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The influence of antimicrobial additives in toothpastes and -brushes on the associated microbiota

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Abstract

Antimicrobials in hygiene products potentially cause microbial diversity loss, which leads to an imbalanced human microbiome and supports the dissemination of multi drug-resistant pathogens.

Therefore, the purpose of this study was to determine how antimicrobials like zinc and silver nanoparticles on toothbrushes, or like triclosan in toothpaste, will affect the growth and diversity of toothbrush-associated microbes.

To investigate that interaction, an artificial mock community out of five isolates, recovered from used toothbrushes, namely *Microbacterium paraoxydans*, *Staphylococcus epidermidis*, *Kocuria rhizophila*, *Staphylococcus warneri* and *Rothia dentocariosa* was designed. The mock community, mixed in artificial saliva, was exposed to unused toothbrushes 29 times on an engineered setup that simulates toothbrushing. In five different conditions the effect of presence and absence of silver and zinc nanoparticles on bristles and triclosan in toothpaste on the mock community was investigated and compared. At seven different time points, the head of toothbrushes were cultivated for non-selective culturing techniques, followed by identification and quantification. Additionally, qPCR was used to quantify the total bacterial biomass.

Cultivation and molecular approaches indicate that toothpaste significantly inhibits the microbial growth, regardless of whether it contains triclosan. Antimicrobial toothbrushes accumulate a higher number of bacteria than standard toothbrushes, which was also shown by both approaches. In fact, cultivation results even show that nanoparticle toothbrushes accumulate almost twice as many bacteria than standard toothbrushes. Results from cultivation dependent experiments additionally suggest that nanoparticles on antimicrobial toothbrushes are only active in the first two days of being used. Comparing the abundance of all isolates, *M. paraoxydans* and *K. rhizophila* seemed to be more robust as they showed a higher survival rate compared to the other isolates.

Our results suggest that toothpaste has a greater inhibiting effect on microbes surviving on toothbrushes than antimicrobial toothbrushes. However, our findings indicate that further studies are needed to corroborate the mentioned interesting observations.

Zusammenfassung

Die Verwendung von antimikrobiellen Substanzen in Hygieneprodukten verursacht möglicherweise einen Verlust der mikrobiellen Vielfalt, was zu einem unausgewogenen menschlichen Mikrobiom führt und die Verbreitung von mehrfach arzneimittelresistenten Krankheitserregern unterstützt.

Ziel dieser Studie war es daher zu bestimmen, wie antimikrobielle Substanzen, wie Zink- und Silbernanopartikel auf Zahnbürsten oder Triclosan in Zahnpasten das Wachstum und die Diversität von Zahnbürsten-assoziierten Mikroben beeinflussen.

Um diese Wechselwirkung zu untersuchen, wurde ein künstliches Konsortium aus fünf Isolaten (*Microbacterium paraoxydans, Staphylococcus epidermidis, Kocuria rhizophila, Staphylococcus warneri* und *Rothia dentocariosa*) zusammengestellt, die von benutzten Zahnbürsten gewonnen wurden. Das Konsortium, gemischt mit künstlichem Speichel, wurde 29-mal mit unbenützten Zahnbürsten in einem technischen Aufbau, der das Zähneputzen simuliert, inokuliert. Dabei wurde in fünf verschiedenen Bedingungen die Wirkung von Zahnbürsten mit silber- und zinkbeschichteten Borsten und Triclosan-haltiger Zahnpasta auf das Konsortium untersucht. Die Zahnbürstenköpfe wurden zu sieben verschiedenen Zeitpunkten nichtselektiv kultiviert, gefolgt von Identifizierung und Quantifizierung. Zusätzlich wurde qPCR verwendet, um die gesamte bakterielle Biomasse zu quantifizieren.

Kultivierung und molekulare Ansätze deuten darauf hin, dass Zahnpasta, unabhängig davon, ob sie Triclosan enthält, das mikrobielle Wachstum signifikant hemmt. Antimikrobielle Zahnbürsten akkumulieren eine höhere Anzahl an Bakterien als Standardzahnbürsten, was auch bei beiden Ansätzen gezeigt wurde. Tatsächlich zeigen die Kultivierungsergebnisse, dass sich auf Nanopartikel-Zahnbürsten fast doppelt so viele Bakterien ansammeln wie auf Standard-Zahnbürsten. Die Ergebnisse legen zudem nahe, dass Nanopartikel auf antimikrobiellen Zahnbürsten nur in den ersten beiden Tagen der Verwendung aktiv sind. *M. paraoxydans* und *K. rhizophila* scheinen robuster zu sein, da sie im Vergleich zu den anderen Isolaten eine höhere Überlebensrate zeigen.

Die Ergebnisse zeigen, dass Zahnpasta eine stärkere Hemmwirkung auf Bakterien hat, die auf Zahnbürsten überleben, als antimikrobielle Zahnbürsten. Jedoch deuten die Resultate darauf hin,

dass weitere Studien erforderlich sind, um die genannten interessanten Beobachtungen zu bestätigen.

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1 Introduction

Bacteria were the first organisms found on our planet (Scoma and Vorholt, 2018) and although not visible with the naked eye, all types of microbes are still an essential part of Earth´s biota (Karoum *et al.*, 1980). Humans, as holobionts, are constantly interacting with billions of microbes, which influence our life and health (van de Guchte, Blottière and Doré, 2018). These microbes, which live on and in our bodies and are collectively referred to as the human microbiota, outnumber our eukaryotic cells by a ratio of around 1.3:1 (Weiman and Torres, 2014).

Establishment of the human microbial composition and influencing factors

The microbial composition of the microbiota can change in response to several variables such as lifestyle, nutrition, host behavior, use of drugs and hygiene. Microbial communities can also be influenced by human genetic and other environmental factors (Blaser and Falkow, 2009; Sender et al., 2016; Blaser, 2018; Hadrich, 2018). For instance, in industrialized countries people usually spend 90% of their time inside of buildings. Through increased urbanization and densely packed structures, a new microbiome composition evolves over time. The indoor microbial community is mainly influenced by the inhabitants living within those structures such as humans, animals and plants but also from the outside air (Mora *et al.*, 2016; Meadow *et al.*, 2014). Houseplants are known to alter biodiversity and to increase microbes associated with human health. Therefore, plants can enhance indoor air quality by stabilizing the overall microbial ecosystem (Berg et al., 2014).

Causes and risks of microbial diversity loss

A high beneficial microbial diversity is important because it can prevent infections (Pham and Lawley, 2014). Alteration of the microbiota might be a reason for the spread of pathogenic strains and the promotion of several common diseases that are linked to inflammation. Since germ theory, humanity is living more and more under sterile conditions. Current practices in hygiene, cleaning, medicine and diet are some potential causes for an imbalanced microbial consortium in humans. These modern, abacterial human behaviors might be the primary factors that lead to global microbial loss.

Antimicrobial chemicals used in modern cleaning and hygiene products cause a decrease of the beneficial microbes in the microbiome while at the same time they are supporting the spread of multi-resistant pathogens. Microbes confronted with antimicrobial chemicals show higher resistance rates against antibiotics, physical and chemical stresses and thus they adapt to this stress-level and more and more multi-resistant strains are able to emerge (Berg, Mahnert and Moissl-Eichinger, 2014) (Blaser, 2018) (Von Hertzen et al., 2011) (Blaser and Falkow, 2009) (Dong, Gupta and Angeles, 2019) (Mahnert *et al.*, 2019). Furthermore antibiotic resistance gene (ARG) dissemination is enhanced by frequent exposure to antimicrobials (Mora *et al.*, 2016; Han *et al.*, 2017), especially to nanoparticles (NPs) (Klümper *et al.*, 2016, 2019; Parra *et al.*, 2019) in hygiene products. Because of that it is also necessary to consider the molecular interactions between bacteria and antimicrobial additives.

Toothbrushes: a source and sink for chemicals and bacteria

One common antimicrobial product, which is used daily, is the toothbrush. Toothbrushes are known to accumulate bacteria from the oral cavity and from the environment (Wetzel *et al.*, 2005; Ferreira *et al.*, 2012; Frazelle and Munro, 2012; Samuel and Ifeanyi, 2015; Okafor *et al.*, 2016; Bds and Bds, 2018). In addition to that, toothbrushes do not only accumulate bacteria but they are also a source and a sink for antimicrobial chemicals (Han *et al.*, 2017). Antimicrobial chemicals are known to have antimicrobial properties; however, the same characteristics can damage and even kill eukaryotic cells. In particular, human exposure to nanoparticles can cause an alteration of the human microbiome, which can have severe health consequences, that are not yet fully understood (Wijnhoven *et al.*, 2009; Li, 2019). Therefore, it is important to characterize (antimicrobial) toothbrushes because they can act as a transporter of bacteria and chemicals to the human oral niche (Ferreira *et al.*, 2012; Frazelle and Munro, 2012; Naik *et al.*, 2015; Okafor *et al.*, 2016).

Antimicrobials used in oral hygiene products

Common antimicrobials used in oral hygiene products include silver (Ag) and sometimes zinc (Zn) nanoparticles on toothbrushes (bristle coating and elastomer infusion), triclosan (TCS) and stannous fluoride in toothpastes, and chlorhexidine in mouthwashes. Sodium lauryl sulfate (SLS), is a commonly used detergent in toothpaste and due to its antimicrobial properties, it is capable

of reducing the formation of dental plaque (Jenkins et al., 1991; Benn et al., 2010; Cascio *et al.*, 2015; Hansen *et al.*, 2015). Microbial culture-dependent analyses have demonstrated that the use of triclosan-containing toothpaste decreased microbial contamination on toothbrushes, selecting for members of *Prevotella*, which are associated with certain oral infections (Warren *et al.*, 2001) (Waaler *et al.*, 1993). Similarly, chlorhexidine spray on toothbrush heads and infusion of toothbrush bristles with Ag NPs has been reported to decontaminate toothbrushes and reduce total bacterial genome counts in supra- and subgingival biofilms after four weeks of brushing (Rodrigues *et al.*, 2011; Do Nascimento *et al.*, 2015a). These studies demonstrate impacts of antimicrobial use on the abundance and diversity of pathogens and normal oral microflora. However, it remains unclear how antimicrobial additives in oral hygiene products may influence the functional capacity of the microbial communities that develop on toothbrushes, which may influence the oral microbiota through exposure and interaction.

