**Detektion intra- und interindividueller
Unterschiede mikrobieller Kompartimente im
humanen Speichel während dreimonatiger
Beobachtungszeit mit Hilfe der
Durchflusszytometrie**

**(Teilprojekt der wissenschaftlichen Arbeit:
A cytometric approach to follow variation and dynamics
of the salivary microbiota)**

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1. Einführung

Die Mundhöhle stellt ein einzigartiges Ökosystem mit einer Vielzahl heterogener Nischen dar. In der warmen, nährstoffreichen und feuchten Umgebung wird das Wachstum einer hoch diversen Mikroflora gefördert [1–5]. Die residenten oralen Bakterien formen komplexe, dynamische Biofilmgemeinschaften, welche beim Vorliegen gesunder Konditionen in mutualistischer Beziehung zum Wirt stehen [6–8]. Als primäre Eintrittspforte zum menschlichen Verdauungstrakt und Haupteingang für Pathogene ist die Mundhöhle jedoch kontinuierlichen Herausforderungen seitens des Wirtes und der Umwelt ausgesetzt [4]. Aufgrund der kurzen Generationsfolge bakterieller Systeme erfolgen Reaktionen auf sich verändernde Bedingungen prompt [9]. Eine resultierende Dysbiose des oralen Biofilms wird mit dem Auftreten lokaler (enoraler) sowie systemischer Erkrankungen in Verbindung gebracht [2, 10, 11]. Bemerkenswerterweise zeigt das orale Mikrobiom in gesunden Zuständen eine hohe Stabilität [12, 13].

Speichel dient dem Erhalt der oralen Homöostase [14]. Er beeinflusst in vielfacher Weise Besiedlung und Wachstum von Bakterien [1]. Als Analysemedium bietet er großes Potenzial, da er neben einer Vielzahl anderer Biomarker auch bakterielle Kompartimente unterschiedlicher Nischen enthält. Darüber hinaus lässt sich Speichel einfach entnehmen und verarbeiten [15, 16].

Das Aufkommen neuer Genomtechnologien wie Next-Generation Sequencing hat zu einem tiefen Einblick in die Diversität der Speichelmikrobiota geführt, doch die Erforschung von Dynamiken in der Mundhöhle stellt noch immer eine Herausforderung dar [17, 18].

Das in der Umweltmikrobiologie bereits etablierte Verfahren der Durchflusszytometrie ermöglicht prospektive Analysen komplexer Gemeinschaftsstrukturen, beispielsweise in Biogasanlagen und Abwassersystemen [19, 20]. Eine Übertragung der Methodik auf die Mundhöhle scheint vielversprechend für die Detektion und Illustration von Fluktuationen mikrobieller Kompartimente im Speichel [18].

1.1 Das orale Mikrobiom

Der menschliche Körper beheimatet eine Vielzahl von Bakterien. Geläufig wird ihre Menge als ein Zehnfaches der humanen Zellen zitiert [21]. Auch wenn neue Studien dieses Verhältnis relativieren und eine nahezu gleichmäßigen Verteilung der Anzahl von Bakterien- und Körperzellen beschreiben, bleibt der Einfluss bakterieller Gemeinschaften auf Gesundheit und Krankheit des Menschen unbestritten [22, 23]. Der Begriff „Mikrobiom“ bezeichnet die Gesamtheit der uns besiedelnden Mikroorganismen bzw. die Gesamtheit aller mikrobiellen Gene [24]. Seine Entschlüsselung und die Untersuchung seiner Auswirkungen sind von großem Interesse und Bestandteil intensiver Forschungen. Als globales und interdisziplinäres Konzept verfolgt dabei das 2007 gestartete *Human Microbiome Project (HMP)* das Ziel der Identifizierung und Charakterisierung des (gesamtheitlichen) menschlichen Mikrobioms [25].

Das Mikrobiom der Mundhöhle ist die am häufigsten untersuchte Mikroflora des Menschen. Dennoch ist der Wissensstand über die dort existierenden Bakterien noch immer limitiert [26]. Wie auch in anderen Bereichen des Körpers bestehen symbiotische Verhältnisse zwischen der residenten oralen Flora und dem Wirt, welche auf einer wechselseitigen Anpassung über Millionen von Jahren (Koevolution) beruhen [27]. So regulieren die kommensalen Bakterien etwa die Aktivität und Entwicklung von Immunzellen, schützen vor der Kolonisation exogener Mikroorganismen und wirken sogar bei Justierungen innerhalb des gastrointestinalen sowie des kardiovaskulären Systems mit [13]. In der *Human Oral Microbiome Database (HOMD)*, <http://www.homd.org/index.php>) werden alle bislang in der Mundhöhle detektierten Spezies in einheitlichen Schemata katalogisiert. Nach aktuellem Kenntnisstand besteht das orale Mikrobiom aus über 700 Bakterienspezies, von denen die Hälfte noch unbenannt und ein Drittel nicht kultivierbar sind [26, 28].

Die Mundhöhle beinhaltet ökologisch verschiedene Lebensräume (Habitats) wie Zähne, gingivale Sulci, Zunge, Wangenepithel, Vestibula, Gaumen, Tonsillen und Rachen [7, 28]. Durch das Vorhandensein weicher und harter oraler Gewebe, schwankender Sauerstoff- und Nährstoffkonzentrationen sowie variierenden Einflüssen von mechanischen Stress und Speichelfluss resultiert eine Vielzahl heterogener Nischen im gesamtheitlichen oralen Ökosystem [5]. Die in der Mundhöhle beheimateten bakteriellen Gemeinschaften weisen entsprechend eine

hohe Artenvielfalt (in anteilig gleichmäßiger Verteilung) auf, was durch den Begriff der „Alpha-Diversität“ beschrieben wird [7, 29]. Im Vergleich dazu zeigen die Mikrobiome anderer Körperbereiche wie der Haut und der Vagina eine deutlich geringere Alpha-Diversität [30–32]. Studien zufolge wird jedes der oralen Habitate von etwa 20-30 dominierenden Spezies kolonisiert [5]. Während manche Bakterien wie Spezies der Gattungen *Gemella*, *Granulicatella*, *Streptococcus* und *Veillonella* in allen Lebensräumen der Mundhöhle residieren, besiedeln andere bevorzugt spezifische Nischen. So finden sich beispielsweise *R. dentocariosa* und *S. gordonii* vorwiegend auf Zahnoberflächen, wohingegen *S. sanguinis* und *S. australis* eine Vorliebe für orale Weichgewebe zeigen [5, 15].

Der Vergleich der oralen Mikrobiome verschiedener gesunder Personen lässt eine hohe Anzahl gemeinschaftlich geteilter Taxa erkennen (definiert als geringe „Beta-Diversität“) [7]. Dies stützt die Annahme eines Kernmikrobioms Mundgesunder, welche Vertreter der Gattungen *Streptococcus*, *Veillonella*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Porphyromonas* und *Fusobacterium* inkludiert [33, 34]. Dennoch ist die mikrobielle Zusammensetzung jeder Mundhöhle einzigartig. Werden die oralen Mikrobiome zweier Personen verglichen, ergeben sich größere Unterschiede als bei der Gegenüberstellung intrapersoneller Differenzen über die Zeit [4, 22, 35–38]. Bei einem Vergleich der Mikrobiome gesunder Probanden mit solchen, die eine Assoziation zu Karies, Gingivitis oder Parodontitis aufweisen, sind ebenfalls große Heterogenitäten feststellbar [39]. So sind die Unterschiede zwischen gesunden und erkrankten oralen Mikrobiota derselben Person sogar größer als interpersonelle Diversitäten beim Vorliegen desselben klinischen Zustands (erkrankt/nicht erkrankt) [35].

Dabei werden diverse Mikroorganismen der Mundhöhle mit dem Auftreten verschiedener oraler Erkrankungen in Verbindung gebracht. Saccharolytische Bakterien wie *Streptococcus spp.*, *Lactobacillus spp.* und *Actinomyces spp.* scheinen im Kariesprozess eine herausragende Rolle zu spielen, während proteolytische Spezies der Gattungen *Prevotella* und *Porphyromonas* mit der Entstehung von Parodontitis assoziiert werden [40–44]. Allerdings stellen Karies und Parodontitis keine klassischen, von externen Spezies verursachten Infektionserkrankungen dar. Auch in der Mundhöhle Nicht-Erkrankter können potenzielle Oralpathogene detektiert

werden, welche zur kommensalen Flora gehören [2, 45–47]. Diese werden auch als „Pathobionten“ bezeichnet [48].

Die oralen Bakterien existieren nicht als unabhängige Entitäten, sondern organisieren sich räumlich sowie metabolisch innerhalb eines polymikrobiellen Biofilms an allen oralen Oberflächen [49, 50]. Physikalische und nahrungsassoziierte Faktoren, wie das Vorliegen bestimmter pH-Werte innerhalb des Biofilms oder die Verfügbarkeit von Glykoproteinen können zur Selektion von Teilpopulationen führen, die ein Ungleichgewicht der Zusammensetzung der hochstrukturierten mikrobiellen Gemeinschaft bedingen [11, 51]. Diese sogenannte Dysbiose wird gemäß der erweiterten ökologischen Plaquehypothese als Schlüsselfaktor für den Ausbruch oraler Erkrankungen begriffen [4, 10, 39, 52, 53]. Um das Verständnis für pathologische Vorgänge in der Mundhöhle und damit auch deren Therapierbarkeit zu erweitern, erscheint die Erforschung der kompositionellen Veränderungen innerhalb des Mikrobioms mundgesunder sowie erkrankter Probanden von großer Relevanz [4, 47].

1.2 Entwicklung des adulten oralen Mikrobioms und Einflüsse auf die mikrobielle Homöostase

Schon innerhalb weniger Stunden nach der Geburt wird die Mundhöhle von Mikroorganismen kolonisiert. Dabei ist der Geburtsvorgang (Kaiserschnitt oder vaginale Geburt) ausschlaggebend dafür, welche Bakterien durch die Mutter übertragen werden [54]. Während der postnatalen Entwicklung beeinflusst auch die Art der Nahrungsaufnahme die mikrobielle Zusammensetzung der Mundhöhle. So können bei gestillten Kindern mehr *Lactobacillus spp.* nachgewiesen werden als bei Babys, die Formulanahrung erhalten [55]. Zudem verändern die Milchzahneruptionen und später der Wechsel zwischen Milch- und permanentem Gebiss die physiologischen Bedingungen des oralen Ökosystems und führen somit zu Verschiebungen innerhalb der mikrobiellen Kompartimente [3, 56].

Nach seiner Reifung bleibt das adulte orale Mikrobiom unter mundgesunden Zuständen bemerkenswert stabil [13, 30]. Doch die Mundhöhle ist kontinuierlich multiplen Einflüssen ausgesetzt, die eine Herausforderung für das mikrobielle Gleichgewicht darstellen: Die enge räumliche Beziehung zwischen Bakterien innerhalb eines Biofilms führt zu Interaktionen, die einerseits synergistischer Art sind, wie Koaggregation [57], Quorum sensing [58], Gentransfer [59] und die Bildung von

Nahrungsketten [60, 61]. Andererseits können Bakterien mithilfe der Produktion von Bakteriziden und anderer inhibierender Substanzen auch gegen konkurrierende Spezies agieren [62–66].

Ferner wirken sich intrinsische biologische Wirtsparemeter auf das orale Milieu aus [39]. Verschiedene Komponenten des Immunsystems in Speichel, Serum und Sulkusflüssigkeit dienen zur Kontrolle der mikrobiellen Kolonisation und somit der Homöostase der Mundhöhle [67, 68]. Indes lassen sich Zusammenhänge zwischen Systemerkrankungen und dem Vorliegen unbalancierter oraler Zustände herstellen: So sind z.B. Hyposalivation, Rheumatoide Arthritis, nicht/schlecht eingestellter Diabetes mellitus und Übergewicht als Risikofaktoren für Karies und parodontale Erkrankungen beschrieben [69, 70]. Physiologische Veränderungen wie Hormonumstellungen in Pubertät und Schwangerschaft können ebenfalls die Stabilität der verschiedenen mikrobiellen Kompartimente stören und zur Einstellung eines neuen Gleichgewichts führen. Die Adaptation gesunder Individuen ist jedoch oft ohne Beeinträchtigung ihrer Mundgesundheit möglich [71].

Demgegenüber stehen extrinsische Faktoren, die das orale Mikrobiom beeinflussen. Die Mundhöhle, als offenes System, ist exogenen Bakterien z.B. aus Luft, Wasser, Nahrung und aus amourösem interpersonellen Kontakt exponiert [2, 72]. Vielfach ist auch die Assoziation zwischen Verhaltensweisen des Wirtes wie Rauch- und Essgewohnheiten auf die orale Homöostase beschrieben [73–78]. Der Konsum von Zucker bedingt u.a. als lokaler Einflussfaktor dysbiotische Zustände zwischen oralen Organismen und Wirt. Diese können im Auftreten und der Unterhaltung oraler Erkrankungen resultieren [79]. Häufigkeit der Zufuhr, absolute Menge des Konsums und Verweildauer in der Mundhöhle sind relevante Parameter für die Entwicklung des pathogenen Geschehens [80, 81]. Karies entsteht aus dem Wechsel eines gesunden oralen Mikrobioms zu einer azidogenen Gemeinschaft [82, 83]. Auch ein erhöhtes Risiko für parodontale Erkrankungen wird mit der häufigen Aufnahme von Zucker in Verbindung gebracht. Allerdings finden die hier zugrundeliegend Pathomechanismen auf Seiten der Wirtsabwehr statt, anstelle die bakterielle Flora zu betreffen [84, 85].

