Rev. Microbiol. 18 (2020) 571–586.

https://doi.org/10.1038/s41579-020-0385-0.

[39] M. Inomata, T. Horie, T. Into, Effect of the Antimicrobial Peptide LL-37 on Gene Expression of Chemokines and 29 Toll-like Receptor-Associated Proteins in Human Gingival Fibroblasts Under Stimulation with Porphyromonas gingivalis Lipopolysaccharide, Probiotics Antimicrob. Proteins. 12 (2020) 64–72. https://doi.org/10.1007/s12602-019-09600-2.

8. Literaturverzeichnis zur Einleitung

[1] A. Brand, L. Allen, M. Altman, M. Hlava, J. Scott, Beyond authorship: Attribution, contribution, collaboration, and credit, Learn. Publ. 28 (2015) 151–155.

[2] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, Clin. Microbiol. Rev. 15 (2002) 167–193.

[3] N. Høiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Antibiotic resistance of bacterial biofilms, Int. J. Antimicrob. Agents. 35 (2010) 322–332.

[4] A. Llama-Palacios, O. Potupa, M.C. Sánchez, E. Figuero, D. Herrera, M. Sanz, Proteomic analysis of Fusobacterium nucleatum growth in biofilm versus planktonic state, Mol. Oral Microbiol. 35 (2020) 168–180.

[5] A. Crabbé, P.Ø. Jensen, T. Bjarnsholt, T. Coenye, Antimicrobial Tolerance and Metabolic Adaptations in Microbial Biofilms, Trends Microbiol. 27 (2019) 850–863.

[6] K.P. Rumbaugh, K. Sauer, Biofilm dispersion, Nat. Rev. Microbiol. 18 (2020) 571–586.

[7] J. Davies, D. Davies, Origins and evolution of antibiotic resistance, Microbiol. Mol. Biol. Rev. 74 (2010) 417–433.

[8] J. Olivares, A. Bernardini, G. Garcia-Leon, F. Corona, M.B. Sanchez, J.L. Martinez, The intrinsic resistome of bacterial pathogens, Front. Microbiol. 4 (2013) 1–15.

[9] D. Herrera, B. Alonso, R. Leon, S. Roldan, M. Sanz, Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm, J. Clin. Periodontol. 35 (2008) 45–66.

[10] J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections, Science. 284 (1999) 1318–1322.

[11] P.N. Papapanou, M. Sanz, N. Buduneli, T. Dietrich, M. Feres, D.H. Fine *et al.*, Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, J. Clin. Periodontol. 45 (2018) S162–S170.

[12] M. Sanz, D. Herrera, M. Kebschull, I. Chapple, S. Jepsen, T. Beglundh *et al.*, Treatment of stage I–III periodontitis—The EFP S3 level clinical practice guideline, J. Clin. Periodontol. 47 (2020) 4–60.

[13] T. Handal, I. Olsen, C.B. Walker, D.A. Caugant, Detection and characterization of β-lactamase genes in subgingival bacteria from patients with refractory periodontitis, FEMS Microbiol. Lett. 242 (2005) 319–324.

[14] A. Mombelli, C. Walter, Antibiotics in Periodontics, Swiss Dent. J. 129 (2019) 835–838.

[15] A. Mombelli, A. Almaghlouth, N. Cionca, D.S. Courvoisier, C. Giannopoulou, Differential benefits of amoxicillin-metronidazole in different phases of periodontal therapy in a randomized controlled crossover clinical trial, J. Periodontol. 86 (2015) 367–375.

[16] Sore throat (acute): antimicrobial prescribing | NICE guideline, (2018). https://www.nice.org.uk/guidance/ng84/.

[17] A. Altiner, R. Berner, A. Diener, G. Feldmeier, A. Köchling, C. Löffler *et al.*, Converting habits of antibiotic prescribing for respiratory tract infections in German primary care - The cluster-randomized controlled CHANGE-2 trial, BMC Fam. Pract. 13 (2012).

[18] L.K. McLellan, D.A. Hunstad, Urinary Tract Infection: Pathogenesis and Outlook, Trends Mol. Med. 22 (2016) 946–957.

[19] N.D. Friedman, E. Temkin, Y. Carmeli, The negative impact of antibiotic resistance, Clin. Microbiol. Infect. 22 (2016) 416–422.

[20] E.M. Antão, C. Wagner-Ahlfs, Antibiotic resistance: A challenge for society, Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz. 61 (2018) 499–506.

[21] J.N. Pendleton, S.P. Gorman, B.F. Gilmore, Clinical relevance of the ESKAPE pathogens, Expert Rev. Anti. Infect. Ther. 11 (2013) 297–308.

[22] W. Thompson, S. Tonkin-Crine, S.H. Pavitt, R.R.C. McEachan, G.V.A. Douglas, V.R. Aggarwal *et al.*, Factors associated with antibiotic prescribing for adults with acute conditions: an umbrella review across

primary care and a systematic review focusing on primary dental care, J. Antimicrob. Chemother. 74 (2019) 2139–2152.