The well-explored oral microbiota

The human oral microbiota, which naturally consists of more than 700 microbial species, plays a significant role in human health (Aas et al., 2015; Aljehani, 2014). Several 16S rRNA gene sequencing-based studies have indicated significant correlations between the diversity of oral microbiota and the development of dental caries and/or periodontal disease (Bik *et al.*, 2010; Teng *et al.*, 2015). Metagenomics-based studies have further demonstrated that the functional dynamics of the microbiota in the oral cavity are rapid (Lloyd-Price *et al.*, 2017) and suggested that alterations in bacterial community structure and function may be associated with risks for developing caries and potentially oral cancer (Belda-Ferre *et al.*, 2012; Börnigen *et al.*, 2017).

Microbial interactions have important implications for antibiotic resistance gene (ARG) dissemination. Mobile genetic element (MGE)-mediated horizontal gene transfer (HGT) is largely responsible for the spread of ARGs, and these processes are enhanced within biofilms (Frost *et al.*, 2005; Madsen *et al.*, 2012). The oral cavity is a hotspot for dynamic biofilms, which may explain why MGEs are frequently detected in oral metagenomes and why several prominent members of the oral microbiota (e.g., *P. gingivalis*) carry multiple ARGs (Seville *et al.*, 2009). Thus, characterizing the dynamics of biofilms and HGT potential within the oral microbiome is essential for mitigating potential health risks.

The unexplored toothbrush microbiome

While the oral microbiome is well-characterized, the toothbrush microbiome remains largely unexplored. Previous studies that have characterized the microbial diversity on toothbrushes used cultivation-based approaches (Nascimento *et al.*, 2014; Morris *et al.*, 2014; Vignesh et al., 2017) or checkerboard DNA-DNA hybridization (Ximénez-Fyvie, Haffajee and Socransky, 2000; do Nascimento *et al.*, 2015a; Do Nascimento *et al.*, 2015b). Thereof arises the question which microbes are present and surviving on used toothbrushes over time.

The effect of Ag and Zn NPs on toothbrush bristles

Ag and Zn are commonly used NPs on antimicrobial toothbrushes and other products such as, clothing, food products, cookware, fitness equipment and baby products (Ahammed, Jayakumar and Vaideki, 2010; Benn, Cavanagh and State, 2010; Taylor *et al.*, 2013; Cascio *et al.*, 2015; Rode *et al.*, 2015; Tulve *et al.*, 2015; Vance *et al.*, 2015).

Metal NPs have been recorded to have bactericidal characteristics. In general, bactericidal mechanisms of NPs are cellular uptake of NPs, cell membrane integration or metal ion release. Cell death can be the result of DNA replication issues, membrane disruption, ATP depletion, protein inactivation or the production of reactive oxygen. If a cell dies depends on the bacterial species and the NP properties, containing size, shape and charge (Slavin *et al.*, 2017).

Products containing NPs are marketed as hygienic with positive effects on human health (Kessler, 2011), however little is known about the products mechanism of microbial growth inhibition, the antimicrobial capabilities and the general effects on health (Bundschuh *et al.*, 2018). The use of NP-containing products is linked to undesirable health issues (Wijnhoven *et al.*, 2009; Royce *et al.*, 2014), environmental consequences (Bundschuh *et al.*, 2018) and influence on the human microbiome (Li, 2019).

The effect of TCS in toothpaste

TCS is one of these chemicals widely applied in oral care products, that acts as a synthetic wide-spectrum antimicrobial. It is a lipid synthesis-inhibiting compound, which was originally registered as a pesticide. At the same time, it is used as an antimicrobial additive in toothpaste in order to reduce plaque, gingivitis and gum bleeding and to provide protection against buildup of oral bacteria through regular toothbrushing. However, the usage of TCS has been linked to

hormone disruption in humans and animals, antimicrobial resistance in bacteria and aquatic toxicity. In the face of widespread concerns over these negative effects of TCS, the U.S Food and Drug Administration (FDA) prohibited the sale of hand wash products containing TCS in the U.S. However antibacterial oral hygiene products, i.e., toothpastes, are still available (Han *et al.*, 2017). Due to these circumstances, it is an imperative to determine how the presence or absence of antimicrobial additives like silver (Ag) and zinc (Zn) on toothbrushes and triclosan (TCS) in toothpaste and may influence the diversity of toothbrush associated microbes.

Experimental design

The conducted study included designing a mock community representing the toothbrush microbiome, construction of a brushing simulation apparatus to expose the mock community with or without antimicrobial additives to unused toothbrushes within a simulation experiment as realistic as possible. The mock community, consisting of five isolates, was selected by cultivating isolates from 19 used toothbrushes. Two different toothbrushes (ProSys, Benco Dental, Pennsylvania, USA), a regular one and an antimicrobial one coated with silver and zinc nanoparticles, were selected as model toothbrushes. For toothpaste, we used a TCS-free mint fluoride gel toothpaste from the same brand. Five different toothpaste-slurry conditions were tested in triplicates and were established to test the given hypothesis. All of the conditions included artificial saliva and the mixture of five isolates representing the designed mock community. Within the first two conditions, the effects of a standard and a toothpaste with nanoparticle-coated bristles were compared to each other. The third condition indicated the effect of a standard toothbrush and toothpaste against the mock community. In the fourth and the fifth condition, the impact of TCS mixed in the toothpaste on a standard as well on an antimicrobial toothpaste was examined. As controls, a set of standard and antimicrobial toothbrushes were included in the simulation experiment without exposing them to anything. These controls showed that there is no risk of contamination while performing the experiment. A set of toothbrushes sampled before any inoculation was treated as a blank. The experiment was performed for 39 days and within this time period the brushing was simulated 29 times. The toothbrushes were sampled, cultivated and analyzed at eight different timepoints.

This thesis used whole genome sequencing to identify five selected members of the mock community, culture-based approaches to quantify and characterize the microbial diversity at the sampling points and a real-time quantitative PCR (qPCR) to quantify abundance of the total bacterial concentrations in the collected samples.

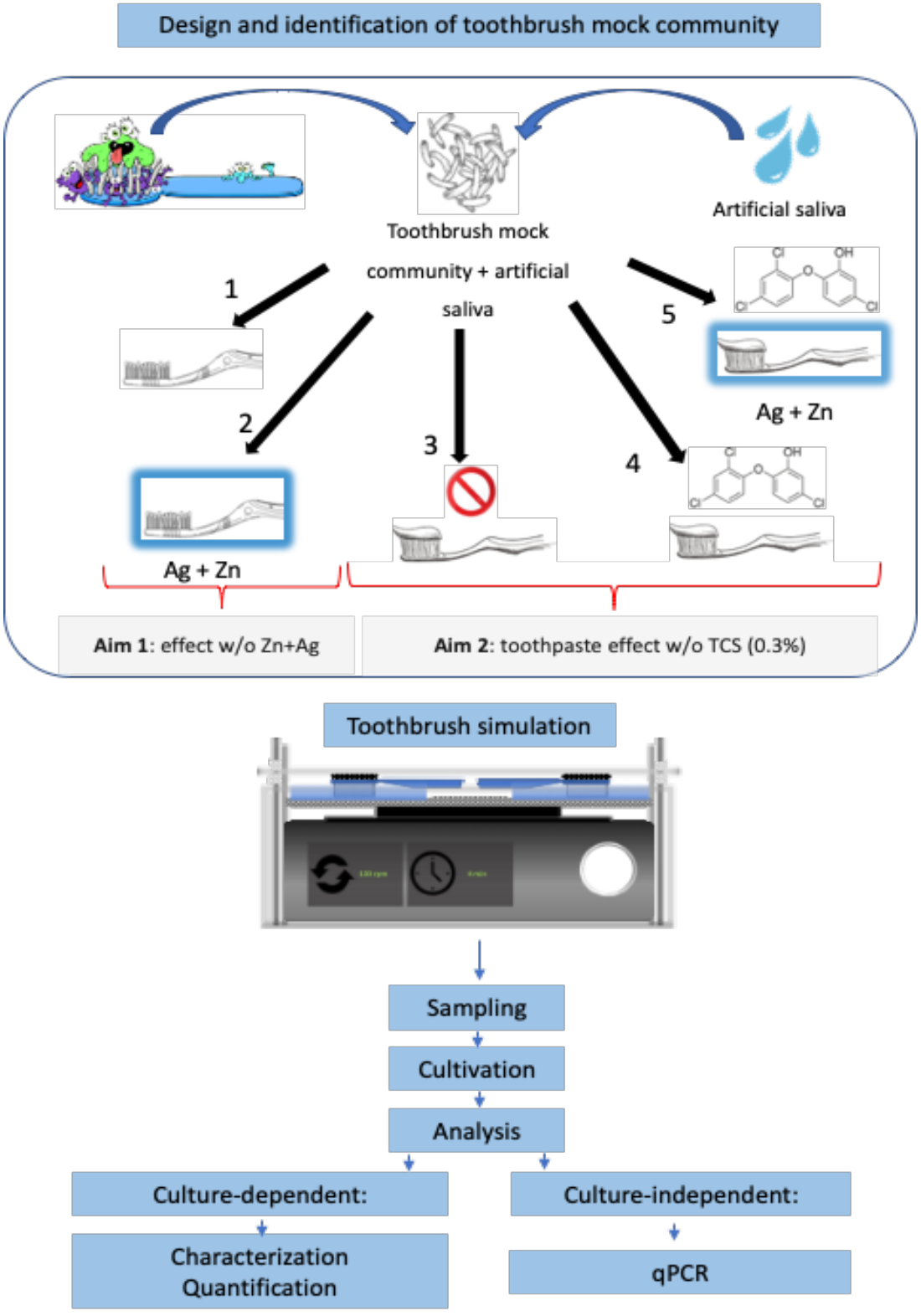


Figure 1: Workflow of the toothbrush simulation experiment. An overview of all working steps is shown. The five different conditions are illustrated in more detail in the scheme. After sampling, probes were cultivated and partitioned for culture-dependent and independent work. Culture-dependent approach include colony morphology characterization and quantification, while as culture independent work qPCR was performed.