Als weiterer extrinsischer, modifizierbarer Faktor wirkt sich die Mundhygiene auf das Gleichgewicht der oralen mikrobiellen Kompartimente aus. Neben der mechanischen Biofilmkontrolle durch Zahnputzmaßnahmen wird dem Einsatz chemischer (antimikrobieller) Substanzen in Mundpflegeprodukten, wie Zahnpasten, -lacken, -gelen oder Mundspüllösungen ein ergänzender Einfluss zugesprochen [86]. Ihre

Wirkweise beruht unter anderem auf der Verhinderung oder Verzögerung der Bildung und Reifung von Biofilmen. Außerdem werden bakterielle Metabolismen wie Zuckertransport, Säureproduktion und Proteaseaktivität inhibiert, die mit der Entwicklung von Karies und parodontalen Erkrankungen in Verbindung stehen [13, 87, 88]. Das Ziel von Mundhygienemaßnahmen ist jedoch keine gänzliche Eradikation der oralen Mikrobiota. Vielmehr muss ein empfindliches Gleichgewicht gewahrt werden, das die Kontrolle des Biofilms auf einem mit Gesundheit vereinbaren Niveau erlaubt, ohne jedoch die benefizielle Flora zu zerstören [13, 87, 88].

Die angeführten Einflussfaktoren (Abb.1) können als Variablen verstanden werden, an die sich das orale Ökosystem in Gesundheit adaptieren kann, ohne die Stabilität des Gesamtsystems und das Gleichgewicht der mikrobiellen Kompartimente einzubüßen [12, 71].

Abbildung 1: Einflüsse auf die mikrobielle Homöostase

Positive Einflüsse auf das Gleichgewicht der mikrobiellen Kompartimente in grüner Umrandung, negative Einflüsse in rot. Grau markierte Parameter können die Homöostase sowohl stabilisieren als auch destabilisieren.

1.3 Untersuchungsmethoden der oralen Mikrobiota

Seit den ersten Beobachtungen von „animalcules“ (kleinen Tieren) innerhalb des Zahnbelags, die Antoni van Leeuwenhoek im Jahr 1680 unter seinem Einlin sen- Lichtmikroskop machte, haben sich die Techniken zur Untersuchung der oralen Organismen rasant weiterentwickelt [89–91].

Die Anzucht von Bakterien unter definierten und reproduzierbaren Bedingungen (Kultivierung) erlaubt die Identifizierung, Benennung und Klassifizierung einzelner Zellen, sowie die Beschreibung deren physiologischer und pathogener Eigenschaften [92]. Als einfaches und ökonomisches Verfahren ist die klassische Kultur ein wesentlicher Eckpfeiler der mikrobiologischen Diagnostik. Die Bestimmung von Lebendzellzahlen über die Zählung von koloniebildenden Einheiten (englisch: *colony-forming units*, CFU) stellt eine Möglichkeit der Quantifizierung von Mikroorganismen dar. Sie birgt jedoch die Gefahr der Unterschätzung, da Zellen, die vital aber nicht kultivierbar sind, der Detektion entgehen [93–95]. Trotz innovativer Methoden, wie der Kultivierung unter anaeroben Bedingungen oder der Bereitstellung verschiedener Nährstoffe, Wachstumsfaktoren, pH-Werte und Temperaturen, ist ein Großteil der oralen Mikroorganismen bislang nicht kultivierbar, da die vielschichtigen interbakteriellen Netzwerke ex vivo nicht adäquat nachgeahmt werden können [92, 96].

Einige Limitationen zur Detektion und Typisierung oraler Spezies sind heute dank molekularbiologischer Ansätze überwunden. Dazu zählen DNA/RNA-Hybridisierung, Polymerasekettenreaktion (PCR) und Sequenzierung. Gemeinsame Zielstruktur dieser kulturunabhängigen Technologien ist die mikrobielle Nukleinsäure. Insbesondere das 16S-rRNA-Gen stellt hierbei einen idealen Marker für die Identifizierung und Klassifizierung von Bakterien dar, da es sowohl hochgradig konservierte als auch hypervariable Regionen enthält.

Hybridisierungsbasierte Untersuchungen nutzen die spezifische Anlagerung eines zum bakteriellen Zielnukleinsäurestrangs komplementären Oligonukleotids, welcher radioaktiv oder mittels Farbstoffen markiert ist und durch geeignete Instrumente bzw. chemische Interaktionen detektiert werden kann. Dabei werden bei der Fluoreszenz-in-situ-Hybridisierung (FISH) mit verschiedenen Fluoreszenzfarbstoffen gekoppelte DNA-Sonden in fixierte Bakterienzellen eingeschleust [97, 98]. Eine Analyse kann anschließend mittels konfokaler Laser-Scanning-Mikroskopie (CLSM) erfolgen [95]. So ist der direkte, simultane in situ Nachweis einzelner Taxa der Mundhöhle sowie

ihre Darstellung in komplexen räumlichen Strukturen möglich [99, 100]. Allerdings lässt sich bei FISH nur eine geringe Anzahl von Spezies innerhalb einer Probe differenzieren, indem verschiedene Farbfilter zur Betrachtung unterschiedlich markierter Sonden Anwendung finden. Die Nukleotidfolge der 16S rRNA, welches das bakterielle Zielmolekül ist, wird für die Herstellung der Gensonden benötigt. Daher sind Mikroorganismen mit unbekanntem Genom nicht detektierbar. Dies stellt neben dem hohen Laboraufwand einen großen Nachteil der Methode dar [98].

Die PCR ist eine in den 1980er Jahren entwickelte Technik zur exponentiellen Vervielfältigung von DNA. Dank ihrer hohen Sensitivität und Spezifität ist sie unter anderem für die Identifikation von Bakterien und den Nachweis auch geringster Mengen bakterieller DNA innerhalb einer untersuchten Multispezies-Probe elementar [92, 101]. Während die Amplifikation artspezifischer hypervariabler Sequenzen des bakteriellen 16S rRNA-Gens die Detektion einzelner Arten ermöglicht, kann beim Einsatz von Universalprimern, die an konservierte Regionen dieses Gens binden, die DNA vieler unterschiedlicher Bakterien vervielfältigt und nachgewiesen werden [102]. Automatisierungen und Standardisierungen der zugrundeliegenden zyklischen Prozesse vereinfachen die Anwendung der PCR und führen zu einem hohen Probendurchsatz. Zudem lässt die sogenannte quantitative Echtzeit-PCR (qPCR) eine kontinuierliche Verfolgung und Quantifizierung der PCR-Produkte durch die Nutzung von DNA-Farbstoffen zu. Diese Fluoreszenzfarbstoffe interkalieren mit den in jedem neuen Zyklus synthetisierten DNA-Molekülen, sodass die Intensität des detektierbaren Fluoreszenzsignals mit der Mengenzunahme der amplifizierten DNA korreliert [103, 104]. Als Limitation dieser Methode ist u.a. die negative Beeinflussung durch Inhibitoren in biologischen Proben zu nennen [105]. Darüber hinaus kann das Vorliegen von DNA toter Zellen und von extrazellulärer DNA zu einer Überschätzung der Anzahl vitaler Zellen in einer Probe führen [106]. Neben der Nutzung als eigenständige Technik dient die PCR auch der Generierung von Untersuchungsmaterial für weitergehende Analysen, wie der nachfolgend beschriebenen Sequenzierung [104].

Als Sequenzierung wird die Bestimmung der Nukleotid-Abfolge in einem DNA-Molekül verstanden. Dieses DNA-analytische Verfahren gilt als Goldstandard für die Untersuchung der Diversität des humanen Mikrobioms [107]. Die Kombination aus 16S-rRNA-Genvervielfältigung, Klonen und klassischer Sanger-Sequenzierung führte zur Entdeckung bakterieller Taxa der Mundhöhle in nie dagewesener Dimension

[108]. Innerhalb öffentlicher Datenbanken wie der „*Human Oral Database*“ werden die ausgelesenen Sequenzen des 16S-rRNA-Gens hinterlegt und sofern möglich mit phänotypischen, phylogenetischen, klinischen und bibliographischen Informationen zu den entsprechenden Arten verlinkt [26]. Bis heute stellen etwa 46% der in dieser Datenbank aufgeführten Taxa allgemeingültig benannte Spezies dar, 14% sind jedoch unbenannt (aber kultiviert) während von 32% nicht mehr als die 16S-rRNA-Gensequenz bekannt ist [26, 28]. Die Anwendbarkeit der klassischen Sequenzierung ist durch ihren hohen ökonomischen und laboratorischen Aufwand sowie niedrigen Geschwindigkeiten limitiert. Unter dem Begriff „*Next-Generation Sequencing*“ (NGS) werden neu entwickelte Sequenzierungstechniken zusammengefasst, die eine Vielzahl von DNA-Fragmenten in einem Durchlauf parallel erfassen können, was einen erhöhten Probendurchsatz erlaubt. Dies resultiert in kostengünstigeren und schnelleren Analysen bakterieller Gemeinschaften [109, 110].

Obgleich mit Hilfe der genannten molekularen Methoden und Sequenzierungstechniken tiefgreifende phylogenetische oder funktionelle Analysen des oralen Mikrobioms durchgeführt werden können, sind sie doch bis heute nicht imstande die Dynamiken in der Zusammensetzung der mikrobiellen Kompartimente der Mundhöhle zu illustrieren [20].

1.4 Durchflusszytometrie

Eine spezielle Methode, um komplexe Ökosysteme schnell und ökonomisch zu untersuchen, ist die mikrobielle Durchflusszytometrie. Sie beruht auf der Vermessung spezifischer optischer Charakteristiken von Zellen, die durch einen Hüllstrom fokussiert einzeln durch den Messbereich eines Lasers geleitet werden. In zweidimensionalen Histogrammen erfolgt die Darstellung der Zellen einer betrachteten Probe gemäß der gewählten Parameter Zellgröße, Granularität und/oder DNA-Gehalt. Die innerhalb des Plots entstehenden Cluster werden als Subgemeinschaften des zugrundeliegenden Mikrobioms verstanden, während die Gesamtheit des zytometrischen Histogramms einen bakteriellen Fingerabdruck der untersuchten Probe repräsentiert (Abb. 2) [111]. Bioinformatische Tools ermöglichen die nahezu automatisierte Analyse der Unterschiede zwischen verschiedenen zytometrischen Fingerabdrücken, sodass eine schnelle Detektion und Illustration von Veränderungen (Shifts) innerhalb bakterieller Kompartimente erfolgen kann. In Bereichen der Umweltmikrobiologie, wie der Untersuchung von Abwassersystemen

und Biogasanlagen, hat sich die Durchflusszytometrie als Methodik bereits etabliert. Hier findet sie Anwendung in der Überwachung von Veränderungen zellspezifischen und abiotischer Parameter von Mikroumgebungen [19, 20].

Die Übertragung des Verfahrens auf die Untersuchung des oralen Mikrobioms scheint vielversprechend zum Erlangen prospektiver Einblicke in die komplexen Zusammenhänge, Interaktionen und Entwicklungen der bakteriellen Gemeinschaften der Mundhöhle.

Abbildung 2: Exemplarisches 2D-Histogramm (zytometrischer Fingerabdruck)

Darstellung der Zellen einer Probe innerhalb des Zellgates gemäß der gewählten Parameter Zellgröße (Forward Scatter) und DNA-Gehalt (DAPI fluorescence). Die Ellipsen markieren einzelne Cluster, die als Subgemeinschaften des vermessenen Mikrobioms verstanden werden.

1.5 Speichel

Obgleich zu über 99% aus Wasser bestehend, enthält der humane Speichel eine Vielzahl sowohl organischer (Proteine/Peptide) als auch anorganischer (Elektrolyte) Stoffe, die maßgeblich am Erhalt der oralen Gesundheit beteiligt sind [112, 113]. Speichel ist ein Sekret der großen und kleinen Speicheldrüsen und setzt sich ferner aus Molekülen des Blutes und der Sulkusflüssigkeit, aus Zellen der oralen Schleimhäute, Nahrungsbestandteilen und Mikroorganismen zusammen [112, 114]. Allein die Anzahl bakterieller Zellen wird dabei auf 10^6 - 10^9 pro ml Speichel datiert [115–117].

Innerhalb der letzten zwei Dekaden gewann die Nutzung des Speichels als diagnostisches Tool an erheblicher Bedeutung. Er stellte sich nicht nur als Quelle für Biomarker oraler Erkrankungen wie Parodontitis und Mundhöhlenkrebs heraus [114, 118, 119]. Auch Indikatoren systemischer und infektiöser Pathologien wie Autoimmunerkrankungen (z.B. M. Crohn), Karzinomen verschiedener Gewebe, Herzkrankheiten und HIV konnten im Speichel nachgewiesen werden [120–122].

Der Begriff „*Salivaomic*“ vereint alle biologischen Fachgebiete, die sich mit der Gesamtheit der diagnostisch relevanten Zielstrukturen, also dem Genom, Epigenom, Transkriptom, Proteom, Metabolom und Mikrobiom im Speichel beschäftigen [123]. Neben seiner kompositorischen Vielfalt gibt es weitere Faktoren, die den Speichel zu einem attraktiven Analysemedium gestalten. Im Gegensatz zu Blutuntersuchungen ist seine Entnahme unkompliziert und non-invasiv möglich, nahezu uneingeschränkt wiederholbar, patientenfreundlich und mit einem niedrigen Infektionsrisiko verbunden [118, 124]. Auch der Versand und die Lagerung von Speichel sind einfach und kostengünstig durchführbar [119, 125].