[23] A. Sebastian, P.G. Antony, M. Jose, A. Babu, J. Sebastian, A. Kunnilathu, Institutional microbial analysis of odontogenic infections and their empirical antibiotic sensitivity, J. Oral Biol. Craniofacial Res. 9 (2019) 133–138.

[24] F. Sgolastra, A. Petrucci, R. Gatto, A. Monaco, Effectiveness of Systemic Amoxicillin/Metronidazole as an Adjunctive Therapy to Full-Mouth Scaling and Root Planing in the Treatment of Aggressive Periodontitis: A Systematic Review and Meta-Analysis, J. Periodontol. 83 (2012) 731–743.

[25] N. Cionca, C. Giannopoulou, G. Ugolotti, A. Mombelli, Microbiologic testing and outcomes of full-mouth scaling and root planing with or without amoxicillin/metronidazole in chronic periodontitis, J. Periodontol. 81 (2010) 15–23.

[26] R.E.W. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.

[27] M. Murakami, T. Ohtake, R.A. Dorschner, R.L. Gallo, Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva, J. Dent. Res. 81 (2002) 845–850.

[28] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395.

[29] A.J. Duplantier, M.L. van Hoek, The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds, Front. Immunol. 4 (2013) 1–14.

[30] S.C. Mansour, O.M. Pena, R.E.W. Hancock, Host defense peptides: Front-line immunomodulators, Trends Immunol. 35 (2014) 443–450.

[31] K. Fukumoto, I. Nagaoka, A. Yamataka, H. Kobayashi, T. Yanai, Y. Kato *et al.*, Effect of antibacterial cathelicidin peptide CAP18/LL-37 on sepsis in neonatal rats, Pediatr. Surg. Int. 21 (2005) 20–24.

[32] N. Mookherjee, K.L. Brown, D.M.E. Bowdish, S. Doria, R. Falsafi, K. Hokamp *et al.*, Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37, J. Immunol. 176 (2006) 2455–2464.

[33] J.D. Heilborn, M. Frohm Nilsson, G. Kratz, G. Weber, O. Sørensen, N. Borregaard *et al.*, The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium, J. Invest. Dermatol. 120 (2003) 379–389.

[34] K. Yamanaka, O. Yamamoto, T. Honda, Pathophysiology of psoriasis: A review, J. Dermatol. 48 (2021) 722–731.

[35] K. Yamasaki, A. Di Nardo, A. Bardan, M. Murakami, T. Ohtake, A. Coda *et al.*, Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea, Nat. Med. 13 (2007) 975–980.

[36] K. Pütsep, G. Carlsson, H.G. Boman, M. Andersson, Deficiency of antibacterial peptides in patients with morbus Kostmann: An observation study, Lancet. 360 (2002) 1144–1149.

[37] S. Davidopoulou, E. Diza, G. Menexes, S. Kalfas, Salivary concentration of the antimicrobial peptide LL-37 in children, Arch. Oral Biol. 57 (2012) 865–869.

[38] H. Wakabayashi, M. Takase, M. Tomita, Lactoferricin derived from milk protein lactoferrin, Curr. Pharm. Des. 9 (2003) 1277–1287.

[39] H. Wakabayashi, K. Yamauchi, T. Kobayashi, T. Yaeshima, K. Iwatsuki, H. Yoshie, Inhibitory effects of lactoferrin on growth and biofilm formation of Porphyromonas gingivalis and Prevotella intermedia, Antimicrob. Agents Chemother. 53 (2009) 3308–3316.

9. Danksagung

An erster Stelle möchte ich mich bei meiner Doktormutter Frau Prof. Dr. Karin Christine Huth bedanken. Sie ermöglichte durch ihr Engagement für dieses Thema, die exzellente Betreuung sowie das Bereitstellen der notwendigen Mittel die Durchführung dieses Projekts.

Weiterhin danke ich Herrn Prof. Dr. Reinhard Hickel, Direktor der Poliklinik für Zahnerhaltung und Parodontologie, für die Möglichkeit, meine Doktorarbeit in seinem Hause durchzuführen zu dürfen.

Einen ganz besonderen Dank möchte ich meinem Mitbetreuer und Kollegen Herrn Dr. Maximilian Kollmuß aussprechen, der mir bedingungslos mit all seinem Wissen und Engagement stets zur Seite stand. Ich danke ihm für seinen unermüdlichen Einsatz im Labor, für unsere anregenden wissenschaftlichen Gespräche und für die kontinuierliche Unterstützung während des gesamten Publikationsprozesses beider Artikel.

Des Weiteren danke ich von ganzem Herzen meinen Brüdern und meinem Partner für die unendliche Geduld und die liebevolle und vielseitige Unterstützung.

Nicht zuletzt richte ich meinen außerordentlichen Dank an meine Eltern, denen ich meinen Zugang zur Wissenschaft und Forschung zu verdanken habe und die mich während meiner gesamten Promotion unermüdlich unterstützten und motivierten.