2 Materials and Methods

2.1 List of Abbreviations

PBS……..phosphate buffered saline

TSA……..tryptic soy agar

OP…… optical property

C……..…..circular

E…… entire

F…… filamentous

U…….. undulate

R…… raised

C…….. convex

F…….. flat

P…….. pulvinate

U……..umbonate

S…….. small

P…….. punctiform

L…….. large

M…… moderate

S…… smooth

R……..rough

S…….. shiny

D…….. dull

C…… cream

Y…….. yellow

W…….. white

T…….. tan

R…….. red

G…… grey

O…… opaque

Tl…… translucent

Tp……transparent

Ag……silver

Zn……zinc

TCS……triclosan

NP……nanoparticle

TB……toothbrush

TP……toothpaste

2.2 Design of mock community

Microbial biomass recovered from 19 used toothbrushes was cultivated as described below. Using the resulting isolates, a mock community of five isolates was selected based on morphological diversity and abundance. Taxonomic classification for each isolate was identified by amplifying and sequencing the 16S rRNA gene using Sanger sequencing. Whole genome sequencing was additionally performed to confirm the Sanger sequencing results. The mock community combined with artificial saliva was then exposed to unused toothbrushes in the absence and presence of antimicrobial additives in toothpaste and on toothbrushes within a simulated brushing experiment.

2.2.1 Collection and cultivation of toothbrush samples

The mock community was established by culturing bacteria from 19 used electric or manual toothbrushes. Toothbrushes were cultivated within one week after they were last used. They were stored in a plastic bag at room temperature until they were sampled. As it was the start of a new project, the cultivation method was optimized during this process. For two toothbrushes, only the bristles were used for cultivating the surviving microbes. For the remaining 17 toothbrushes, the whole head of the toothbrush was used for cultivation. Due to practical

reasons and because microbes may also be attached to the toothbrush-head itself and to obtain a higher biomass concentration, it was decided to use the whole head for detaching remaining microbes.

The head of the toothbrush was cut off with a pair of shears, which were flamed before each use. Under sterile conditions, the toothbrush-head was directly cut into a 50 mL conical tube. Electric toothbrushes were treated the same way. Occasionally when the toothbrush contained a metal component inside, which was the case for electric toothbrushes, the plastic housing was removed, and the metal part remained connected to the head. The 50 mL sample tube was filled with 15 mL phosphate buffered saline solution (PBS buffer) containing filter-sterilized (Whatman 0.2 µm nylon syringe filters) 0.01% Tween (Tween 80, Fisher Scientific, USA). After 10 sec vortexing, the sample was shaken in a shaking incubator for 10 min at 25°C and 180 rpm. Afterwards it was vortexed again for 10 sec to detach and collect microbes from the bristles and the head elastomer. For each processed toothbrush sample, the buffer solution containing eluted microbes will be partitioned for microbial culture work and for microbial DNA analyses.

2.2.2 Microbial culture analysis

30 µl of the PBS buffer solution containing eluted microbes were plated on tryptic soy agar (TSA). The undiluted sample and three dilutions (10^{-2}, 10^{-4}, 10^{-5}) were plated to ensure isolated colony growth on TSA plates. Plates were incubated at 25°C and 37°C for at least 48h under aerobic conditions until growing colonies were observed. Plates were stored at 4°C until colony morphology was characterized.

2.2.3 Bacterial colony morphology characterization of isolates

Isolated colonies were classified based on their morphotype. Characterization properties are colony shape, margin, elevation, size, texture, appearance, pigmentation, optical property and effect on growth medium (Figure 2).

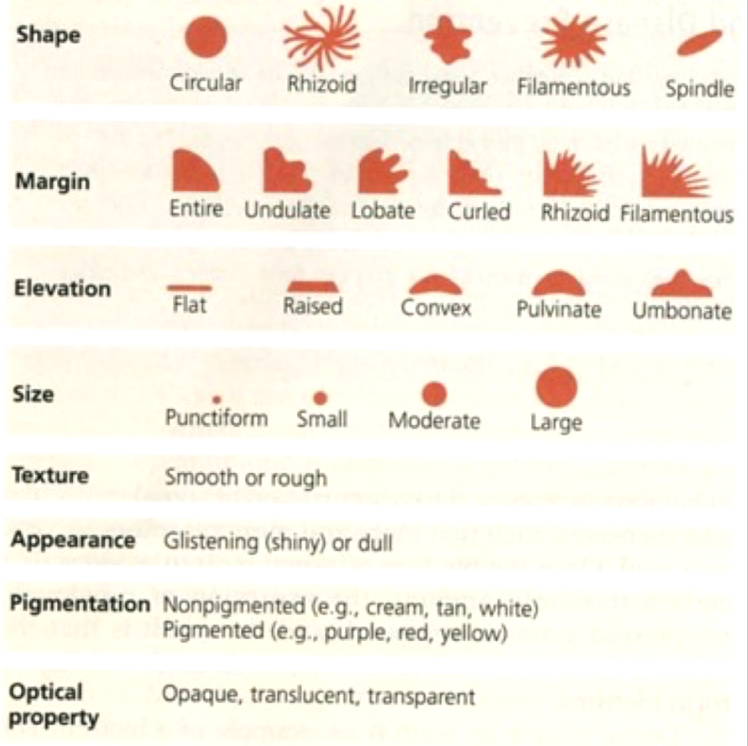


Figure 2: Characteristics of bacterial colonies. Shape, margin, elevation, size, texture, appearance, pigmentation and optical properties are described by several terms. Quelle: http://spot.pcc.edu/~jvolpe/b/bi234/lab/differentialTests/ColonyMorphology.htm (accessed Oct 4, 2019)

2.2.4 Picking and storing isolates

Plates were observed by visualization, and different colony types were counted when possible to count. One colony of each morphotype was labelled with a number and characterized regarding the previous described properties. 102 isolates were characterized and then picked with a sterile bacteriological loop.

Each picked isolate was streaked out on a sterile TSA plate to ensure it was a pure culture. If single colony isolation was successful, one colony was picked from the streaking plate with a flamed loop and inoculated in 1 ml tryptone soy broth (TSB). Tubes were incubated overnight either at 25°C or 37°C depending at which temperature colonies had grown on TSA plates. If the solution was turbid on the following day, the tube was briefly vortexed and 750 µl of the culture were transferred to a cryotube filled with 750 µl of a 50% glycerol stock solution. The final

concentration of glycerol stock solution was 25% (Fisher scientific, USA). Samples were gently vortexed and stored at -80°C.

2.2.5 Selection of mock community members

The goal was to design a mock community consisting of five unique isolates, representing the toothbrush microbiome. In total, 102 isolates were characterized found on 19 examined toothbrushes. From those 102 isolates, 66 isolates were unique based on morphology characterization properties (Table 1). A selection of five isolates was made based on morphological characterization. Therefore, isolates were clustered into groups consisting of the same morphotype (Coghlan, 2015). The most abundant group contains seven isolates with the same observed morphotype (Nr. 1 in Table 1) and the less common one consists of two uniform isolates (Nr.9-19). Three isolates were randomly selected from the five biggest clustered groups (Nr.1-5). Two more were chosen randomly from unique isolates, showing a 1 in the frequency column in Table 1. This selection was made aiming to represent as much of the diversity of the toothbrush microbiome as possible.

Table 1: Colony morphology characterization of 102 isolates. OP= optical property. Frequency refers to how often this specific colony type occurs, f.e. isolate Nr.1 occurs seven times and represents therefore the most abundant morphotype. Frequency: 1 stands for a unique isolate, which appears just once within 102 characterized isolates. Single letters represent the first letter of the morphology characteristics in Figure 1, f.e. c in column shape stands for (c)ircular. Isolates with additional information (isolate identity) in the frequency column are the five selected isolates for representing the toothbrush microbiome.

Nr.	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	OP	frequency
1	**c**	**e**	**r**	**s**	**s**	**s**	**c**	**o**	**7 (TB 21/06)**
2	**c**	**e**	**c**	**s**	**s**	**s**	**y**	**o**	**5 (TB 18/05)**
3	c	e	c	p	s	s	w	o	4
4	c	e	c	p	s	s	y	o	4
5	c	e	r	s	s	s	w	o	4
6	c	e	c	s	s	s	w	o	3
7	c	e	f	p	s	s	c	tl	3
8	c	e	p	s	s	s	y	o	3
9	c	e	c	p	s	s	w	tl	2
10	c	e	c	s	s	s	c	o	2
11	c	e	f	p	s	s	t	tl	2
12	c	e	f	p	s	s	w	o	2
13	c	e	f	p	s	s	w	tl	2

14	c	e	p	s	s	s	c	o	2
15	c	e	p	s	s	s	w	o	2
16	c	e	r	p	s	s	c	tl	2
17	c	e	r	s	s	d	w	o	2
18	**c**	**e**	**u**	**s**	**s**	**s**	**y**	**o**	**2 (TB 07/03)**
19	c	m	f	p	s	d	c	tl	2
20	c	c	c	s	s	s	r	o	1
21	c	c	r	m	s	s	t	o	1
22	c	c	r	s	s	s	t	tl	1
23	c	e	c	c	s	s	w	o	1
24	c	e	c	l	s	s	c	o	1
25	c	e	c	p	s	s	c	o	1
26	c	e	f	m	r	s	c	o	1
27	c	e	f	p	r	d	c	tl	1
28	c	e	f	p	s	d	c	tl	1
29	c	e	f	p	s	d	w	tl	1
30	c	e	f	p	s	s	c	tp	1
31	c	e	f	s	r	d	c	tl	1
32	c	e	f	s	r	s	w	tl	1
33	c	e	f	s	s	d	w	tl	1
34	c	e	f	s	s	s	c	tl	1
35	c	e	f	s	s	s	t	o	1
36	c	e	f	s	s	s	w	tl	1
37	c	e	p	s	s	d	t	o	1
38	c	e	p	s	s	d	y	o	1
39	c	e	p	s	s	s	g	o	1
40	c	e	p	s	s	s	t	tl	1
41	c	e	p	s	s	s	y	tl	1
42	c	e	r	m	s	s	c	tl	1
43	c	e	r	p	s	s	c	o	1
44	**c**	**e**	**r**	**p**	**s**	**s**	**g**	**tl**	**1 (TB 22/02)**
45	c	e	r	p	s	s	t	tl	1
46	c	e	r	p	s	s	w	o	1
47	c	e	r	p	s	s	w	tl	1
48	c	e	r	p	s	s	y	o	1
49	c	e	r	p	s	s	y	tl	1
50	c	e	r	s	s	s	r	o	1
51	c	e	r	s	s	s	t	o	1
52	c	e	r	s	s	s	y	o	1