Die Funktionen des Speichels sind vielfältig und eng mit seinen Komponenten verknüpft: Remineralisation, Pufferwirkung, antimikrobielle Abwehr, Bolusbildung und Spülfunktion seien beispielhaft als wichtige, die Mundgesundheit positiv beeinflussende Faktoren genannt [41, 117, 126, 127]. Die komplexe biologische Flüssigkeit wirkt sich in mehrfacher Hinsicht direkt auf Kolonisation, Wachstum und Aktivität von Bakterien aus [1, 10, 117, 128]. Das Mikrobiom des Speichels weist in gesunden Zuständen eine höhere Alpha-Diversität (phylogenetische Diversität) als andere oralen Nischen auf [4, 26, 29, 129]. Dabei gehören die verschiedenen Bakterienspezies vornehmlich den 5 Stämmen *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* und *Fusobacteria* an [17, 130]. Das Speichelmikrobiom

zeigt große Analogien zum Mikrobiom oraler Schleimhäute (Zunge, Rachen, Tonsillen), da es deren abgeschilferte Bakterien enthält [4, 17, 46, 74, 131]. Es wird darüber hinaus als Reservoir der mikrobiellen Kompartimente aller oralen Nischen verstanden, fungiert doch der Speichel als Schnittstelle und Transportmedium für Bakterien zwischen Zunge, Zahnhartgeweben und oraler Schleimhaut [16, 17, 132, 133]. So erklärt sich die Annahme, dass die Zusammensetzung des Speichelmikrobioms (auch) lokale Zustände widerspiegelt [16, 134]. Mit oraler Gesundheit sind dabei eine vergleichsweise geringe phylogenetische Diversität und gleichmäßige Verteilung bakterieller Gattungen im Speichel assoziiert [46, 47, 135–137]. Sequenzierungsbasierte longitudinale Betrachtungen zeigen eine hohe Stabilität gesunder Speichelmikrobiome [129, 138]. Zeitlich engmaschig entnommene Proben einer Person ähneln sich dabei nicht zwangsläufig mehr als jene Entnahmen mit einem größeren Zeitintervall [139]. Dies verweist auf das Vorhandensein personenspezifischer bakterieller Taxa und Muster im Speichel, die geringen Fluktuationen unterliegen [4, 139]. Der Tageszeitpunkt der Entnahmen spielt für die Unterschiede innerhalb der bakteriellen Zusammensetzung eher eine untergeordnete Rolle [140]. Darüber hinaus zeigen die Untersuchungen von stimulierten und unstimulierten Speichelproben derselben Probanden vergleichbare Ergebnisse [141]. Wie auch für das gesamte orale Mikrobiom zutreffend, sind die Abweichungen zwischen den Speichelgemeinschaften verschiedener oral Gesunder größer als die gemessenen intraindividuellen Differenzen. Daraus resultiert die Annahme, dass die Diskrimination zweier Personen anhand der bakteriellen Kompartimente ihres Speichels möglich ist [130].

Beim Vorliegen oraler Erkrankungen wie Karies oder Parodontitis können im Speichel spezifische Bakterienprofile mit einem signifikant erhöhten Anteil krankheitsassoziierten Gattungen identifiziert werden [129, 134, 136, 137, 142]. Die aktuelle Studienlage basiert vornehmlich auf zeit- und kostenintensiven Sequenzierungsmethoden zur Untersuchung des phylogenetischen Aufbaus des Speichels. Die Erkenntnis, dass kompositionelle Shifts und Unterschiede der bakteriellen Kompartimente im Speichel bedeutungsvoll für interpersonelle Unterscheidungen und für die Differenzierung zwischen gesunden und kranken oralen Bedingungen sind, rechtfertigt die Suche nach einem Analyseverfahren, welches ein schnelles Screening von Speichelproben zur Detektion und Visualisierung solcher Änderungen ermöglicht.

1.6 Zielsetzung und Fragestellung der vorliegenden Studie

Die vorliegende Pilotstudie verfolgte das Ziel, die Durchflusszytometrie als praktikables Tool zur umfassenden Untersuchung und Darstellung des Speichelmikrobioms mundgesunder Probanden zu validieren. Es wurde ein standardisierter und reproduzierbarer Arbeitsprozess erarbeitet. Dabei sollte die Methode unter verschiedenen Fragestellungen angewendet und geprüft werden. Die Realisierung des Gesamtprojektes erfolgte in drei Teilprojekten durch die Doktoranden Susanna van Gelder und Nicola Röhrig, wobei die Projektbereiche wie folgt bearbeitet wurden:

- I. **Longitudinalstudie** (Inhalt der vorliegenden Dissertationsschrift):
Teilziel: Analyse von Diversität und Dynamik der Speichelmikrobiome mundgesunder Probanden innerhalb einer dreimonatigen Longitudinalstudie.
Hypothese: Die Speichelmikrobiome mundgesunder Probanden sind trotz autonomer dynamischer Schwankungen über einen mehrmonatigen Untersuchungszeitraum intraindividuell stabil und interindividuell divers. Detektion und Visualisierung der wiederkehrenden individuellen Grundstrukturen sowie von Dynamiken innerhalb der bakteriellen Zusammensetzung im Speichel sind mit Hilfe der Durchflusszytometrie möglich.
- II. **Stressversuche** (Inhalt der Dissertationsschrift von Frau Susanna van Gelder):
Teilziel: Detektion individueller Veränderungen des Speichelmikrobioms mundgesunder Probanden in Reaktion auf intensiven temporären Zucker- bzw. Säurestress.
Hypothese: Die hochfrequente orale Aufnahme zucker- bzw. säurehaltiger Nahrungsmittel führt zu individuell verschiedenen Modulationen innerhalb der bakteriellen Zusammensetzung des Speichels. Diese Dynamiken sind mit Hilfe der Durchflusszytometrie nachweisbar.
- III. **Einsatz antibakterieller Mundspüllösungen** (Inhalt der Dissertationsschrift von Frau Susanna van Gelder):
Teilziel: Untersuchung des Einflusses verschiedener Mundspüllösungen auf die Zusammensetzung des Speichelmikrobioms mundgesunder Probanden

bei einmaliger Applikation. Zusätzlich Messung von Bakterienzellzahlen im Speichel und Analyse des Einflusses der chlorhexidinhaltigen Mundspüllösung auf die Bakterienlast.

Hypothese: Einmalig applizierte Mundspüllösungen modifizieren die Zusammensetzung der mikrobiellen Kompartimente im Speichel mundgesunder Probanden nicht. Neben dem Nachweis von Dynamiken innerhalb des Speichelmikrobioms sind auch Zellzahlmessungen mit Hilfe der Durchflusszytometrie möglich. Die chlorhexidinhaltige Mundspüllösung reduziert die Zellzahlen im Speichel bereits nach einmaliger Anwendung.

2. Publikationsmanuskript

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A cytometric approach to follow variation and dynamics of the salivary microbiota

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ABSTRACT

Microbial flow cytometry is an established fast and economic technique for complex ecosystem studies and enables visualization of rapidly changing community structures by measuring characteristics of single microbial cells. Cytometric evaluation routines are available such as flowCyBar which are useful for automatic data processing. Here, a cytometric workflow was established which allows to routinely analyze salivary microbiomes on the example of ten oral healthy subjects. First, saliva was collected within a 3-month period, cytometrically analyzed and the evolution of the microbiomes followed as well as the calculation of their intra- and inter-subject similarity. Second, the respective microbiomes were stressed by exposition to high sugar or acid concentrations and immediate changes were recorded. Third, bactericide solutions were tested on their impact on the microbiomes. In all three set ups huge intra-individual variations in cytometric community structures were found to be largely absent, even under stress, while inter-individual diversity was obvious. The bacterial cell counts of saliva samples were found to vary between 3.0×10^7 and 6.2×10^8 cells per sample and subject in undisturbed environments. The application of the two bactericides did not cause noteworthy diversity changes but the loss in cell numbers by about 50% was high after treatment. Illumina[®] sequencing of whole microbiomes or sorted sub-microbiomes revealed typical phyla such as *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Fusobacteria*. This approach is useful for fast monitoring of individual salivary microbiomes and automatic calculation of intra- and inter-individual dynamic changes and variability and opens insight into ecological principles leading to their sustainment in their individual environment.

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1. Introduction

Microorganisms tend to assemble to complex three-dimensional structures in which they are spatially organized as intimately interacting microbial communities. Embedded in an exo-polysaccharide-matrix these constructs form biofilms [1–4]. They are ubiquitous and can grow on natural as well as artificial surfaces [5]. The oral cavity as a highly organized ecological system provides all conditions to enable the indigenous bacteria to form and mature such complex structures [1,3,6]. Currently, roughly 700 phylotypes have been detected in the oral bacterial microbiome. Species can be characterized by being specific for a site

(e.g. tongue, palate, buccal mucosa, tonsils, plaque) as well as promoting health or disease [7–9].

Multitudes of modulating factors like varying bacterial and molecular interactions, micro-geographical characteristics (protective niches), nutrient availability, diet and host defense cause a heterogeneous ecosystem within the oral cavity which is further supported by distinct micro-niches [4,6,10–12]. High dynamics within this system can be assumed as microorganisms adapt rapidly to altering environmental conditions because of the generally short generation times of microorganisms under such growth supporting conditions [13]. Caries and periodontitis, as the most common oral pathologies are classic examples for primarily biofilm-related diseases but are in entirety multifactorial conditioned [1,4,14,15]. Not the appearance of single pathogens (specific infection) is responsible for the etiology of these oral diseases but the disturbance of the oral homeostasis that triggers the predominance of facultative pathogenic species (opportunistic infection) [16,17].

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The homeostasis of the oral cavity is preserved by saliva [18,19]. It is an elementary part of this environment and the substance that links the different niches [20]. Therefore it provides a representative analysis medium for the global assessment of the oral microbiome [21–23]. The examination of the salivary microbiome is greatly relevant since it serves as a reservoir of the overall oral microbiota in its planktonic phase. Saliva as the planktonic suspension mirrors changes that are associated with oral diseases like caries, gingivitis and periodontitis [9,21–25]. It is indispensable to investigate the salivary microbiome of the healthy in its entirety before shifts that promote oral diseases are detectable [7]. An advantage of analyzing the saliva is its simple, non-invasive and economical collection [19,26].

The study of oral microbial communities has changed from the first report of it by Leeuwenhoek in 1676 using single-lens microscopy [27] to the characterization using the current molecular biology techniques. Fluorescence *in situ* hybridization (FISH) enables a direct, simultaneous surveillance of bacterial taxa, but is confined to a few species [28,29]. The present gold standard for analyzing human microbiomes diversity are sequencing methods. Based on these techniques, a large-scale project including approximately 200 healthy subjects was launched with the result of the human oral microbiome database (HOMD) with global information on approximately 700 bacterial species detected in the oral cavity [30]. However, sequencing is still time- and labor-intensive. Another approach for analyzing complex ecosystems in a fast and economic way is microbial flow cytometry [31]. This is an established technique in environmental microbiology and enables the visualization of rapidly changing community structures in cytometric histograms [13,32,33]. For the evaluation of the microbial cytometric data bioinformatic tools such as Cytometric Barcoding (flowCyBar) are available that allow a nearly automatic interpretation of the data [13,34].

Aim of this study was to establish a cytometric protocol for the analysis of saliva microbiota to enable fast monitoring of changes in and between diverse microbiota. Thus, the project focused on balance or dys-balance rather than qualitative, taxonomic composition. Existing bioinformatic evaluation pipelines were tested for their resolution depth and their ability to reveal saliva community variation. Detectability of individual profiles within this methods study was hypothesized as well as the feasibility of monitoring changes during time and different stress impacts (sugar and acid, bactericide mouth-rinses).

2. Material and methods

2.1. Subjects

Ten subjects with the following inclusion criteria were selected for the study: representing both genders, ranging in age from 21 to 40, and with no signs of oral disease. The subjects were examined orally prior to sampling and did not have any active caries lesions, nor showed any signs of periodontal diseases. During the examination, a general anamnesis was recorded, which assessed the presence of any general diseases, medications or further oral health related parameters (e.g. smoking habits). Furthermore, a dental examination using the decayed-, missing- and filled-teeth status (DMF-T) was performed to detect carious teeth showing a cavitation of the surface (D-T). Furthermore, the PSR/PSI was executed which detects periodontal probing depth and bleeding on probing and thus reflects the periodontal treatment need. Exclusion criteria were: general diseases, antibiotic treatment six months prior to and during sample collection or any medication that could influence the saliva secretion. Instructions of the subjects regarding the individual oral hygiene were given. For detailed information

see [Supplementary material Table S1](#). Each experiment was run twice (for the results of the second run see [Supplementary material Fig. S3–S5](#)). The study was approved by the ethics committee of the medical faculty of the University of Leipzig, Germany (069/17-ek). All participants were informed verbally and in writing about the study and gave their written informed consent.

Trouble shooting: Different oral hygiene habits (techniques, frequency), dissimilarity of oral health state, subjects' eating habits, lack of examination of intimate partners, current respiratory tract infections

2.2. Sample collection

The subject was instructed to carry out the last oral hygiene procedure until 12 am of the day before collection, unless otherwise indicated. One hour (h) prior to the sample collection, the subject was asked to refrain from eating and drinking. The collection of saliva samples was conducted in accordance to the standardized spitting method protocol by Navazesh, 1993 [35]. The subject was seated comfortably for a rest of five minutes to adapt to the situation. After a mouth rinse with distilled water for 30 s the 5-min-collection period for unstimulated saliva followed. The subject was instructed to minimize orofacial movements during this time. The whole saliva was spit into a sterile graduated test tube on ice. Unstimulated saliva was collected to minimize potential impacts of additional process steps and due to reduced costs of materials for saliva-stimulating agents (e.g. paraffin).

Trouble shooting: Lacking certainty of following all guidelines given in the test design, unintentional stimulation of saliva, increased/decreased rate of secretion (stress, fluid intake, temperature etc.), loss of sample material (accidental swallowing, missing the collecting tube)

Reagents: crushed ice, distilled water, collection tubes (Corning, New York, USA)

2.3. Sample fixation

Sterile, cooled glycerol as cryoprotective agent was added to the saliva samples in a concentration of 15% (v/v). After 10 min incubation time on ice and division into 2–3 aliquots the samples were shock frozen in liquid nitrogen and afterwards stored at -80°C .

Trouble shooting: Inaccuracy of volume determination (disruptive element: foam), heterogeneity of saliva, non-compliance with time and temperature requirements

Reagents: sterile, cooled glycerol, crushed ice, liquid nitrogen

2.4. Cell staining

Deep frozen and glycerol fixed saliva samples were put on crushed ice to defrost. The optical density (OD) of the cells was adjusted to 0.1 ($d_{\lambda 700\text{nm}} = 0.5\text{ cm}$) with PBS (6 mM Na_2HPO_4 , 1.8 mM NaH_2PO_4 , 145 mM NaCl with bi-distilled H_2O , pH 7) and well mixed. After centrifugation of 2 ml of this solution for 10 min at 4°C and 3200g the supernatant was discarded. The cell-pellet was resuspended in 0.5 ml of permeabilization buffer (0.1 M citric acid, 4.1 mM Tween 20, bi-distilled water) and incubated for 20 min at room temperature. After a further centrifugation step the supernatant was discarded and the cells were resuspended in 1 ml DNA staining solution (0.68 μM 4',6-di-aminodino-2-phenyl-indole (DAPI, Sigma-Aldrich, St. Louis, USA), in 417 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (289 mM Na_2HPO_4 , 128 mM NaH_2PO_4 with bi-distilled H_2O , pH 7)) for subsequent staining overnight at 6°C until cytometric measurement. A biological standard (*Escherichia coli* BL21 (DE3), stationary phase of growth curve (16 hs cultivation time), fixed with PFA (2%)/EtOH (70%)) was

stained as above except the OD was adjusted to 0.035 ($d_{470nm} = 0.5$ cm).

Trouble shooting: Pipetting inaccuracies, processing aberrations and inhomogeneity due to high viscosity of saliva, instability of the cell-pellet, disruptive factors (big human cells, extrinsic soiling)

Reagents: PBS, permeabilization buffer, DNA staining solution, biological standard (UFZ strain collection, Germany), crushed ice

2.5. Flow cytometry

Cytometric data were generated using the MoFlo Legacy cell sorter (Beckman-Coulter, Brea, California, USA). It is equipped with two lasers. The blue laser Genesis MX488-500 STM OPS (Coherent, Santa Clara, California, USA) (488 nm, 400mW) was used to determine the forward scatter (FSC; bandpass filter 488 nm $\pm$ 5 nm, neutral density filter 1.9) which is an optical characteristic containing information related to cell size and the side scatter (SSC; bandpass filter 488 nm $\pm$ 5 nm, neutral density filter 1.9, trigger signal) which is an optical characteristic containing information related to cell density. The UV laser Xcyte CY-355-150 (Lumentum, Milpitas, California, USA) (355 nm, 150mW) was used for exciting the DAPI fluorescence (bandpass filter 450 nm $\pm$ 32.5 nm), an optical characteristic that is used for quantification of cellular DNA-content. Photomultiplier tubes were purchased from Hamamatsu Photonics (Models R928 and R3896; Hamamatsu City, Japan). The fluidic system was run at 56 psi (3.86 bar) with sample overpressure at maximum 0.3 psi and a 70 μ m nozzle. The sheath fluid was composed of 10 $\times$ Sheath buffer (19 mM KH_2PO_4 , 38 mM KCl, 166 mM Na_2HPO_4 , 1.39 M NaCl with bi-distilled H_2O) diluted with 0.1 μ m filtrated bi-distilled H_2O to a 0.2 $\times$ working solution (for cell sorting: 0.5 $\times$ working solution). Prior to all measurements, daily and in-between-day calibration of the instrument was performed linearly with 1 μ m blue fluorescent beads (FluoSpheres F8815 (350/440), lot No.: 69A1-1) and 2 μ m yellow-green fluorescent beads (FluoSpheres F-8827 (505/515), lot No.: 1717426), both from Molecular Probes (Eugene, Oregon, USA). Blue fluorescent beads (0.5 μ m and 1 μ m, both Fluoresbrite BB Carboxylate microspheres, (360/407), lot No.: 552744 and 499344, PolyScience, Niles, Illinois, USA) were used for calibration of logarithmic scale and added to each sample for measurement stability. A biological standard (*Escherichia coli* BL21 (DE3)) was measured as a biological adjustment. The stained samples were filtered using 50 μ m CellTrics filter (Sysmex Partec GmbH, Görlitz, Germany) before measurement to

prevent clogging of the cytometer nozzle. By measuring the samples, logarithmically scaled 2D-dot plots with FSC (cell size) against DAPI-fluorescence (chromosome content) as parameters were generated (Fig. 1). During these measurements, a parent gate has been created with Summit 4.3 (Beckman-Coulter, Brea, CA) comprising all stained cells and excluding noise and beads. Every sample was measured at a maximum speed of 3000 events/s until 150,000 cells within this parent gate were detected. Raw cytometric data can be found under: <https://flowrepository.org/id/RvFrzrE6fOGuSmf7NqWmPVGkY1rKfqFnB3hXuii6qghQ63b9PDJPM8qlxRqEQZW>

Trouble shooting: Precipitation of the buffer, air-bubbles in the system, insufficient sample amount, high sample intrinsic noise

Reagents: 1 μ m and 2 μ m yellow green fluorescent beads, 0.5 μ m and 1.0 μ m blue fluorescent beads

2.6. Cell counting

Cell numbers of the cell suspensions were determined by SYBR GreenI staining of DNA to stain all cells. In comparison to DAPI staining no centrifugation steps are necessary to avoid cell loss. Staining was performed using OD 0.1 ($d_{470nm} = 0.5$ cm) adjusted sample suspensions, which were filtered using 50 μ m CellTrics filter (Sysmex Partec GmbH, Görlitz, Germany). 925 μ l filtered sample solution were stained with 50 μ l 100% ethanol (working concentration 5%) and 25 μ l 20 $\times$ SYBR GreenI solution (working concentration 0.5 $\times$) (ThermoFisher Scientific, Waltham, Massachusetts, USA) for at least 30 min. 1 μ m yellow-green fluorescent beads (FluoSpheres F-13081 (505/515), lot No.: 63B2-1, Molecular Probes (Eugene, Oregon, USA)) with a microscopically determined concentration were added to the staining solution prior to measurement. SYBR GreenI was excited at 488 nm with the Genesis MX488-500 STM OPS laser and logarithmically scaled 2D-dot plots with red (bandpass filter 670 nm $\pm$ 15 nm) against green fluorescence (bandpass filter 530 nm $\pm$ 20 nm) were created according to Hammes et al., 2012 [36]. Gates to determine cells and beads were created (Supplementary material Fig. S1) and cell counts/ml calculated as follows:

$$\text{Cell number ml}^{-1} = \frac{f \cdot C(\text{parent}) \cdot B \cdot V}{B(\text{YG}) \cdot V(\text{sample})}$$

f: Dilution rate of sample for counting

C(parent): Virtual cell number in the parent gate

Fig. 1. Exemplary cytometric 2D-histogram and gate template. A 2D-histogram created by the generated data of MoFlo measurements is depicted. The two parameters Forward scatter (cell size) vs. DAPI fluorescence (DNA content) were chosen. A: Cell gate, defined in FlowJo V10 (FlowJo, LLC, Oregon USA) excluding noise and 0.5 μ m and 1.0 μ m beads, depicted with an exemplary saliva sample (subject 6, taken at baseline for the mouth rinse experiment). For detailed information see Section 2.8. B: Gate template, generated in FlowJo V10. It consists of 37 gates and must be valid for all samples.

B: Defined concentration of 1 μm yellow-green fluorescent beads

V: Volume of defined concentration 1 μm yellow-green fluorescent beads

B(YG): Number of beads in the gate 1 μm yellow-green fluorescent beads

V(sample): Defined volume of DNA-stained cells sample

Cell count/sample volume was determined using the estimated sample volume.

Trouble shooting: Pipetting inaccuracies, processing aberrations and inhomogeneity due to high viscosity of saliva

Reagents: Ethanol (100%), SYBR GreenI solution, 1 μm yellow-green fluorescent beads

2.7. Cell sorting

The cell sorting procedure was done according to the protocol by Koch, 2013 [33]: A representative number of samples were chosen under the premise of comprising 500,000 cells per gate and selected for sorting. The most accurate sort mode of the MoFlo (single and one-drop mode: highest purity 99%) at a rate not higher than 2500 events/s was adjusted. After sorting, cells were harvested by a centrifugation step (20,000g, 4 °C, 25 min), and the pellet was frozen at $-20\text{ }^{\circ}\text{C}$ for later DNA isolation and Illumina® sequencing. For detailed information regarding the chosen samples and gates, see [Supplementary material Table S4](#).

Trouble shooting: Insufficient sample amount, precipitation of the buffer, air-bubbles in the system, high sample intrinsic noise, insufficient cooling due to long sorting time of low abundant gates, loss of cells by centrifugation after sorting, cell disruption of vulnerable cells during sorting

Reagents: 1 μm and 2 μm yellow-green beads, 0.5 μm and 1.0 μm blue fluorescent beads

2.8. Data analysis

The measurements of each sample were visualized with Summit 4.3 (Beckman-Coulter, Brea, CA) and FlowJo V10 (FlowJo, LLC, Oregon USA) using the dot-plot option FSC (cell size) against DAPI fluorescence. Subsequently, a cell gate bearing to the already defined parent gate in Summit has been defined in FlowJo excluding noise and beads ([Fig. 1, A](#)). Events within the resulting 2D-histogram can be interpreted as recorded virtual cells whose optical characteristics are represented by chosen parameters such as FSC and DAPI fluorescence. Those with similar optical properties are consequently clustered and defined as subcommunities. Visible differing clusters were marked with separate ellipsoid gates. Previous studies revealed that subcommunities may consist of only single or few phylotypes but can also contain a huge variety of phylotypes [31]. The more precisely one gate is defined the higher is the probability that one single genus dominates this gate in a high percentage. Nevertheless, the gate must be designed with a tolerance for biological deviations within the sample pool. It must be valid for all samples within the setup of samples that needs to be evaluated together. All gates of all samples in their entity generated the gate template ([Fig. 1, B](#)), which consists of 37 gates in this study.

Trouble shooting: Operator and experience dependent (individual gating procedure according to Günther et al., 2015 [37]), necessary compromises by creating the mastergate due to biological variations and technical sensibility that cause deviation, missing of low abundant species

2.9. Data evaluation

Gates of interest can be compared regarding the recorded events that correspond to the virtual cell abundance. All gate values from each sample were extracted using FlowJo, saved as an Excel file and transformed into a text file. These data are normalized and can be used by flowCyBar [38] to create unique barcodes ([Fig. 2, A](#)), where every bar/column corresponds to one specific gate and every row corresponds to one sample. This procedure is called cytometric fingerprinting [33]. In [Fig. 2, A](#) the samples were put together in four different groups. A color key gradient displays the variation of the normalized gate abundances ([Fig. 2, A](#)): dark blue corresponds to low and red to high virtual cell abundance compared to the average of the appropriate gate. The average of virtual cells per gate is indicated by white color. Thus, the barcode shows an in-/decrease of gate-cell abundances, but no interpretation of the cells' percentage in this specific gate. On the top of each barcode a cluster dendrogram illustrates the clustering of all gates as a result of a hierarchical approach using the Euclidean metric. A second depiction of the cytometric data is possible by creating box-plots that show the distribution of the relative abundances of gate cell numbers of each gate ([Fig. 2, B](#)).

Furthermore, non-metric multidimensional scaling (NMDS) was performed based on the relative gate cell abundances of all samples. The distance measure used for this approach was the Bray-Curtis dissimilarity. The NMDS plots were created using the NMDS method of the R package flowCyBar [38] which is based on the metaMDS method of the R package vegan [39]. The distance between two points/samples describes their (dis-) similarity ([Fig. 2, D and E](#)). Thus, two samples with high similarity are ordinated closer together than those with a low similarity. All dynamics of an oral community can be illustrated representing the direction of the evolution of samples over time ([Fig. 2, D](#)). The similarity or dissimilarity of a defined group of samples is mirrored by the deviation between all points of this group ([Fig. 2, E](#)). Consequently, larger deviations between points represent high dissimilarity between samples. In [Fig. 2, E](#) two sample groups were defined and connected by a solid line. Instead of connecting the points of one group it is also possible to add ellipsoid lines showing the standard deviation of this group.

Trouble shooting: over-interpretation of distances due to lacking standard gradation of NMDS-plots

2.10. Sequencing

The workflow for the sequencing procedure is presented in detail in the [Supplementary Appendix \(Supplementary material, pages 4–7\)](#). Several steps need to be undertaken starting with the creation of mock strains and a mock community for control, DNA extraction and quality testing, library preparation for Illumina® sequencing, and finally, the sequencing data evaluation procedure.

2.11. Controls

2.11.1. Technical replicates

The reliability of cytometric measurements was assured by creating technical replicates. A possible impact of the cytometric workflow can cause variation in measured data sets. To ensure a minimum of possible influences connected to protocols or equipment, independent measurements of the same sample were performed. Therefore, one sample was split into three parts and processed separately according to the cytometric workflow and each was measured three times. Their dissimilarity is extremely slight as shown in [Fig. 3 \(TR\)](#).

Fig. 2. Gate dependent variations in cell abundancies, evolution of microbiome structure and similarity analysis. Two different subjects are compared (subjects 6 and 4). Four samples were collected within 8 h (time points: 0 h, 5 min, 2 h and 8 h). All gates of all samples were used to compare the similarity of the samples. A: Barcodes show in-/decrease of normalized gate cell abundancies. B: The boxplots show the distributions of the relative cell abundancies per gate. C: For a clearer depiction of the changes between two time points (0 h and 5 min), exemplary gates were chosen and colored corresponding to the color key of the barcode that describes lower abundancies (blue color), higher abundancies (red color) or average abundancies (white color). D: NMDS-plots show the evolution of the person's individual salivary microbiome within 8 h. Intra-individual changes are depicted. The size of the dots increases from 0 h up to 8 h. E: Intra- and inter-individual similarity analyses of two persons is demonstrated. Both subjects create their own cluster in this NMDS-plot. The size of these clusters can be used to describe the (dis-) similarity between the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.11.2. Fixation stability

Fixation of samples is a critical issue in order to keep the cells in stasis at a particular point and to avoid deterioration. In this study, N₂ fixation (Glycerol 15% (v/v), –80 °C; see Section 2.3) was used to

stabilize and fix all cells, because after testing two further different fixatives N₂, Glycerol 15% (v/v), –20 °C and formaldehyde/alcohol (PFA 2%/EtOH 70%) it showed the highest quality preservation. The stability was tested for up to 135 days using flow cytometry.