53	c	e	r	s	s	s	y	tl	1
54	c	e	u	s	r	d	g	o	1
55	c	e	u	s	s	s	c	o	1
56	c	f	f	s	r	d	w	tl	1
57	c	u	c	m	s	d	y	o	1
58	c	u	f	m	r	s	c	tl	1
59	c	u	f	p	r	s	w	o	1
60	c	u	p	m	s	s	y	o	1
61	c	u	r	s	r	d	y	tl	1
62	**c**	**u**	**r**	**s**	**s**	**s**	**w**	**o**	**1 (TB 17/04)**
63	c	u	r	s	s	s	y	o	1
64	c	u	u	l	s	s	c	o	1
65	c	u	u	s	r	d	w	o	1
66	c	u	u	s	s	d	c	tl	1
								Sum	**102**

2.3 Identification of the mock community

2.3.1 DNA extraction of the mock isolates

To obtain genomic DNA from test toothbrushes, the QIAGEN DNAeasy Kit (Quiagen, Venlo, Netherlands) was used. Cultures were grown in 6 mL TSB broth and incubated overnight in a shaking incubator. The growth temperature was either 25°C or 37°C, depending on the individual isolate. 1 mL of the active growing culture was used for imaging the sample in order to avoid fungal contamination. 5 mL of the overnight culture was centrifuged for 3 min at 4200 rpm. The supernatant was discarded, and the resulting pellet was resuspended in 250 µL bead-tube solution. Extraction was performed according to the manufacturer´s protocol. DNA quality was determined using Synergy HTX Multi-Mode Reader (Biotek, Winooski, VT). DNA concentration was measured using the QuantiT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA).

2.3.2 DNA extraction optimization

The collected toothbrushes were used as test toothbrushes for finding the propriate DNA pretreatment method and DNA extraction protocol in order to get the highest yield and for

finding the ideal protocol for further simulation experiments (2.2.1 DNA extraction of the mock isolates).

Sonication (Model 120 Sonic Dismembrator, Fisher Scientific, USA), boiling at 95°C for 10 min, bead beating for 10 min (Vortex Adapter for 24 (1.5–2.0 ml) tubes, Quiagen, Venlo, Netherlands) and Proteinase K (50ug/uL, Lucigen, Middleton, WI) were tested as DNA-pretreatment methods. We further tested Qiagen DNAeasy Kit, phenol/ethanol extraction (Köchl, Niederstätter and Parson, 2005), Fecal DNA Extraction (Evans *et al.*, 2014), and MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI) for optimized DNA extraction methods.

Showing the highest yield, sonication and the Master Pure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI) were selected as DNA extraction method for simulation experiment samples.

2.3.3 DNA quantification

A Biotek Synergy™ HTX Multi-Mode Microplate Reader and Take3™ Multi-Volume Plate (Biotek, Winooski, VT) were used for quantifying DNA concentrations of the samples. The Take3™ Multi-Volume Plate was cleaned with ethanol and deionized water on a Kimwipe (Kimtech Science, USA). The plate has 16 microspots arranged as eight rows and two columns. Samples were always pipetted in duplicate. 2 µL of a sample were pipetted to each of the microspots without touching the plate surface with the pipette tip. Elution buffer was used as a blank. After all samples were loaded, the plate was closed carefully. Before setting the plate on the plate reader tray, the loaded spots were checked for air bubbles looking from underneath through the spots. Then absorbance measurements were recorded with the specific program for measuring nucleic acid concentrations on the Biotek Synergy™ HTX Multi-Mode Microplate Reader.

DNA concentration determined by fluorimetry was confirmed using the Quant-iT^{TM} PicoGreen® dsDNA Reagent and Kits (ThermoFisher Scientific, Waltham, MA). The ultrasensitive fluorescent nucleic acid stain is used for detecting small amounts of DNA, which was very useful for the low concentration samples. The assay was performed according to the manufacturer´s manual.

2.3.4 16S rRNA PCR

To identify the unknown bacterial isolates, Sanger sequencing of the 16S rRNA gene was processed. To prepare the product for sequencing, PCR (Table 2) was performed to amplify 16S rRNA gene using universal bacterial primers (Table 3). The PCR was performed according to the program described in Table 4 using a Mastercycler nexus (Eppendorf, Hamburg, Germany).

Table 2: PCR reaction mix (reaction volume 50 µL)

Volume (µL)	Reagent
30.75	H_2O (nuclease free PCR water)
10.0	5x buffer
4.0	$MgCl_2$
1.0	dNTP
1.0	27f-Primer (10 µM)
1.0	1391r-Primer (10 µM)
0.25	Go-Tag Flexi DNA (Promega)
2.0	DNA

Table 3: List of universal primers

Primer Label	Nucleotide sequence (5´-3´)
Forward 27F	AGRGTTYGATYMTGGCTCAG
Reverse 1391R	GACGGGCGGTGWGTRCA

Table 4: Thermocycler program for PCR

	Temperature (°C)	Time	
Initial DNA denaturation	95	2:00	
DNA denaturation	95	1:00	x25
Primer annealing	55	0:45	
Elongation	72	1:30	
Final Extension	72	5:00	
End	4	∞	

To ensure that the amplification was successful, the PCR products were loaded onto a 2% agarose gel at 120 V for 60 min (Owl™ EasyCast™ B1 Mini Gel Electrophoresis Systems; 1xTAE buffer-Fisher Scientific, USA).

2.3.5 DNA preparation for Sanger sequencing

The PCR products were purified with MinElute PCR purification Kit (Quiagen, Venlo, Netherlands). The steps were conducted according to the manufacturer´s manual. The only deviation was that the elution step was proceeded with nuclease free PCR water. After purification the DNA quality and concentration were determined using the methods described above. Samples were packed in an envelope and sent to the NUSeq core facility for 16S Sanger Sequencing.

2.3.6 DNA preparation for whole genome sequencing

Out of 10 previously chosen isolates, five were finally selected to represent as much of the diversity of the toothbrush microbiome as possible. Whole genome sequencing (WGS) was performed with five chosen isolates to ensure the results of 16S Sanger sequencing.

Isolates were grown for 24-48 hours in TSB media at 25°C with continuous shaking. Cells were then pelleted by centrifugation at 10,000 *g* for 3 min. DNA was extracted using a MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI) following the manufacturer's instructions. DNA quality was measured using a Synergy HTX Multi-Mode Reader (Biotek, Winooski, VT) and found to be of acceptable quality if the 260/280 ratio was found to be between 1.8-2.0. DNA was quantified using the QuantiT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Massachusetts, USA). DNA was stored at -20°C for further tests.

Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) following manufacturer´s instructions. Fragment lengths of prepared libraries were measured on an Agilent 2100 Bioanalyzer (Santa Clara, CA). Libraries were then normalized and pooled in preparation for a MiSeq (Illumina, San Diego, CA) run. Prepared samples were sent to a collaborating lab for sequencing.

2.3.7 Growth curve

TSB medium was inoculated with the isolates and culture was grown overnight at 37°C to saturation. The inoculums were diluted 1:100 that the initial OD_{600} results between 0.05 and 0.1. 200 µL of each dilution was filled into a 96-well plate in triplicate. The plate reader manufacturer´s instructions for programming were followed. The temperature was set at 37°C.

The plate was shaken continuously to keep the culture aerated, to ensure adequate oxygen for growth and that sediments on the bottom of the plate were mixed before each reading. The OD_{600} was measured every 15 min. The total run time of the growth curve assay was 24 h, during which all cultures were able to reach a plateau in OD. 200 µL TSB medium, the same culture medium as in the experimental wells, was used in a blank well in order to zero the absorbance reading. During the experiment, the 96-well plate (MicroAmp™ Optical 96-Well Reaction Plate, Applied Biosystems™, Thermo Scientific, Waltham, USA) was closed with a lid to avoid evaporation of the culture.

2.3.8 Kirby-Bauer disk diffusion susceptibility test

For the disk diffusion test the Kirby Bauer method was applied (Bauer et al., 1966). Tetracycline, Colistin, Gentamicin, Chloramphenicol, Vancomycin and Ampicillin (BD BBL™ Sensi-Disc™, USA) were tested.

2.3.9 Screening on different agar plates

All 5 isolates were screened on multiple agar plates, including MSA (Acumedia, USA), Mac Conkey (BD Difco, USA), R2A (BD Difco, USA), M9, CAN (CHROMagar) and Blood agar (TSA with 5% sheep's blood, BD BBL, USA). For each plate, a sterile wooden stick was soaked into each overnight culture. The agar plate was touched with this wooden stick and afterwards it was streaked out with a sterile loop for a single colony. Plates were incubated at 25°C and 37°C.

2.4 Simulated brushing experiment

2.4.1 Toothbrush and toothpaste

In the experiment two types of manual toothbrushes and a toothpaste were used from the same brand (PRO-SYS, USA). One type is labelled as an original toothbrush with a compact-size head and white soft DuPont™ Tynex bristles. To explore the impact of chemical additives on bristles, an antimicrobial-advertised toothbrush from the same manufacturer was selected as the second type of toothbrush. Containing a compact-size head, this toothbrush uses soft and blue DuPont StaClean bristles, featuring StaClean technology with silver and zinc nanoparticles. Mentioned

nanoparticles are said to eliminate growth of microorganisms, including bacteria, fungi and yeast. Both toothbrushes have bristles made of 612 nylon filaments, that have an abrasive grit additive. Compared to other nylons, this type is stiffer when wet and absorbs less moisture (DuPont USA, 2019). Both toothbrushes have a rubber-free handle to keep the toothbrush clean and free of residue. The brand was chosen because they have comparative toothbrush models with the same material, both with and without nanoparticles. As a toothpaste a mint tooth gel containing fluoride but no TCS was tested. It was selected because it was possible to add TCS manually and because it contains neither any essential oils nor stannous fluoride, which are also known as antimicrobials.

2.4.2 Mixing five isolates to represent the mock community

From an overnight culture of each isolate 1:2, 1:4, 1:8, 1:16, 1:32 dilutions were made (serial dilutions I). The OD_{600} of each of those dilutions was measured in duplicate. The particular media that the cells were growing in was always used as a blank. In this case TSB was used. Another four serial dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}=serial dilutions II) were created out of each previously prepared dilution. All of these dilutions were plated on TSA plates and incubated at 37°C for at least 48 h. Colonies were counted and recorded. Using plate count data, colony-forming units per milliliter (CFU/mL) were calculated using following formula:

cfu/ml = (number of colonies x dilution factor) / volume of culture plate

The average value of the determined duplicate OD_{600} of the isolates and the blank were calculated. With this data, the corrected average was determined. The CFU/mL (*y*-axis) values were plotted against the absorbance (*x*-axis) in order to create a scatter graph, which shows the slope formula. This slope formula represents the standard equation.

The total natural bacterial density in the mouth is 1.00E+06-1.00E+08, therefore it was decided to aim a total density of 1.00E+07 microbes (Loesche, 1982) in the mixed mock community. Dividing this number by five means each isolate should be represented by a number of 2.00E+06 cells.

For the experiment, the mock community mix was created every day anew. For this purpose, a fresh overnight culture was made every day. OD_{600} of all isolates and blanks were measured in

duplicate. The average was calculated, and the average blank was subtracted from the average values of the isolates to find the corrected average value. Entering the resulting average value into the given formula (y=k*x+d) as x, it was possible to calculate y, representing the CFU/mL. Then the appropriate amount of each isolate was calculated to yield 2.00E+06 CFU of each isolate in the total volume of the mock community mix. The necessary volume that needs to be prepared was dependent on the number of toothbrushes needed to be inoculated on the particular day in the experiment.

2.4.3 Preparation of the mock community-artificial saliva mix

Artificial saliva was prepared following the Klimek method which is commonly used for imitating saliva (Han *et al.*, 2017). The mock community consisted of five isolates, which will be described subsequently in more detail in 3.1. The five isolates were streaked out on TSA plates to obtain single colonies once a week from the frozen glycerol stock in order to have freshly growing isolates to inoculate overnight cultures. TSA plates were incubated at 37°C at least 48-72h until isolated colonies were observed. Plates were stored in an incubator at 25°C to keep them fresh and actively growing.

The OD_{600} of the overnight cultures of all isolates was measured in duplicates in a plate reader. To take the measurements, a tube containing the culture was shaken manually and vortexed gently in case sediment formed on the bottom of the tube. 200 µL of each homogeneous mixed overnight culture was transferred in a well of a 96 well plate. Measured OD_{600} values were used to calculate the appropriate volume of overnight culture needed to obtain the correct CFUs per isolate in order to imitate a total number of 1.00E+07 microbes total, meaning 2.00E+06 CFU of each isolate. Calculated volume of each isolate was transferred into a new tube. Samples were centrifuged for 3 min at 10000 rpm in a microcentrifuge. The supernatant was discarded by pouring it out manually, and the pellet was resuspended in 1 mL artificial saliva and centrifuged again. This washing step was repeated twice in order to remove media residues from the cells. After the last centrifugation step, the supernatant was discarded, and the cell pellet was resuspended in artificial saliva. The resuspended samples were mixed into a fresh autoclaved

bottle and the missing amount of artificial saliva was added to attain the properly volume for preparing required slurries. The solution was mixed by gently shaking.

2.4.4 Preparation of toothpaste slurries

The base of each slurry consisted of 3 mL artificial saliva and 1.00E+07 microbes representing the mock community. 1.0 g toothpaste or toothpaste with 3 mg TCS (0.3% TCS in 1.0 g toothpaste) was added for whichever condition (Figure 3) (Han *et al.*, 2017). 1.0 g toothpaste per brushing event is a standardized usage amount for adults (Jensen *et al.*, 2012). The toothpaste- artificial saliva ratio (1:3; w:w) was adapted from previous studies (Alshara *et al.*, 2014). For each of the five conditions, specific slurries were prepared for a set of three toothbrushes individually in 50 mL conical tubes. 3 ± 0.02 g freshly extruded toothpaste was weighed directly into a 50 mL conical tube. For weighing TCS a weighing paper was folded and TCS was put on the resulting fold of the paper to avoid losing any powder. Due to the low amount, weighing was performed with an analytical balance. Toothpaste and TCS was added to the mock community-artificial saliva mix shortly before the simulation experiment started. The prepared slurry solution was vortexed for 10 sec and also shaken manually until a homogeneous slurry solution was obtained. Slurries were preheated to 37°C in a shaking incubator before use to simulate the temperature in the mouth. After the heating step, slurry tubes were gently vortexed again.

2.4.5 Five test conditions

In order to investigate the effects of chemicals, five conditions in presence and absence of the antimicrobials were tested out and compared to each other. Condition 1 was a standard toothbrush without toothpaste. Condition 2 was an antimicrobial toothbrush without toothpaste. Condition 3 was a standard toothbrush with toothpaste. Condition 4 was a standard toothbrush with toothpaste and added TCS. Condition 5 was an antimicrobial toothbrush with toothpaste and added TCS. Conditions were visualized in Figure 3.

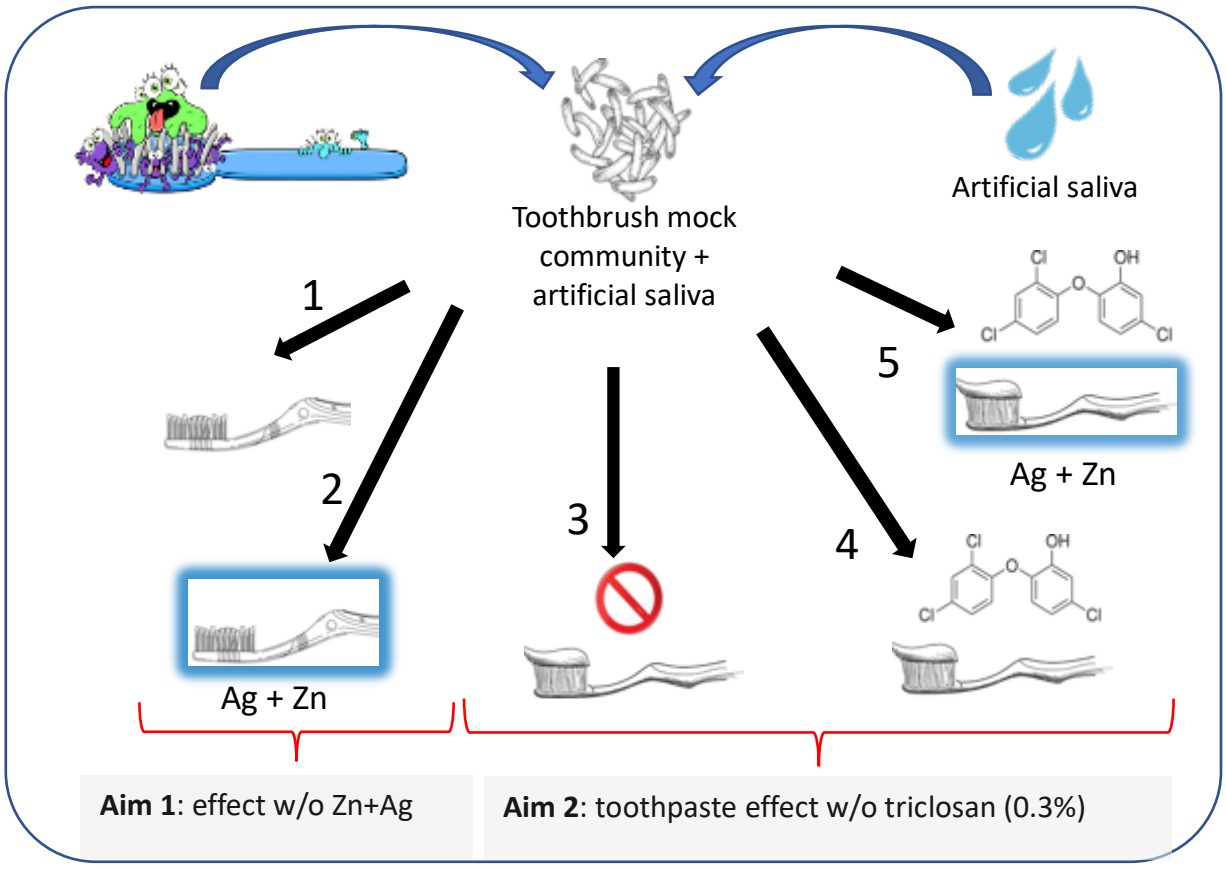


Figure 3: Overview of experimental conditions. 1: standard toothbrush without toothpaste. 2: antimicrobial toothbrush without toothpaste. 3: standard toothbrush with ProSys toothpaste. 4: standard toothbrush with ProSys toothpaste and added triclosan (TCS). 5: antimicrobial toothbrush with ProSys toothpaste and added TCS. Conditions are represented in Table 5 as well. Ag=Silver; Zn=Zinc.

2.4.6 Design of the experimental brushing setup

The goal was to design an apparatus to simulate realistic brushing in a way that was practical and reproducible in a laboratory setting. The most important conditions for designing the set up was that the bristles were soaked in the slurries completely, that the bristles were touching the glass petri dishes evenly during the simulation experiment and that the two different types of toothbrushes were located apart from each other.