Fig. 3. Technical replicates and fixation stability. Dissimilarity analysis of three parallels (including fixation, washing and staining) which were measured three times each as technical replicates (TR) and of fixation stability, measured at days 0, 1, 3, 7, 15, 50, 135 (FIX, open circles in ascending size according to progressive time points). For reference, a long-term experiment (DV) was added: 11 samples of one subject, collected within 3 months (closed circles in ascending size according to progressive time points). The ellipsoids show standard deviations of the sample groups (DV, TR, FIX).

A sample was repeatedly prepared and analyzed at different measuring days to ensure the comparability of measurements. The fixation stability was proven as shown in Fig. 3 (FIX) and a high similarity between all samples was discernible.

2.11.3. Negative controls

Negative controls of the used products were performed to ensure that a measured change in community structure is not caused by these products and that they do not perform a distortion on the measured events within the cell gate. Therefore, PBS buffer, the tooth brushes Elmex inter X mittel (GABA Group, Therwil, Swiss) and Sensodyne Mikro Aktiv extra sanft (GlaxoSmithKline plc., London, Great Britain), the tooth pastes Sensodyne Multicare (GlaxoSmithKline plc., London, Great Britain) and Meridol (GABA Group, Therwil, Swiss), and the mouth rinses Listerine® Total Care Clean Mint (Johnson & Johnson, New Brunswick, USA) and Dynexan Proaktiv 0,2% CHX (Kreussler Pharma, Wiesbaden-Biebrich, Germany) were proceeded according to the cytometric workflow. The contribution of the stained particles to the events of the defined cell gate was found to be not significant. For further information see [Supplementary material Fig. S2](#).

Trouble shooting: Determination of the maximum amount of product that one saliva sample could contain

Reagents: PBS buffer

2.11.4. Sorting controls

To exclude possible distortions by the sorting procedure one sample was split. One part was sequenced directly, the other part after cytometric measurements. Only slight differences became apparent (Fig. 9, D).

Trouble shooting: See Section 2.7 Cell sorting and workflow for Illumina® sequencing

3. Results and discussion

3.1. Workflow

A summary of the performed experiments as well as the cytometric workflow is illustrated in Fig. 4. Unstimulated saliva samples were collected from ten oral healthy subjects. Three different experiments were designed, including a long-term experiment where the variations of the respective personal microbiota were followed over three months; a short-term experiment where the influence of both free-sugar containing sweets (represented by Toffifee (Storck, Berlin, Germany)) and dietary acid soft drinks (represented by Coca Cola Zero (The Coca-Cola Company, Atlanta, USA)) on the microbiota was tested; and finally two bactericide solutions were imparted and their impact on the microbiota analyzed. After sampling and fixation, the samples were processed immediately or to a later time point. Fixation stability was tested and found stable for up to 135 days (Fig. 3, FIX). Washing and DNA-staining of the samples was followed by flow cytometric community analysis. Subsequently, the generated data were evaluated by using bioinformatic tools. Although this workflow has been used to investigate samples from different environments in the past, it has never been used for saliva before. The number of cells per ml saliva is rather low (3.4 Mouth-rinses and cell numbers; [Supplementary material Tables S2 & S3](#)) which is different for e.g. samples from a wastewater treatment plant [40,41] or mice feces [31]. In addition, the observed appearance of human cell debris after biocide treatment (not shown) and the viscous consistency of the saliva that contained a certain particle matrix needed a particular careful sample handling such as a new fixation technique, parallel sample testing, surplus control set ups and creation of a specific parent gate that contained all stained cells excluding noise and beads (Section 2). Therefore, the reliability of the workflow was proven by the successful formation of almost identical cytometric community patterns of three parallels (including fixation, washing and staining) which were measured three times each (Fig. 3, TR).

3.2. Person dependent oral microbiota

There have been several studies to investigate the human oral microbiome by sequencing approaches [7,24,25,30,42,43]. The human oral microbiome project (HOMP) [30,43] studied seven intra-oral and two oropharyngeal sites from approximately 200 healthy donors and created a vast database (<http://www.homd.org/>). It covers 185–322 bacterial genera belonging to 13–19 phyla, of which *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* were the most dominant ones. The project revealed that saliva as one oral habitat showed the highest median alpha-diversity but one of the lowest beta-diversities. The total number of organisms (richness of measured OTU (operational taxonomic unit) numbers) and/or the respective relative abundances of organisms (evenness) within a sample were used to characterize alpha-diversity. Beta-diversity in contrast describes the comparison between samples from the same habitat among subjects. Hence, concerning saliva samples, the measured OTU level richness within one sample was described to be high, but samples of different subjects shared similar organisms [30,43]. Moreover, when it comes to time-linked saliva community studies it seems that intra-individual variation occurred only subtly but differed in succession from other saliva microbiome structures [44]. Also other studies found that samples collected over longer intervals were more similar, while those taken at shorter intervals were often more dissimilar as possible deviation from the normal state was regarded with higher weight [9,30]. Such data point to the existence of a highly diverse

Fig. 4. Routine workflow for analyzing salivary microbiomes based on flow cytometric measurements. In this figure, the experimental design is illustrated schematically. The pilot study implemented ten oral healthy subjects. Unstimulated saliva samples were collected within 3 different experiments: a long-term experiment, testing of sugar and acid influences and the use of bactericide solutions. Single steps of the following processing of the saliva samples within the cytometric workflow are depicted: Fixation procedure enables a stability (for further information see Section 2.11.2), so further processing can be delayed if necessary. Once washed and stained with DNA staining solution (DAPI), the flow cytometric community analysis of the samples followed. Duration of measurements can vary from several minutes to nearly half an hour until 150,000 cells per cell gate are detected. Data analysis can be performed immediately by using bioinformatic tools (step by step explanation see Sections 2.8 and 2.9).

'personal microbiome' that undergoes only modest fluctuations when it is not heavily affected.

Using the cytometric workflow, the findings of the long-term experiment in the current study (details in [Supplementary material Table S1](#)) correspond to the findings discussed before [9,30,43,44]: Subject specific microbiome patterns showed intra-individual constancy but were different between each other, although partly overlapping in similarity, thus revealing inter-individual diversity (Figs. 5, S6). Samples of some of the subjects showed an especially high constancy over time (e.g.: subjects 6 and 10), as their respective microbiomes are arranged closely together within the dissimilarity plot. The salivary microbiota of others showed less permanent patterns within this 3-month period (e.g.: subjects 3 and 8; also revealed by an independent parallel set up represented in Fig. S3). To statistically confirm the findings an ANOSIM test was performed based on the cytometric gate abundances of all 10

subjects for the long-term experiments. *R* values of approx. 0.5 describe higher inter-individual than intra-individual variance with a significance value of 0.001 for the first long-term experiment and for the parallel set up (S6A, S7A). Notched boxplots were created to visualize these test results (S6A, S7A). To confirm the cytometric measurements and the obtained community structure information, Illumina® sequencing was performed exemplarily of two samples from the microbiome of subject 1 taken with a time delay of three weeks. Only few changes in abundance were observed by an increase of 15–22 OTUs and 10–16 genera between the two samples. All 10 genera found in week 1 were also present in week 4 and the other 6 genera increased in abundance to only about 1% each (Fig. 9, A). The genus *Prevotella* was more than 2 times more abundant in the sample taken at 7 days while e.g. the genus *Neisseria* was about 4 times more abundant at the 4 week-sampling. In contrast to the microbiome of subject 1, the sequencing data of

Fig. 5. Intra- and inter-individual microbial cytometric diversity. The NMDS-plot compares the microbial cytometric diversity of saliva samples collected from 10 subjects, each represented by an individual color. The sampling started at baseline (0 h, smallest points) and continued for three months (points increase in size). From each subject 8–11 samples were measured (see [Supplementary material Table S1](#)). The larger the distance between two points, the more dissimilar was the cytometric structure of the respective microbial communities. The depiction of the standard deviation occurs by ellipsoids. The expanse of an ellipsoid relates to the intra-individual variability: If it is small, the variability is low. The more distant two ellipsoids are located, the higher is the inter-individual diversity. Stress of the NMDS-plot: 0.12.

other microbiomes (subjects 6 and 10) showed more different compositions of the OTU types although both were taken at the same time point (see [Fig. 9, A](#)). *Lachnospiraceae* were only represented in the sample of subject 6 while *Brevundimonas* as well as *Lactococcus* were found only in the sample of subject 10. Other phylotypes detected in both samples were represented in different abundancies, for example *Prevotella* was more abundant in the sample of subject 6. The difference in the OTU types and abundance between the three microbiomes, however, was lower as described in literature [43] due to sequencing depth, but nevertheless, also the cytometric analysis and similarity calculation positioned those three microbiomes in near vicinity ([Fig. 5](#)). Thus, the data obtained by Illumina® sequencing strongly supported the trends calculated on the basis of cytometric data by representing the high intra-individual constancy of OTU types in the microbiome of subject 1 and inter-individual diversities (variation of OTU types) of microbiomes of subjects 1, 6 and 10. The beta-diversity among the microbiomes of those three subjects was existent, but low.

3.3. Oral microbiota and stress

It is verified that an excessive intake of sugars and acids leads to an increased risk for oral diseases like caries and dental erosion [45–47]. This is accompanied by a microbial shift from oral homeostasis to a dysbiosis due to selection of facultative pathogenic phylotypes such as *Streptococcus* spp., *Prevotella* spp., *Veillonella* spp., and *Lactobacillus* [23,48]. In healthy oral microbiomes these phylotypes are also frequently represented, but minor abundant

[22,49]. Saliva serves as a protection against stress influences due to its functions as cleansing solution, buffer and source for remineralization [50,51]. A previous sequencing project revealed an association between salivary bacterial profiles and oral health and disease, thus bacteria from local oral diseased sites are also detectable in saliva [23]. Nonetheless, the presence of such pathogenesis-associated genera does not obligatorily implies an existing disease. Objective of this experimental set up was to imitate a dietary lifestyle by taking up of sugars or acids several times within an 8 h period to avoid recovery of the salivary microbiome. The data of this study suggest that a short-term intensive extrinsic stress such as treatment with small molecular sugars liberated from sweets or acids from soft drinks did not change the intra-individual constancy of microbiomes that much ([Fig. 6](#) and [Supplementary material Fig. S4](#)). ANOSIM testing for variance and significance was performed and results documented in S6B,C and S7B,C. As before, the similarity analysis showed clear differences between the microbiomes of different subjects taken at baseline (before a stress impact) similar to the microbiomes taken for the long-term experiment (see Section 3.1). After the stress impact (8 h) the microbiomes were still in the vicinity to their earlier position in the similarity analysis. These findings were found for the two different stress situations (influence of low molecular sugars and acids) and also the two independent experimental replicates. The data therefore suggest that the intra-individual constancy of the microbiomes seem to be a central phenomenon as the microbiomes act with high resilience and operated the stress with only slightly changed basic structures. The microbiomes did not evolve

Fig. 6. Effect of sugars and acids on microbial community structures. The NMDS-plots compare the microbial cytometric diversity of samples collected at baseline (0 h, small points) and after stress (8 h, big points) caused by sugars (A) or acids (B). The color of the points represents the involved subjects (see Fig. 5). The ellipsoids show standard deviations of samples collected at baseline (continuous line) and samples collected after the stress influence (broken line). In both experiments the two ellipsoids show high overlaps, hence no major shifts in respective community structures was caused by the applied stress. Stress values of the NMDS-plots: 0.13 (A), 0.2 (B).

Fig. 7. Decrease in bacterial cell number after CHX mouth-rinse application. The cell number of the samples collected at 0 h (baseline), 5 min, 2 h, and 8 h after treatment with 0.2% CHX mouth-rinse is demonstrated per ml and per entire sample amount for two subjects (subjects 6 and 10). All samples show a clear decrease in cell number after 8 h. Both the cell number per entire sample and per ml decreased after rinsing for about 1.8 and 2.6 times (subject 6) and 1.2 and 2.1 times (subject 10), respectively, indicating a successful effect of CHX.

collectively to a united structure as could have been suggested by an assumed dominance of acidogenic phylotypes under such conditions. Though data in literature are controversé, most authors describe that excessive and long-term dietary sugar intake and low pH leads to a shift of the healthy oral microbiota to a decreased microbial diversity encouraging acidogenic phylotypes, such as *Streptococcus*, *Lactobacillus* and *Veillonella* [22,23,52,53]. In this study sequencing was performed exemplarily for one subject (subject 6) at baseline and after 8 h of stress impact (sugar). In addition, selected gates of the two microbiomes were sorted and sequenced (Fig. 9, B). Some gates of the baseline microbiome were dominated by key subpopulation such as gate 3 by *Prevotella* (almost 70%) or gate 37 by *Alloprevotella* (almost 85%). Instead of the promotion of only a few acidogenic phylotypes an increased number of phylotypes was detectable after the sugar stress impact (Fig. 9, B, 8 h). The alpha-diversity within samples increased, for example the genera *Aminobacteria*, *Lactococcus* or *Myroides* turned up. As in literature a decrease of the microbial diversity of caries disease is

explained [23,52], initially these results appear controversial, yet the healthy state of the salivary microbiome of the examined subject and the short-term impact of only 8 h have to be considered. Furthermore, abundances of phylotypes changed. An increase of the genera *Streptococcus* by the factor 3.5 is interesting, because some *Streptococcus* spp. are associated with caries pathogenesis [22,48]. Interestingly, gate 4 was mainly occupied by *Streptococcus* (37%) but also other phylotypes were present: *Prevotella* (16%), *Porphyromonas* (9%), *Gemella* (8%). However, since only one microbiome structure under stress was resolved by Illumina® sequencing, no detection on species level was performed and, additionally, the existence of *Streptococcus* spp. does not mandatory reflects caries, no further statement regarding a potential disease etiology can be made. As stated before, the cytometric microbiome data suggested only a minor change after short term impact of sugars and acids but without affecting the general structures of the individual microbiomes that much and thus saliva seems to accomplish its function as protective reservoir of a healthy microbiome for such periods.

3.4. Mouth-rinses and cell numbers

Mouth-rinses are used with the intention to reduce the microbial load in the oral cavity [54–56]. The agents contained therein are responsible for the effectiveness of meeting this requirement [54,57,58]. Mouth-wash containing 0.2% Chlorhexidine (CHX) is well investigated for several times and its significant antibacterial effect is confirmed [59]. The cationic nature of the active substance CHX enables an adherence on anionic oral surfaces and persistence at effective concentrations. This characteristic is defined as substantivity, enabling a prolonged antibacterial activity [60,61]. This parameter can be evaluated by observing the decrease of salivary bacterial numbers over a time period. Different methods, like epifluorescence microscopy investigating bacterial vitality or cultivation on agar plates for counting colony forming units (CFU)/ml are already described in literature. The resulting findings describe an immediate potent reduction of the salivary flora after application of CHX followed by a progressive recovery in bacterial vitality, still showing an incomplete return to baseline hours later [60,62,63]. The intention of this study was to determine the absolute cell numbers by flow cytometry rather than using cultivation techniques

Fig. 8. Effect of CHX and Listerine® mouth-rinse on microbial community structure. The NMDS-plots represent the microbial cytotmetric diversity of saliva samples of five subjects each, who used two different mouth rinses: 0.2% Chlorhexidine (A) and Listerine® (B). The color of the points represents the involved subjects (see Fig. 5) and the size of the points demonstrates the times of sample collection: baseline (0 h = small points; 5 min = medium small points; 2 h = medium big points; 8 h after rinsing = big points). The respective four areas compare the samples from one collecting time point. The ellipsoids show standard deviations of samples collected at baseline (continuous line), after 5 min (broken line), 2 h (pointed line), and 8 h (mixed line). Both chemicals did not cause noteworthy shifts in the community structure, since there is high overlap of the ellipsoids. Stress values of the NMDS-plots: 0.14 (A), 0.18 (B).

that miss almost all the species in the microbiome due to unknown cultivation conditions. First, the cell number of microbiomes collected at baseline was determined for all subjects (Supplementary material, Tables S2 & S3) by SYBR GreenI staining (2.6. Cell counting) to avoid cell loss due to washing- and centrifugation-steps that are not necessary in contrast to DAPI-staining. In addition, the cell number was determined per ml, but also per entire sample because the saliva production is a highly heterogeneous secretion process and the range of the individual saliva quantities can be wide [64]. The flow cytometric measurements provide bacterial cell numbers ranging from 3.0×10^7 to 6.2×10^8 per sample and 1.5×10^7 to 5.1×10^8 per ml in undisturbed environments. The generated data overlaps with the findings in literature [65], though comparable source material is rare. For instance, quantitative real-time PCR of bacterial counts in untreated saliva samples revealed values ranging from 1.56×10^6 to 1.08×10^8 cells/sample [65]. Second, cell numbers of two subjects (subjects 6, 10) that rinsed with CHX mouth-rinse were taken before treatment (0 h) and at three time points (5 min, 2 h, 8 h) afterwards (Fig. 7). Both the cell number per entire sample and per ml decreased within 8 h after rinsing for about 1.8 and 2.6 times (subject 6) and 1.2 and 2.1 times (subject 10), respectively, indicating a successful effect of CHX. The second run showed similar trends concerning reduction of bacterial cell counts by CHX, but it was also clear that the general abundance of cells per ml or sample varied between subjects (Fig. 7, Supplementary material Tables S2 & S3, Fig. S5).

Additionally, the influence of CHX and Listerine on the cytometric structure of the salivary microbiomes of five subjects was investigated in two independent experiments, respectively (Fig. 8, Supplementary material Fig. S5). The cytometric data were grouped according to the microbiome collection times (0 h, 5 min, 2 h, 8 h). The high overlaps in all four experiments indicated that the salivary microbiomes maintained their structure despite the mouth-rinse treatment and did not collectively evolve in a united direction as could be assumed if only distinct phylotypes would survive the treatment. ANOSIM testing for variance and significance was performed and results documented in S6D,E and S7D,E. Therefore, the losses in cell number seem to be the domi-

nant effect although some phylotypes seem to be specifically affected. Illumina® sequencing was performed exemplarily for one subject (subject 6) at baseline and 8 h after rinsing with 0.2% CHX (Fig. 9, C). Again, due to the stress situation the 8 h sample showed higher alpha-diversity with upcoming phylotypes such as *Fusobacterium*, *Neisseria* or *Rothia*, which was in contrast to the high abundancies of *Prevotella* at 0 h. The genera *Streptococcus* increased of a factor of 2-fold (from 3.5% to 6.6%) while *Prevotella* decreased by 5 times (from 48.9% to 10.6% abundance). The intention of mouth-rinse solutions is rather to cause a general and random reduction in bacterial cell counts [54,55] as was verified by the cell count measurements and the similarity comparison of the cytometric data. But shifts in microbial composition as were observed in this one exemplary microbiome of subject 6 with Illumina® sequencing can be dangerous because also pathogens might find unoccupied niches in a rinsed mouth ecosystem. Further studies are necessary in this regard.

4. Conclusion

Flow cytometry and connected bioinformatics tools visualize changes in the salivary microbiome with economic swiftness and can thus serve as screening methods preceding in depth analysis by sequencing techniques which then can be applied more selectively and only when needed. In the study, the dynamics of the saliva microbiomes of ten subjects within a 3-month period revealed individual fingerprint-like profiles with high intra-community constancy. Short term stress caused no noteworthy changes in cytometric structure of the individual microbiomes, independent if the stress was caused by sugars or acids as well as bactericides. The mouth-rinse CHX, however, caused drastic reductions in cell counts even after 8 hs recovery time. The cytometric data suggested in general random elimination of bacteria while the sequencing data of (only) one microbiome alert also for niche colonization of formerly infrequent phylotypes. But largely the data obtained by Illumina® sequencing, as qualitative analysis, supported the intra-community constancy especially for subject 6 because the variations in number of phylotypes and their abundan-

cies did not change much during all experimental set ups. Illumina® sequencing also confirmed that cells measured by flow cytometry were typical members of the salivary microbiome and that some gates might serve as indicator gates for phylotypes such as *Prevotella*.

In this study, microbiomes of healthy subjects were investigated according to particular calibration-guidelines (Supplementary material Table S1) but with no further control of nutritional habits. Therefore, additional factors (state of health, oral hygiene measures) that influence the salivary microbiome can't be excluded. Furthermore, diurnal variation could also affect the bacterial composition, but there is little or no evidence [44]. Nevertheless, under realistic clinical conditions patients are not standardized regarding these aspects either. The sparse number of included subjects requires caution, a fact that could be overcome by large-scale studies. Extended investigation of other oral niches, like periodontal pockets or the inclusion of subjects with oral diseases can yield further insights on the relation between changes of the microbial composition, community dynamics, and pathogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jymeth.2017.08.009>.

Fig. 9. Illumina® sequencing of exemplary saliva samples. The figure depicts the distribution of phylotypes per sample detected by Illumina® sequencing in a way that the colors mark the genera and the rare faction curves the OTU numbers. Whole community sequencing as well as sequencing of sorted gates was conducted. Gates for sequencing have been chosen on the basis of increasing and decreasing cell numbers, respectively. A: Samples of the long-term experiment. Sequencing was performed exemplarily for subject 1 (orange) at two different time points (1 week, 4 weeks) as well as subject 6 (blue) and subject 10 (pink) at the same time point (6 weeks). B: Samples of the sugar experiment. Sequencing was performed exemplarily for subject 6 (blue) of samples taken at baseline and after 8 h of stress impact (sugar). In addition, different subsets of the community of subject 6 were sorted such as G3 and G37 (both visible in the 2D-histogram at baseline, but diminished at 8 h), which represented the key phylotypes *Prevotella* and *Alloprevotella*. Other subcommunities showed higher diversity such as G4, G9 (both intensified after the stress impact), and the joint sorting of gates G8, 26 and 29 (all three intensified after the stress impact). C: Samples of the mouth-rinse experiment. Sequencing was performed exemplarily for subject 6 at two different time points (0 h and 8 h after applying 0.2% CHX mouth-rinse). Cell sorting was performed for the combined gates G5 and G6 (both diminished after the use of mouth-rinse from 11.33% to 5.25%), which also evolved as key gate for *Prevotella*. D: To exclude possible distortions by sorting procedure, a sample of subject 6 was split and sequenced unsorted and sorted. Only slight differences become apparent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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3. Zusammenfassung der Arbeit

Dissertation zur Erlangung des akademischen Grades Dr. med. dent.

Detektion intra- und interindividueller Unterschiede bakterieller Kompartimente im humanen Speichel während dreimonatiger Beobachtungszeit mit Hilfe der Durchflusszytometrie

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Die humane Mundhöhle beheimatet als hoch komplexes Ökosystem eines der vielfältigsten Mikrobiome des gesamten Körpers. Das Vorhandensein diverser Nischen wird durch abiotische und biotische Parameter wie mikrogeographische Charakteristiken, die Verfügbarkeit von Nahrung und Sauerstoff, spezifische pH-Werte und Temperaturen, Abwehrsysteme und Essgewohnheiten des Wirtes sowie molekulare und bakterielle Interaktionen bedingt. Zum derzeitigen Kenntnisstand umfasst das orale Mikrobiom etwa 700 bakterielle Phylotypen, die zum Teil als orts- und/oder wirtsspezifisch klassifiziert werden können. Aufgrund kurzer Generationsfolgen innerhalb des bakteriellen Wachstums adaptieren die mikrobiellen Kompartimente schnell an sich verändernde Umweltbedingungen. Dabei ist ein dynamisches Gleichgewicht zwischen der residenten Mikroflora und dem Wirt ohne die Prädominanz potenziell pathogener Spezies für die Mundgesundheit elementar. Dem Erhalt dieser oralen Homöostase dient u.a. der Speichel mit seinen vielfältigen Funktionen. Als globales Reservoir der Mikrobiota der Mundhöhle in ihrer planktonischen Phase reflektiert er zudem Veränderung innerhalb der bakteriellen Zusammensetzung, welche mit oralen Erkrankungen wie Karies, Gingivitis und Parodontitis assoziiert sind. Die einfache, non-invasive und kostengünstig durchführbare Entnahme gestaltet den Speichel zum attraktiven Analysemedium. Derzeit stellen hierfür Sequenzierungstechniken den Goldstandard zur Untersuchung der phylogenetischen Vielfalt des oralen Mikrobioms dar. Allerdings sind diese

Methoden mit einem hohen finanziellen, zeitlichen und laboratorischen Aufwand verbunden. Ein bereits in der Umweltmikrobiologie etabliertes Verfahren zur Betrachtung komplexer Ökosysteme ist die Durchflusszytometrie. Sie ermöglicht - gemäß eines Screenings - die Visualisierung und Bewertung sich schnell verändernder Gemeinschaftsstrukturen. Außerdem können quantitative Analysen im Sinne von Zellzahlmessungen der betrachteten Proben durchgeführt werden.

Die vorliegende Arbeit verfolgte das Ziel, die Anwendbarkeit der Durchflusszytometrie auf Untersuchungen bakterieller Kompartimente im humanen Speichel zu evaluieren. Dafür sollten im Rahmen des Teilprojektes der vorliegenden Dissertationsschrift zunächst intra- und interindividuelle Unterschiede der bakteriellen Zusammensetzung innerhalb eines dreimonatigen Entnahmezeitraums untersucht werden. In einem weiteren Teilprojekt verfolgte Frau van Gelder die Reaktion der Bakteriengemeinschaften auf verschiedene extrinsische Stresseinflüsse (Zucker, Säure und antibakterielle Mundspüllösungen).

Für die geplante Untersuchung erfolgte die Auswahl von zehn oral und allgemein gesunden Probanden beider Geschlechter im Alter über 18 Jahren. Alle Teilnehmer der Studie erhielten eine einheitliche Zahnbürste (Elmex InterX mittel) und die Zahnpasta Meridol (beides CP GABA Hamburg, Deutschland). Die standardisierten Mundhygieneartikel sollten während des gesamten Zeitraums der Untersuchungen verwendet werden. Die Probanden wurden angewiesen, zweimal täglich ihre Zähne zu putzen. Für die Analyse zeitabhängiger intra- und interindividueller Unterschiede der Speichelmikrobiome erfolgten 11 Probenentnahmen in festgelegten Intervallen: 0 Stunden (h), 8h, 24h, 3 Tage (d), 7d, 2 Wochen (w), 4w, 6w, 8w, 10w und 12w nach Beginn der Untersuchung. An den Tagen der Speichelgewinnung wurden die Probanden angehalten, ihre Zähne bis zur Entnahme nicht zu putzen. Das letzte Zähneputzen sollte spätestens bis 24 Uhr des Vortages erfolgt sein. Ferner sollte eine Stunde vor Probengewinnung keine Nahrungs- oder Getränkeaufnahme stattfinden.