On square plates cut out of ¼" clear acrylic sheets (6.35 mm - Mc Master-Carr Supply Company, Illinois, USA), five sets of three toothbrushes were fixed with Velcro™ tape. The five sets of toothbrushes, representing the five different conditions in the experiment, will be explained later in more detail (Figure 7).

A box of the same material was built around the shaker, so that plates with the toothbrushes on it could be bolted to the box. Brushing was simulated using the aid of the rotation movement of the shaker (Figures 4, 5).

Parts of the box and the toothbrush plates were designed with Autodesk Inventor. The single pieces were cut with a laser cutter, called Universal Laster Systems ILS.75. Parts were assembled and attached with an appropriate glue for acrylic plates (Scigrip, smarter adhesive solutions, North Carolina, USA).

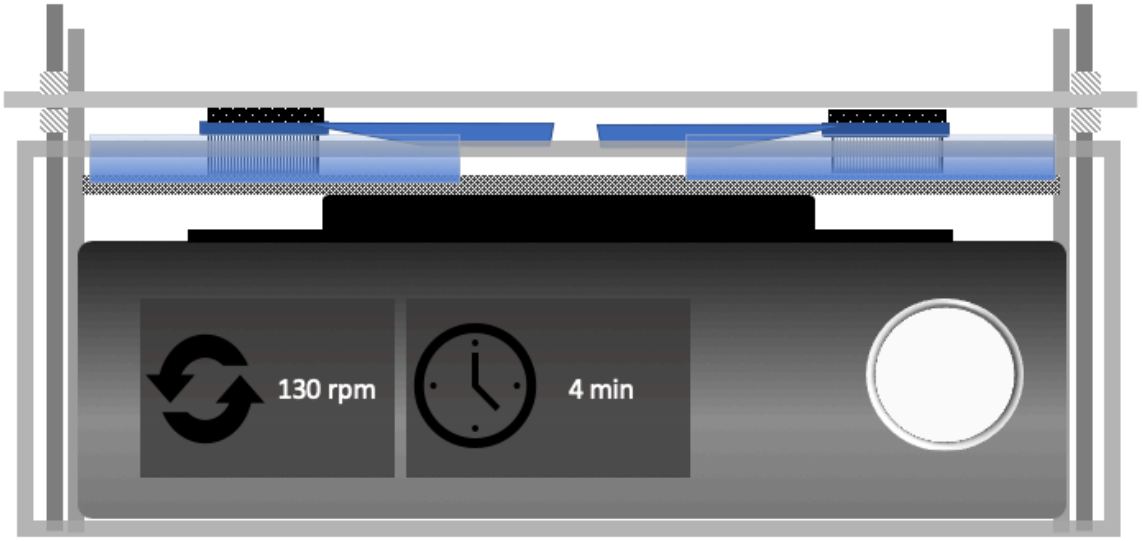


Figure 4: Designed simulation set up with toothbrush plate. Bristles soaked in the slurries filling the petri dishes.

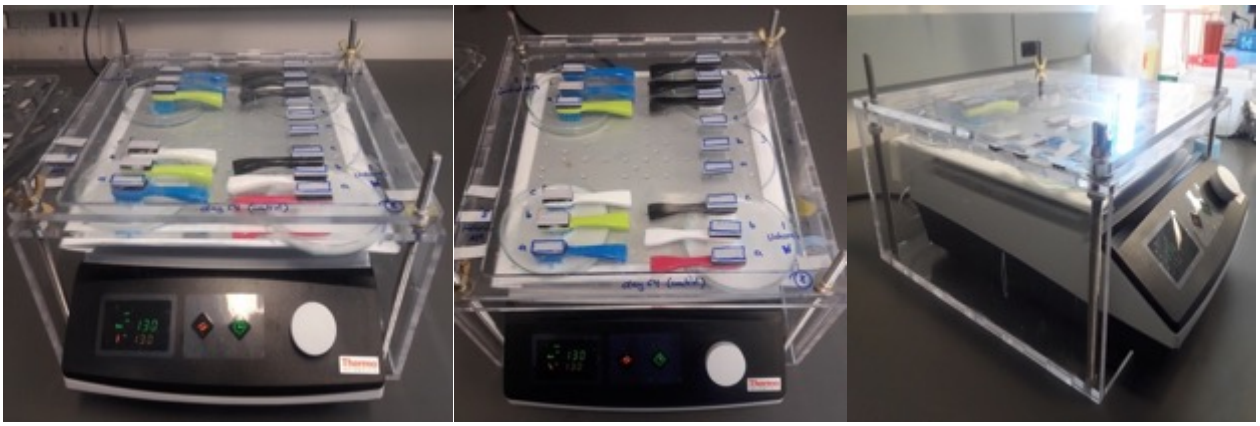


Figure 5: Pictures of the experimental brushing set up

2.4.7 Preparing toothbrush plates

First the autoclave pans, which were used as a cover for the toothbrush plates, scissors, a role of Velcro tape (Velcro, United Kingdom) and a pair of pliers were sterilized for 45 min. The acrylic plates were wiped clean with 70% ethanol. Both sides of the plates were sterilized for 45 min under UV light in the biosafety cabinet. All toothbrushes, used for the 6-week experiment, (Pro-Sys, Pennsylvania, USA) were unwrapped in the biosafety cabinet and the toothbrush handle was cut off with pliers. The pliers were wiped clean with 70% ethanol between every new toothbrush. Antimicrobial toothbrushes and standard toothbrushes without nanoparticles were collected separately in two autoclave pans and sterilized for 45 min using UV light. The Velcro tape was cut into 1 cm pieces. The protective foil was carefully removed from the adhesive, and the Velcro was taped on the back of the toothbrush head and on the predefined position on the acrylic plate (Figure 6, 7). Toothbrushes were finally attached to the acrylic plate using Velcro. Toothbrushes were inoculated in triplicates in a specified slurry. To that end, three toothbrushes were placed next to each other at a distance of 1 cm. The next set of three was placed adjacent to the first at a distance of 2 cm. On one side of the acrylic plate three standard toothbrush sets were positioned. The other side consisted of two sets of three toothbrushes, which represented the antimicrobial toothbrushes. The goal was to keep the two different types of toothbrushes apart, therefore they were physically separated on the acrylic plates (Figure 7). When fully assembled, one experimental toothbrush plate consisted of nine standard and six antimicrobial toothbrushes. One toothbrush plate was prepared for each sample point (0, 1, 2, 7, 13, 19, 23, 29). Control plates consisted of six standard and six antimicrobial toothbrushes. Controls

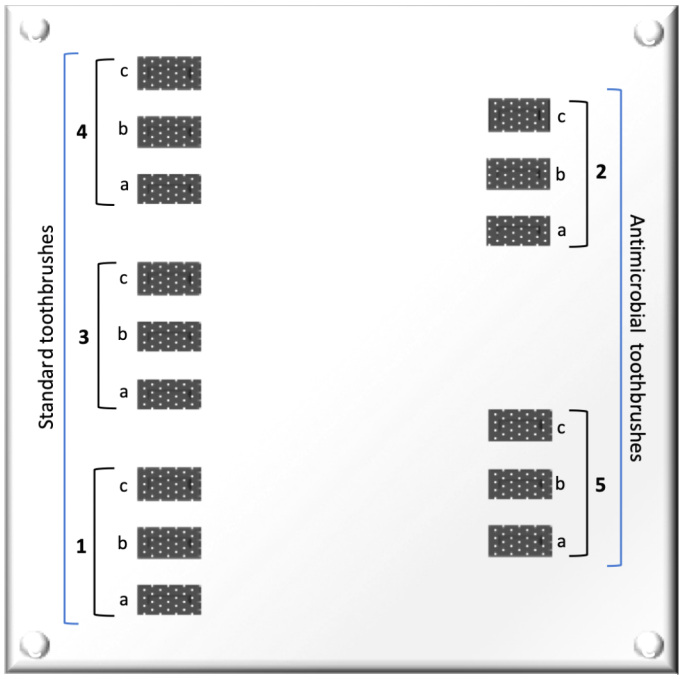


Figure 6: Acrylic plate without toothbrushes but with Velcro tape, it will be upside down on the shaker (mirrored on the left side).

represent negative controls which were not inoculated during the experiment; however, brushing was performed on the simulation apparatus in clean and dry glass petri dishes to imitate the complete process of brushing. This was done to estimate the risk of contamination during the whole experiment period in this unsterile simulation environment.