Die Gewinnung unstimulierten Speichels wurde gemäß eines standardisierten und validierten Protokolls durchgeführt: Hierfür spülten die Probanden nach einer fünfminütigen Adaptationszeit für 30 Sekunden mit destilliertem Wasser und sammelten dann innerhalb weiterer fünf Minuten den unstimulierten Speichel im Mund. Im Anschluss an die Speichelprobengewinnung erfolgten die Fixierung des Gesamtspeichels mit Glycerol, eine Schockgefrierung in flüssigem Stickstoff und die

Lagerung bei -80°C. Für die weiterführende laboranalytische Auswertung wurden die Speichelproben aufgetaut und ihre Zellen entsprechend eines definierten Färbeprotokolls mit dem DNA-Farbstoff DAPI (4',6-Di-Amidin-2-Phenyl-Indol) behandelt bzw. gefärbt. Nachfolgend konnten die Proben im Durchflusszytometer (MoFlow Legacy cell sorter) vermessen und bezüglich der optischen Parameter Zellgröße (Vorwärtsstreulicht, „Forward Scatter“) und DNA-Gehalt (DAPI-Fluoreszenz) analysiert werden. Mit Hilfe der Programme Summit Ver. 4.3, FlowJo und flowCyBar erfolgte die bioinformatische Auswertung der zytometrischen Daten.

Die Profile der Speichelmikrobiome zeigten über den untersuchten Zeitraum intra-individuelle Stabilitäten, aber unterschieden sich zwischen den Probanden, mit Ausnahme partieller Überlappungen. Personenspezifische Muster waren nachweisbar. Dabei stellten sich die Mikrobiomstrukturen einiger Probanden sehr beständig dar, während die bakteriellen Kompartimente anderer Probanden größere Schwankungen aufwiesen. Die zytometrischen Messungen wurden exemplarisch mittels Illumina®Sequenzierung verifiziert. Die phylogenetischen Unterschiede (Anzahl und Verteilung der operational taxonomic units) der Proben einer Versuchsperson zu unterschiedlichen Entnahmezeitpunkten waren hierin geringer als die der Proben zweier Teilnehmer zur selben Entnahme.

Die unter Teilprojekt I formulierte Arbeitshypothese wurde durch die Ähnlichkeitsanalysen der Longitudinalstudie bestätigt.

Auf dieser Basis können folgende Schlussfolgerungen getroffen werden:

Die Durchflusszytometrie ermöglicht eine schnelle Detektion komplexer bakterieller Kompartimente und deren Veränderungen im Speichel mundgesunder Probanden. Dabei sind individuelle, personenspezifische Bakterienprofile innerhalb der untersuchten Probanden graphisch darstellbar. Diese Profile weisen eine hohe innergemeinschaftliche Beständigkeit auf. Als qualitative Analyse bestätigt die Illumina®-Sequenzierung die Messung typischer Vertreter des Speichelmikrobioms im Zytometer sowie das Vorliegen intraindividuelle Stabilitäten. Zur Verifizierung der Ergebnisse ist eine Ausweitung der Studie auf einen größeren Probandenpool erforderlich.

4. Ausblick

Die vorliegende Studie stellt als Pilotprojekt die Grundlage für weitere Untersuchungen dar. Nachdem die Anwendbarkeit der Durchflusszytometrie zur Detektion bakterieller Kompartimente im humanen Speichel mundgesunder Probanden bestätigt werden konnte, ist eine Ausweitung der Analysen auf großangelegte Studien sinnvoll. Hierin ließe sich untersuchen, inwiefern sich sozioökonomische Einflüsse, Gender, Lifestyle-Faktoren (z.B. Stress, körperliche Aktivität, Nikotin- und Alkoholkonsum) und Ernährungsgewohnheiten auf das Bakterienprofil (Mundgesunder) auswirken. Diese Parameter fanden in der vorliegenden Pilotstudie bisher keine Berücksichtigung.

In einem weiteren Schritt ist auch der Einschluss oral Erkrankter sinnvoll. Die zytometrische Analyse ermöglicht das schnelle Erfassen globaler mikrobieller Shifts und verspricht somit prospektive Einblicke in die Ätiologie von Karies und Parodontitis – Krankheiten, die auf der Beeinflussung des oralen Mikrobioms beruhen. Wünschenswert wäre die Entwicklung einer Screening-Methode, die noch vor der Manifestation pathologischer Zustände auf deren Entstehung hinweist oder innerhalb des Therapieverlaufs zum Monitoring angewandt werden kann. Im Zuge dessen sind Studien denkbar, die den Einfluss von Pre- und Probiotika auf die bakterielle Zusammensetzung in der Mundhöhle zum Gegenstand haben. Die Reetablierung eines dynamischen, mit Gesundheit assoziierten mikrobiellen Gleichgewichts durch die Verdrängung potenziell pathogener Spezies könnte ein neuer, zukunftsweisender Therapieansatz sein.

Darüber hinaus wäre es konstruktiv, die Anwendbarkeit der Durchflusszytometrie auf die Untersuchung anderer orale Nischen, wie der parodontale Tasche, zu überprüfen. Hierbei kann auch eine Ausweitung der von Frau van Gelder vorgenommenen Studien bezüglich des Einflusses von Stressfaktoren angedacht werden. Sowohl eine Verlängerung des Applikations- und Beobachtungszeitraums auf mehrere Wochen als auch das Aussetzen oraler Hygienemaßnahmen stellen vielversprechende Ansätze dar. Daraus gewonnene Einblicke in die Beständigkeit bzw. Veränderungen der Zusammensetzung des subgingivalen Biofilms könnten einen großen Nutzen für das Verständnis parodontaler Pathogenese darstellen.

5. Anhang

5.1 Supplementary Data

A. Table S1: Experimental set up

	Sugar-experiment	Acid-experiment	Mouth-rinse-experiment	Person dependent oral microbiota
Products	<ul style="list-style-type: none"> • Toffifee¹, 15 pieces • Tooth brush: „Sensodyne Mikro Aktiv extra sanft“² • Toothpaste: „Sensodyne Multicare“² 	<ul style="list-style-type: none"> • Coca Cola Zero³, 1l, pH-value: 2,9 • Tooth brush: „Sensodyne Mikro Aktiv extra sanft“ • Toothpaste: „Sensodyne Multicare“ 	<ul style="list-style-type: none"> • Dynexan Proaktiv 0,2% CHX⁴ • Listerine® Total Care Clean Mint⁵ 	<ul style="list-style-type: none"> • Tooth brush: „Elmex inter X mittel“⁶ • Toothpaste: Meridol⁶
Taking intervals	3 toffees each time 1. subsequent after calibration of the subjects (teeth brushing) 2. half an hour later 3. rest must be taken in a steady 2h-interval	200 ml each time 1. subsequent after calibration of the subjects (teeth brushing) 2. half an hour later 3. rest must be taken in a steady 2h-interval	subjects have been divided in two groups; first group: 10-15 ml Dynexan (CHX); second group: 20 ml Listerine; each group rinsed for 30 seconds (according to manufacturer 's information)	integration in daily oral hygiene
Sample collection time points	1. before tooth brushing 2. 20-30 minutes after tooth brushing 3. 1 hour after the last taking	1. before tooth brushing 2. 20-30 minutes after tooth brushing 3. 1 hour after the last taking	1. before rinsing 2. 5 minutes after rinsing 3. 2 hours after rinsing 4. 8 hours after rinsing	1. 20-30 minutes after tooth brushing 2. 8 hours later 3. following the saliva collecting protocol in an interval of: 1 day, 3 days, 1 week, 2/4/6/8/10/12 weeks

This table shows detailed information concerning the experimental set-up. The used products, taking intervals and sample collection time points are described.

B. Table S2: Cell numbers at baseline; first run

	1	2	3	4	5	6	7	8	9	10
per ml	5.5x10 ⁷	2.8x10 ⁷	1.9x10 ⁷	2.0x10 ⁷	5.8x10 ⁷	6.0x10 ⁷	1.7x10 ⁷	4.1x10 ⁷	1.9x10 ⁷	6.6x10 ⁷
per sample	3.3x10 ⁷	1.1x10 ⁸	4.3x10 ⁷	3.5x10 ⁷	1.5x10 ⁸	2.3x10 ⁸	3.4x10 ⁷	8.6x10 ⁷	3.0x10 ⁷	1.1x10 ⁸

For the mouth-rinse experiment, the cell number of the first collected sample was determined for all subjects by SYBR GreenI staining. The cell number was determined by referring to both: ml and entire sample amount. The cell numbers ranged from 1.9x10⁷ to 6.6x10⁷ per ml and from 3.0x10⁷ to 2.3x10⁸ per sample.

¹ Storck, Berlin, Germany

² GlaxoSmithKline plc. (GSK), London, Great Britain

³ The Coca-Cola Company, Atlanta, USA

⁴ Kreusler Pharma, Wiesbaden-Biebrich, Germany

⁵ Johnson & Johnson, New Brunswick, USA

⁶ GABA Group, Therwil, Swiss

C. Table S3: Cell numbers at baseline; second run

	1	2	3	4	5	6	7	8	9	10
per ml	4.3×10^8	7.0×10^7	2.7×10^7	2.3×10^7	5.1×10^8	1.7×10^8	1.5×10^7	1.1×10^8	2.2×10^7	3.8×10^7
per sample	3.4×10^8	2.3×10^8	7.5×10^7	3.5×10^7	6.2×10^8	5.8×10^8	3.0×10^7	1.6×10^8	4.0×10^7	6.4×10^7

For the mouth-rinse experiment, the cell number of the first collected sample was determined for all subjects by SYBR GreenI staining. The cell number was determined by referring to both: ml and entire sample amount. The cell numbers ranged from 1.5×10^7 to 5.1×10^8 per ml and from 3.0×10^7 to 6.2×10^8 per sample.

D. Table S4: Samples for sequencing

Experiment	Subject	Sampling time point	Sorted Gates
Longterm-diversity	1	5 (7d)	
Longterm-diversity	1	7 (4 weeks)	
Longterm-diversity	6	8 (6 weeks)	
Longterm-diversity	10	8 (6 weeks)	
Sugar	6	1 (0 h, baseline)	3, 4+9, 37
Sugar	6	3 (8h)	4, 8+26+29, 9
Mouth-rinse	6	1 (0h, baseline)	5+6
Mouth-rinse	6	4 (8h)	

This table lists all samples that have been chosen for whole community sequencing as well as for sorting of selected gates.

E. Sequencing workflow*Mock strains and communities*

Illumina® 16S rRNA gene amplicon sequencing was done to verify flow cytometric data. Two mock species (*Rhodococcus* sp. RAH1 and *Pseudomonas putida* KT2440) and a mock community MBARC26 [1] were used as positive control for the sequencing MiSeq run as well as positive controls for the cytometric data analysis pipeline. MBARC26, composed of 26 cultivable species in different abundances, was designed to simulate the diversity of a natural microbial community. With our protocol, we found the two mock species and 23 species out of the 26 species of the mock community. The three missing strains (*N. dassonvillei*, *C. thermocellum*, and *S. enterica*) were probably lost due to their low abundance level and the sequencing technique we used for the metaprofiling (MiSeq, V3 kit, 2 x 300 bp (V3 kit, Illumina, USA)). Different from our workflow Singer et al., 2016 [1] conducted a higher resolving metagenomic approach by using PacBio and HiSeq. Nonetheless, the data of the mock species and mock community confirmed the reliability of our workflow and the raw data are available under the BioProject accession number: PRJNA387753. In parallel we used the data to set the confidant OTU threshold for cleaned reads to 0.71 % as was recommended by Bokulich et al., 2013 [2]. Species with abundance below this 0.71 % OTU threshold were not included in the data evaluation, but this threshold allowed us to study the most abundant genera in our samples.

DNA Extraction and quality testing

DNA extraction: To extract a comparable amount of DNA from each fixed but not sorted control sample, cells were diluted in 70 μ l PBS to a final optical density of 0.01 ($d=5$ mm,

$\lambda=700\text{nm}$). In parallel, by cell sorting, 500,000 cells were separated from each sample of interest. Following the protocol of Koch et al., 2013 [3], the cells were pelleted by a centrifugation step (25 min, 20,000 g, 4°C), respectively and the pellets were frozen at -20°C. After this freezing step, 70 μl of 10 % Chelex solution (Biorad, Hercules California, U.S.A.) was added to each pellet, followed by a heating step (45 min, 90°C). A last centrifugation step (5min, 7,000 g, 4°C) was needed to avoid cell walls debris in the collected supernatant which contained the purified DNA. The extracted DNA samples were stored at -20°C until library preparation by a double PCR step.

DNA quality testing: The DNA extracted both from 500,000 sorted cells and the non-sorted communities was too low in quantity to be detected by the Qubit® 3.0 (Life Technologies, Carlsbad, California, USA) for quality control. Therefore, a PCR step was performed to evaluate the quality of the isolated DNA by testing their amplified products by gel-electrophoresis. The PCR step was done with 35 cycles in a S1000 Thermal cycler (Biorad) by using the universal primers Forward 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse 1492R 5'-TACGGYTACCTTGTTACGACTT-3' following the recommendations of Lane et al., 1991 [4]. All tested DNA samples showed high quality results after amplification.

Library preparation for Illumina®

The V3-V4 region of the bacterial 16S rRNA gene region was the target of the used primers Pro341F 5'-CCTACGGGNBGCASCAG-3' [5] and Pro805R 5'-GACTACNVGGGTATCTAATCC-3' [6] synthesized by Eurofins (Eurofins Scientific, Luxembourg City, Luxembourg). The library was performed by using a unique combination of sample-affiliated dual-barcodes which allowed us to sequence multiple samples in the same sequencing run. The barcodes were composed by 6 bp nucleotides designed as follows: 5'-NNNN-6nt-primer-Forward-3' and a 5'-NNNN-6nt-primer-Reverse synthesized by Eurofins (Eurofins Scientific, Luxembourg City, Luxembourg).