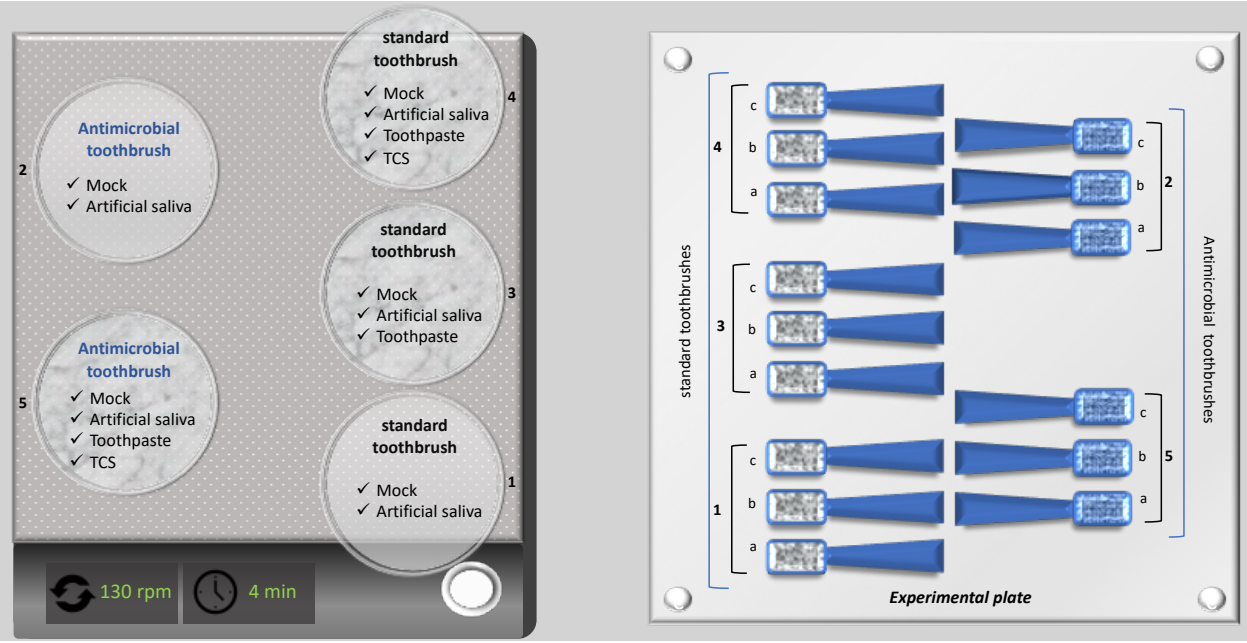


Figure 7: Experimental plate and slurry composition. Left: Shaker with glass petri dishes filled with slurries and individual type of toothbrush to be brushed in the slurry. 1= condition 1, standard toothbrush, mock community in artificial saliva; 2= condition 2, antimicrobial toothbrush, mock community in artificial saliva; 3= condition 3, standard toothbrush, mock community in artificial saliva, toothpaste; 4= condition 4, standard toothbrush, mock community in artificial saliva, TCS; 5= condition 5, antimicrobial toothbrush, mock community in artificial saliva, TCS. Right: toothbrushes taped on acrylic plate using Velcro tape. 1-5 refer to the different type of slurries. a, b, c: representing triplicates for each slurry. The left side consists of standard toothbrushes and the right side of antimicrobial toothbrushes. During brushing, the acrylic plate was upside down on the shaker, such that the numbers of the toothbrush set corresponded to the petri dishes filled with appropriate slurries.

Table 5: Conditions representing slurry compositions for experimental plates. Check mark/minus sign indicates the presence/absence of the specific component in the particular condition.

Experimental plates	Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
Toothbrush	Standard	Antimicrobial	Standard	Standard	Antimicrobial
Mock	✓	✓	✓	✓	✓
Artificial saliva	✓	✓	✓	✓	✓
Toothpaste	-	-	✓	✓	✓
TCS	-	-	-	✓	✓

2.4.8 Simulated brushing experiment – experimental protocol

The autoclaved glass petri dishes were attached at the marked locations on the shaker plate with double-sided adhesive tape. The preheated slurries were manually shaken a final time and poured into the appropriate petri dish. The autoclave pan, which was used as a cover for the toothbrush heads, was removed from the acrylic toothbrush plate. The toothbrush plate was placed on the set-up box while bolts were going through the holes in the acrylic plates. Plates were fixed on the box with wing nuts. The shaker was switched on for 4 min at 130 rpm in order to simulate brushing twice a day (2x2 min). The recommended practice of brushing twice a day and 2 min per brushing was adopted (Jensen *et al.*, 2012). After 4 min brushing, shaker was switched off and the wing nuts were removed. After completion of one brushing event, the toothbrush plate was lifted from the slurry and placed on the top of the bolts. Slurry residuals remaining on the toothbrushes were able to drip off sufficiently before transferring plates to the washing station. Toothbrush plate was removed from the set up and placed on a desk with the toothbrushes facing upwards. Autoclave pan was placed on the toothbrush plate to cover the TB heads. Remaining slurries in the petri dishes were either sampled or discarded. To discard the slurries, petri dishes with the slurries were carefully removed from the shaker to avoid spilling slurries. They were soaked in an autoclaved pan half filled with 10% bleach (Clorox, California, USA). Afterwards they were twice washed manually with a washing powder (Alconox, New York, USA) and rinsed twice with deionized water. After drying overnight, they were wiped again with 70% ethanol, wrapped into aluminum foil and autoclaved.

2.4.9 Washing step

The whole washing step was performed at the sink. Autoclaved 60 mL plastic syringes were filled with 60 mL autoclaved tap water under sterile conditions. A square cut piece of parafilm was wrapped around the tip of the syringe to avoid contamination of the water filled in the syringe. For collecting the water during washing, an autoclave pan was half filled with 10% bleach solution to kill microbes which are leaching from the toothbrushes while washing. The prepared pan was placed in the sink. The washing step was performed with every plate directly after the toothbrush simulation. Parafilm was removed from the syringe-tip before use. Each toothbrush-head was rinsed individually with 30 mL tap water. Water was sprinkled very gently directly on the bristles.

The distance between toothbrush head and tip of the syringe was about 10-15 cm. The washing step was performed as steadily as possible. The toothbrush was shake dried for a few seconds. Afterwards the back of the toothbrush head was wiped dry with a Kim wipe. Toothbrushes were placed back on the same position on the toothbrush plate. After the washing step was performed with every toothbrush the plate was covered again with an autoclave pan. The toothbrush-plate is always covered except for experiment events. After washing, the plunger was pulled out of the syringe, so both parts of the syringe were able to dry overnight. Dried syringes and plungers were wrapped into aluminum foil and autoclaved. Cooled syringes were filled with water for further use.

2.4.10 Sampling and Cultivation

Toothbrush sampling was performed according to the experimental schedule listed in Table 6. First, toothbrushes were removed from the plate and the Velcro tape was removed from the back of the toothbrush-head in the biosafety cabinet. Glue residuals were wiped off with a Kimwipe and DNA away (Andwin Scientific, USA) to remove possible DNA contaminations from the toothbrush-head. All other steps were performed as with the test-toothbrushes, which is described in 2.2.1 Collection and cultivation of toothbrush samples and 2.2.2 Microbial culture analysis.

Exceptions to the protocol are that undiluted, 10^{-2} and 10^{-3} dilutions were plated and incubated only at 37°C. Colonies were classified as one of the five isolates, counted and recorded.

Table 6: Experimental Schedule. Plan containing exposure times, washing steps and sampling timepoints. Exposure describes the process of exposing the mock community in artificial saliva to the toothbrushes within the simulation experiment. Break means no exposure on this particular day. The numbers in the sampling plate column refer to the amount of inoculation/exposure times at the moment of sampling and at the same time this particular plate was sampled on this particular day. F.e. Plate 0 and 1 were both sampled on day 0, which is visible in the second row. Plate 2 f.e. was sampled on day 2 before the third exposure time.

Week	Day	Date	Day#	Exposure#	Washing	Sampling plates*
Week 1	Mon	08.27.18	Day 0	1		0 ; 1
	Tue	08.28.18	Day 1	2		
	Wed	08.29.18	Day 2	3		2
	Thu	08.30.18	Day 3	Break	Break	
	Fri	08.31.18	Day 4	4		
	Sat	09.01.18	Day 5	Break	Break	
	Sun	09.02.18	Day 6	Break	Break	
Week 2	Mon	09.03.18	Day 7	Break	Break	
	Tue	09.04.18	Day 8	5		
	Wed	09.05.18	Day 9	6		
	Thu	09.06.18	Day 10	7		
	Fri	09.07.18	Day 11	8		7
	Sat	09.08.18	Day 12	9		
	Sun	09.09.18	Day 13	Break	Break	
Week 3	Mon	09.10.18	Day 14	10		
	Tue	09.11.18	Day 15	11		
	Wed	09.12.18	Day 16	12		
	Thu	09.13.18	Day 17	13		
	Fri	09.14.18	Day 18	14		13
	Sat	09.15.18	Day 19	15		
	Sun	09.16.18	Day 20	16		
Week 4	Mon	09.17.18	Day 21	17		
	Tue	09.18.18	Day 22	18		
	Wed	09.19.18	Day 23	19		
	Thu	09.20.18	Day 24	20		19
	Fri	09.21.18	Day 25	21		
	Sat	09.22.18	Day 26	Break	Break	
	Sun	09.23.18	Day 27	Beak	Break	
Week 5	Mon	09.24.18	Day 28	22		
	Tue	09.25.18	Day 29	23		
	Wed	09.26.18	Day 30	24		23
	Thu	09.27.18	Day 31	25		
	Fri	09.28.18	Day 32	26		
	Sat	09.29.18	Day 33	Break	Break	
	Sun	09.30.18	Day 34	Break	Break	
Week 6	Mon	10.01.18	Day 35	Break	Break	
	Tue	10.02.18	Day 36	27		
	Wed	10.03.18	Day 37	28		
	Thu	10.04.18	Day 38	29		
	Fri	10.05.18	Day 39	30		29; control plate

2.4.11 DNA extraction

Pretreatment before DNA extraction

Sonication was performed after the cultivation step and after a sample was taken for microbial culture analysis. The 50 mL tube containing PBS-buffer with 0.01% Tween and the toothbrush head (Cultivation of toothbrush samples) was put on ice to avoid overheating during the sonication step. Before using the sonicator, the sonotrode was cleaned with ethanol and deionized-water. Then the tip of the sonotrode was carefully plunged into the sample without touching the tube itself. The total time for sonication was 2 min, alternating between 10 sec pulsing (sonication) and a 10 sec pause. The amplitude was set on 40% at 4 Watts. After usage the sonotrode was cleaned again with 70% ethanol.

DNA extraction with MasterPure Complete DNA and RNA Purification Kit

The lysis protocol for cell samples (e.g., mammalian cell culture, buccal cells, *E. coli*) and the Precipitation of Total DNA (for all biological samples) were performed according to the manual of MasterPureTM Complete DNA and RNA Purification Kit.

2.4.12 Real time quantitative PCR (qPCR)

In addition to the cultivation-based approach to analyze microbial diversity on toothbrushes, qPCR was performed to determine the copy number of the 16S ribosomal RNA gene, the target gene of interest. The 16S rRNA codes for the RNA component of the 30S subunit of the bacterial ribosome. As it is widely present in all bacterial species, it represents total bacterial DNA in the samples.