The PCR was performed in 10 μl containing 10 pmol of Forward and Reverse primers, 2 nmol of dNTP mix (Promega, Fitchburg, Wisconsin, USA), 2 μl 5x Phusion® GC solution, 20 nmol of MgCl_2 , (both provided in the polymerase kit), 0.2 units of Phusion® High-Fidelity Polymerase (New England Biolabs, Ipswich, Massachusetts, USA) and 1 μL DNA solution. Nuclease free water (Qiagen, Velno, Netherlands) was added to adjust the final reaction volume to 10 μL . For PCR, the following conditions were chosen: 3 min of initial denaturation at 95°C, and a cycle of denaturation at 95°C for 30 sec, annealing at 52°C for 60 sec, extension for 1 min at 72°C and a final extension at 72°C for 10 min before storage at 4°C. The sorted samples were run for 20 PCR cycles and the non-sorted samples for 15. This difference was due to a lower DNA content in the sorted cell DNA solution. For each reaction, a negative control without any DNA was amplified for 35 cycles and visualized via gel electrophoresis (1.5 % agarose) to ensure that no contamination was present. In absence of contamination, the procedure continued by the purification of the amplicons by using the Agencourt® AMPure® XP-Kit (Beckman Coulter, Brea, California, USA) following their recommended protocol. The purified DNA fragments were resuspended in nuclease free water (Qiagen, Velno, Netherlands). The sample-affiliated dual-barcodes were added and 8 PCR cycles were performed at the same conditions as mentioned above. Again, negative controls were run in parallel without DNA by 35 PCR cycles to test for contaminations. Samples free of contamination were purified as described before; this DNA was quantified by using the Qubit® 3.0 and the HS DNA kit (both Life technologies, Carlsbad, California, USA), and finally equimolarly pooled to be sequenced on a MiSeq sequencing machine (Illumina, San Diego, California, USA) at the Helmholtz Center for Infection Research Braunschweig by the Genome Analytics Group (GMAK, HZI, Braunschweig, Germany). To minimize the technical biases, every PCR was done in triplicates and the DNA pooled before quantification.

Sequencing data evaluation procedure

The Illumina dataset was processed and evaluated using BBDuk (trimq= 25 <http://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/>). The remaining sequences

were demultiplexed and merged by using Mothur version 1.39 [7]. The pre-clustering step helped us to remove the singletons from the data set and the chimeras were removed by using UCHIME [8]. The OTU classification was done by using the Mothur's average neighbour clustering algorithm with a 97 % sequence similarity cut off on the SILVA database version 128 [9].

The obtained data sets comprised between 13,568 cleaned reads for the sample 'P10_Long_term_experiment_6_weeks (whole community without sorting)' (ref.: SAMN07163516, NCBI database) which was kept as subsampling threshold for the normalization procedure to allow us to compare diversity between samples, and 41,763 cleaned reads obtained from the sample 'P6_stress_Sugar_8_hours_Flow-cytometry_sorting_procedure_on_Gate_8_AND_26_AND_29' (ref.: SAMN07163651, NCBI database). The rarefaction curves were plotted using ggplot2 package in R [10]. The sequences of the 17 samples analyzed in this study are placed in the BioProject accession number: PRJNA387918. The gamma diversity we obtained from these 17 samples, represented by 230,656 forward-reverse overlapped subsampled sequences, comprised 48 different OTUs at an OTU threshold of 0.71 %. Instead, the raw data provide 810 OTUs, hinting at a huge diversity in the rare biosphere of our samples.

Rarefaction curves of 17 raw data sets samples, (unsorted, sorted and control cells) with a maximum diversity of 469 OTUs for the sample 'P6_stress_Sugar_8_hours_Flow-cytometry_sorting_procedure_on_Gate_8_AND_26_AND_29'. The green line marks the lowest cleaned data set comprising 13,568 reads for the sample 'P10_Long_term_experiment_6_weeks (whole community without sorting)'.

Rarefaction curves of the 17 samples (unsorted, sorted and controls) at a threshold of 0.71 % OTU. Due to the OTU threshold the curves show the diversity of the most abundant OTU.

F. Figure S1: Cell counting

Logarithmically scaled 2D-dot plot representing nucleic acid contents (green vs. red fluorescence). An exemplarily sample with the cell gate created for SYBR GreenI staining of cellular nucleic acids and a defined gate for the 1.0 μm yellow-green fluorescent beads is depicted. The cell gate was created excluding noise and beads and including measured cells.

G. Figure S2: Negative controls for cytometric experiments

A=Phosphate buffered saline (PBS), B=0,2% Chlorhexidine mouth rinse (CHX), C=Listerine© mouth rinse (LIST), D=Elmex inter X mittel (tooth brush), E=Sensodyne Mikro Aktiv extra sanft (tooth brush), F=Meridol (tooth paste), G=Sensodyne Multicare (tooth paste)

All products were processed according to the cytometric workflow by using the potential maximum amount that one saliva sample could contain: 2 ml PBS, 15 ml CHX/LIST, abrasion of tooth brush from 3 min brushing in 2 ml PBS, tooth paste according to manufacturers' recommendation. Standardized concentration of 0.5 μm and 1.0 μm UV beads were added. The samples were measured until 2,500 events in the 0.5 μm bead gate were detected. The measured events are eventually over-represented due to measurement of undiluted solutions of test materials. In addition, in a cell-free test solution ADCs select abiotic particles and noise with higher preference. F/G: For these samples the analysis was terminated earlier due to the high amount of particles measured outside of the cell gate. The same measuring time was set with lower sample pressure due to high sample thickness. Consequently, less than 2,500 events at the 0.5 μm bead gate were detected. All measured negative controls did not produce a significant distortion of the samples.

H. Figure S3: Second run: person dependent oral microbiota

The NMDS-plot compares the microbial cytometric diversity of samples collected from 10 subjects (independent parallel samples to data shown in Fig. 5), each represented by an individual color. The sampling started at baseline (0 h, smallest points) and continued for three month (points increase in size). From each subject 8-11 samples were measured (see Tab. S1). The larger the distance between two points, the more dissimilar was the cytometric structure of the respective microbial communities. The depiction of the standard deviation occurs by ellipsoids. The expanse of an ellipsoid relates to the intra-individual variability: If it is small, the variability is low. The more distant two ellipsoids are located, the higher is the inter-individual diversity. Stress value of the NMDS-plot: 0.16. Stress value of the NMDS-plot: 0.16.

I. Figure S4: Second run: sugar- and acid-experiments

The NMDS-plots compare the microbial cytometric diversity of samples collected at baseline (0 h, small points) and after stress (8 h, big points) caused by sugars (A) or acids (B). The color of the points represents the involved subjects (see Fig. S3). The ellipsoids show standard deviations of samples collected at baseline (continuous line) and samples collected after the stress influence (broken line). In both experiments the two ellipsoids show high overlaps, hence no major shifts in respective community structures was caused by the applied stress. However, the inter-community variation was lower after the stress impact. Stress values of the NMDS-plots: 0.10 (A), 0.2 (B).

J. Figure S5: Second run: mouth-rinse experiments

The NMDS-plots represent the microbial cytometric diversity of samples of five subjects each, who used two different mouth rinses: 0.2 % Chlorhexidine (A) and Listerine® (B). The color of the points represents the involved subjects (see Fig. S3) and the size of the points demonstrates the times of sample collection (baseline = 0 h; 5 min, 2 h, 8 h after rinsing). The respective four areas compare the samples from one collecting time point. The ellipsoids show standard deviations of samples collected at baseline (continuous line), after 5 min (broken line), 2 h (pointed line), and 8 h (mixed line). Both chemicals did not cause noteworthy shifts in the community structure. Stress values of the NMDS-plots: 0.17 (A), 0.21 (B).

The cell number of the samples collected at 0 h (baseline), 5 min, 2 h, and 8 h after treatment with 0.2 % CHX mouth rinse is demonstrated per ml and per entire sample amount for two subjects (test persons 6 and 10). All samples show a clear decrease in cell number after 8 h. Both, the cell number per entire sample and per ml decreased within 8 h after rinsing for about 1.6 and 2.6 times (subject 6) and 1.4 and 1.7 times (subject 10), respectively, indicating a successful and huge effect of CHX.

K. Figure S6: Results of the ANOSIM tests, first run

The ANOSIM test was performed based on the cytometric gate abundances of all 10 subjects (A) of the long-term experiment. The outcome of the ANOSIM test confirmed the findings. A R value of 0.546 describes a bigger inter-individual than intra-individual variance with a significance value of 0.001. Most of the subjects show a low variance in gate abundances (e.g. subjects 2, 7, 9, 10) but the variance between all subjects is relatively high. The ANOSIM test was performed based on the cytometric gate abundances of both sample groups of the stress experiments (B, C). The outcome of the ANOSIM test showed that there is nearly the same variance within both the sample groups 0h and 8h as well as the combined sample groups for sugar and acids, respectively. This is described by a calculated R value of 0.031 with a p-value of 0.261 for the stress caused by sugar (B) and a calculated R value of 0.113 with a p-value of 0.037 for the stress caused by acids (C). The ANOSIM test was performed based on the cytometric gate abundances of each sample group of the mouth-rinsing experiments (D, E). The outcome of the ANOSIM test showed that there is nearly the same variance within all sample groups (0h, 5min, 2h, 8h) both for CHX and Listerine. This is described by a calculated R value of 0.221 with a p-value of 0.009 for the mouth-rinsing with CHX (D) and a calculated R value of -0.006 with a p-value of 0.497 for the mouth-rinsing with Listerine (E).

L. Figure S7: Results of the ANOSIM tests, second run

The ANOSIM test was performed based on the cytometric gate abundances of all 10 subjects (A) of the long-term experiment. The outcome of the ANOSIM test confirmed the findings. A R value of 0.546 describes a bigger inter-individual than intra-individual variance with a significance value of 0.001. Most of the subjects show a low variance in gate abundances (e.g. subjects 2, 7, 9, 10) but the variance between all subjects is relatively high. The ANOSIM test was performed based on the cytometric gate abundances of both sample groups of the stress experiments (B, C). The outcome of the ANOSIM test showed that there is nearly the same variance within both the sample groups 0h and 8h as well as the combined sample groups for sugar and acids, respectively. This is described by a calculated R value of 0.031 with a p-value of 0.261 for the stress caused by sugar (B) and a calculated R value of 0.113 with a p-value of 0.037 for the stress caused by acids (C). The ANOSIM test was performed based on the cytometric gate abundances of each sample group of the mouth-rinsing experiments (D, E). The outcome of the ANOSIM test showed that there is nearly the same variance within all sample groups (0h, 5min, 2h, 8h) both for CHX and Listerine. This is described by a calculated R value of 0.221 with a p-value of 0.009 for the mouth-rinsing with CHX (D) and a calculated R value of -0.006 with a p-value of 0.497 for the mouth-rinsing with Listerine (E).

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5.2 Abkürzungsverzeichnis

CFU	koloniebildende Einheit
CLSM.....	konfokale Laser-Scanning-Mikroskopie
DAPI	4',6-Di-Amidin-2-Phenyl-Indol
DNA	Desoxyribonukleinsäure
FISH	Fluoreszenz-in-situ-Hybridisierung
HMP.....	Human Microbiome Project
HOMD.....	Human Oral Microbiome Database
NGS.....	Next-Generation Sequencing
OTU	Operational Taxonomic Unit
PCR.....	Polymerasekettenreaktion
qPCR.....	quantitative Echtzeit-Polymerasekettenreaktion
RNA.....	Ribonukleinsäure
rRNA.....	ribosomale Ribonukleinsäure

5.3 Abbildungsverzeichnis

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7. Wissenschaftliche Präsentationen

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Detektion von Veränderungen bakterieller Kompartimente im humanen Speichel mit Hilfe der Durchflusszytometrie

Kurzvortrag: Young Scientists in Dentistry – Symposium für Nachwuchswissenschaftler; 5. und 6. Mai 2017; Dresden, Deutschland

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Kurzvortrag (ID 1318): DGZ-Gemeinschaftstagung mit der DGPZM, DGR²Z und DGET; 23.-25.11.2017; Berlin, Deutschland

8. Darstellung des eigenen Beitrags

Erklärung über den wissenschaftlichen Beitrag des Promovenden zur Publikation

Name, Vorname: Röhrig, Nicola
Institut: Poliklinik für Zahnerhaltung und Parodontologie
Angestrebter Doktorgrad: Dr. med. dent.
Thema der Dissertation: Detektion intra- und interindividueller Unterschiede bakterieller Kompartimente im humanen Speichel während dreimonatiger Beobachtungszeit mit Hilfe der Durchflusszytometrie

Hiermit erkläre ich, dass ich bei der Erstellung und Veröffentlichung der Publikation:

van Gelder S*, Röhrig N*, Schattenberg F, Cichocki N, Schumann J, Schmalz G, Haak R, Ziebolz D, Mueller S;

A cytometric approach to follow variation and dynamics of the salivary microbiota

Methods. 2017 Aug 24. pii: S1046-2023(17)30161-5.
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folgenden wissenschaftlichen Beitrag geleistet habe:

- Administrative Vorbereitung und Organisation eines Teilprojektes der Pilotstudie (Longitudinalstudie)
- Erstellung des Studienprotokolls zur Beurteilung des Forschungsvorhabens durch die Ethik-Kommission
- Konzeptanfertigung zur Versuchsdurchführung der genannten Teilprojekte
- Erarbeitung eines standardisierten und reproduzierbaren Arbeitsflusses zur Probenentnahme und -fixierung
- Probenentnahme und -verarbeitung gemäß des erarbeiteten Protokolls und etablierter Färbeprotokolle
- Auswertung der nach der Vermessung im Durchflusszytometer erhaltenen Daten mit Hilfe bioinformatischer Tools (Summit 4.3, FlowJoV10, flowCyBar)
- Verfassung des Manuskripts

Fulda, 10.10.2018

Ort, Datum

Nicola Röhrig

Hiermit bestätige ich als Mitautor o.g. Publikation die von Frau Nicola Röhrig
abgegebene Erklärung:

Leipzig, 16.10.2018

Ort, Datum

Prof Dr. Dirk Ziebolz, M.Sc.

Leipzig, 17.10.2018

Ort, Datum

Prof. Dr. Rainer Haak, MME

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Leipzig, 05.11.18

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Leipzig, 5.11.2018

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9. Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

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10. Lebenslauf

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11. Danksagung

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