A primer set that targets the 16S rRNA gene present in all isolates was optimized. Gene copy number determinations were made by comparing sample Ct-values to Ct-values of a standard curve. The standard curve was created by making samples containing varying concentrations of a plasmid containing the 16S rRNA gene and determining the Ct-values for said samples. The concentration range of the standard curve covered the full range of sample 16S rRNA concentrations.

The qPCR was performed according to Table 7 and 9 using a Quant Studio 3 Real-Time PCR System (Applied Biosystem, California, USA). 96 well plates were used, and each sample was measured in triplicate. The resulting data was analyzed using Quant Studio software v1.4.1.

Table 7: qPCR reaction mix (reaction volume 20 µL)

Volume [µL]	Reagent
10.0	PowerUp SYBR Green Master Mix
7.0	Nuclease free H_2O
0.5	341f primer (100 µM)
0.5	534r primer (100 µM)
2.0	Template DNA

*Nuclease free H_20 was used as template in the negative control.

Table 8: Universal primer set targeting 16S rRNA gene

Primer Label	Nucleotide sequence (5´-3´)
Forward 314F	CCT ACG GGA GGC AGC AG
Reverse 534 R	ATT ACC GCG GCT GCT GGC A

Table 9: QuantStudio 3 cycler program. Thermal profile.

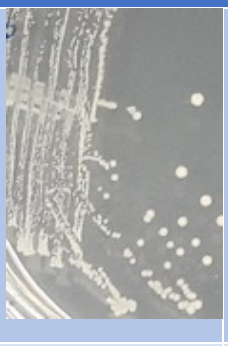
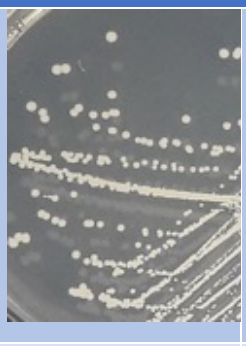
	Temperature (°C)	Time	
Stage 1	**50.0**	**2 min**	
	95.0	**2 min**	
Stage 2	**95.0**	**15 sec**	
	55.0	**15 sec**	x40
	72.0	**1 min**	
Melt curve	**95.0**	**15 sec**	
	60.0	**1 min**	
	95.0	**15 sec**	

3 Results

3.1 Mock community

As described in 2.2.3 isolated colonies, were characterized based on their morphotype. Characterization properties are shape, margin, elevation, size, texture, appearance, pigmentation and optical property. Five isolates, which represent the artificial mock community, were selected out of 66 unique isolates cultivated from 19 test toothbrushes (Table 10). The criteria for the selection was based on a factor of diversity. "TB" in the labelling ID refers to toothbrush, the first number stands for the number of the toothbrush (1-19) and the second number shows the number of the chosen isolate for the particular toothbrush.

Table 10: Colony morphology characterization of five isolates forming the mock community. Shape, **m**argin, **e**levation, **s**ize, **t**exture, **a**ppearance, **p**igmentation and **o**ptical properties are described for each isolate.

	TB 07/03	TB 17/04	TB 18/05	TB 21/06	TB 22/02
					
	Microbacterium paraoxydans	***Staphylococcus epidermidis***	***Kocuria rhizophila***	***Staphylococcus warneri***	***Rothia dentocariosa***
S	circular	circular	circular	circular	circular
M	entire	undulate	entire	entire	entire
E	umbonate	raised	convex	raised	raised
S	small	small	small	small	punctiform
T	smooth	smooth	smooth	smooth	smooth
A	shiny	shiny	shiny	shiny	shiny
P	yellow	white	yellow	cream	grey
O	opaque	opaque	opaque	opaque	translucent

TB= toothbrush; S=shape; M=margin; E=elevation; S=size; T=texture; A=appearance; P=pigmentation; O=optical property

After 16S rRNA Sanger sequencing and whole genome sequencing, the five isolates were identified. The mock community consists of a *Microbacterium paraoxydans*, *Staphylococcus epidermidis*, *Kocuria rhizophila*, *Staphylococcus warneri* and *Rothia dentocariosa.*

M. paraoxydans, a yellow pigmented, gram-positive bacterium, which is able to cause disease in fish, was first isolated from Nile tilapia in Mexico (Table 11) (Komen and Culture, 1995). *M. paraoxydans* are nowadays found in different environmental sources, such as water and soil samples and can cause bacteremia in immunocompromised patients (Chorost *et al.*, 2018). In general, *Microbacterium* species, which can cause severe systemic infections, were detected in dairy goods and in oral samples from subjects wearing dentures. However there is no evidence that the genus *Microbacterium* is part of the natural oral microbiome (Tsuzukibashi *et al.*, 2015). *K. rhizophilia*, similar to Micrococci and Staphylococci, belongs to the family *Micrococcaceae*. They usually form small whitish, round convex colonies and might turn yellowish after prolonged incubation (Table 10). They are common skin commensals in mammals, they also occur in soil, chicken meat, fresh water or food and are associated with fish health disorders (Pękala *et al.*, 2018). Genera of *Kocuria* and *Micrococcus* have been identified on human skin and in the oral cavity, but *K. rhizophilia* in particular was not identified in oral niches (Kandi *et al.*, 2016).

Staphyloccoci are commonly associated with skin and mucous membranes of mammals. *S. epidermidis* is the most common species occurring on human epithelia (Otto, 2009). The coagulase-negative *staphylococci*, called *S. warneri* is part of the human skin microflora and can cause bacteremia, infective endocarditis and other infectious diseases (Buttery *et al.*, 1997) (Schleifer and Kloos, 1975). Species of staphylococci, which have been most frequently recorded from the mouth are amongst others, *S. epidermidis* and *S. warneri* (Smith, Jackson and Bagg, 2001). An anaerobic gram-positive *coccobacillus*, named *R. dentocariosa* is part of the oral cavity microflora and can potentially lead to negative health consequences (Broeren and Peel, 1984; Zaura *et al.*, 2009; Utter et al., 2016; Eriksson, Lif Holgerson and Johansson, 2017).

More details about each isolate are summarized in Table 10. All isolates grow best at 37°C. Their GC content varies from 31% to 70%. GC content explains the percentage of guanine and cytosine molecules in DNA or RNA. Regarding oxygen demand, all isolates are aerobic, except *S. epidermidis,* which is a facultative anaerobic bacterium. Facultative anaerobic means that an

organism builds ATP by aerobic respiration if oxygen is available. If oxygen is absent it is able to switch to anaerobic respiration (Namvar, 2014).

A large part of the oral microbial community consists of a large number of anaerobic bacteria (Sultan *et al.*, 2018), which possibly also occur on toothbrushes. However, the focus for this project was on aerobic bacteria.

Table 11: Members of the mock community and its properties. ID refers to the name of the original isolate; OGT: Optimal growth temperature on TSA agar plates; GC: guanine-cytosine content on the DNA; G+: gram-positive and G-: gram-negative; oxygen refers to the o oxygen requirement for growth; source explains where the isolate originates from.

Isolates	ID	OGT [°C]	GC (%)	G+/G-	Oxygen	Source
Microbacterium paraoxydans	TB07/03	37	70.1	G+	aerobic	Fish Nile tilapia in Mexico
Staphylococcus epidermidis	TB17/04	37	31.9	G+	facultative anaerobic	Water and soil; skin flora in human
Kocuria rhizophila	TB18/05	37	70.8	G+	aerobic	Water and soil; human skin
Staphylococcus warneri	TB21/06	37	32.6	G+	aerobic	Normal human skin microflora
Rothia dentocariosa	TB22/02	37	53.8	G+	aerobic	Oral cavity

3.1.1 Growth curves of each isolate

Bacterial growth curves were assessed in order to understand the growth behavior of the five isolates and to determine if the growth performance plays a role in the abundance and the accumulation of the isolates on toothbrush bristles.

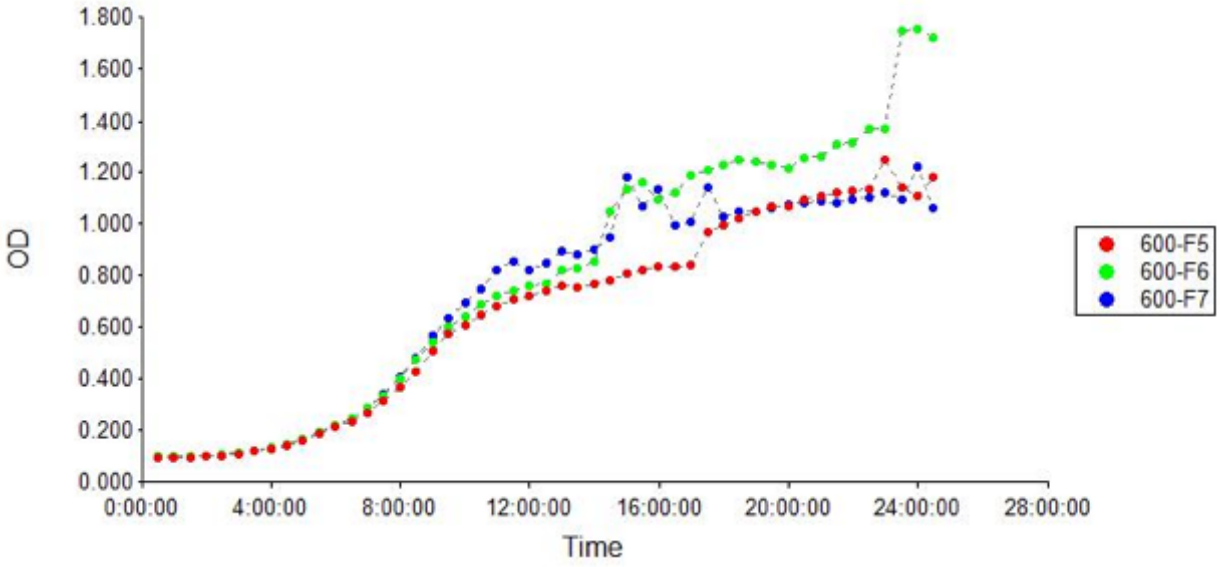


Figure 8: Growth curve for *M. paraoxydans* (TB 07/03). OD_{600} as a function of time in TSB incubated at 37°C.

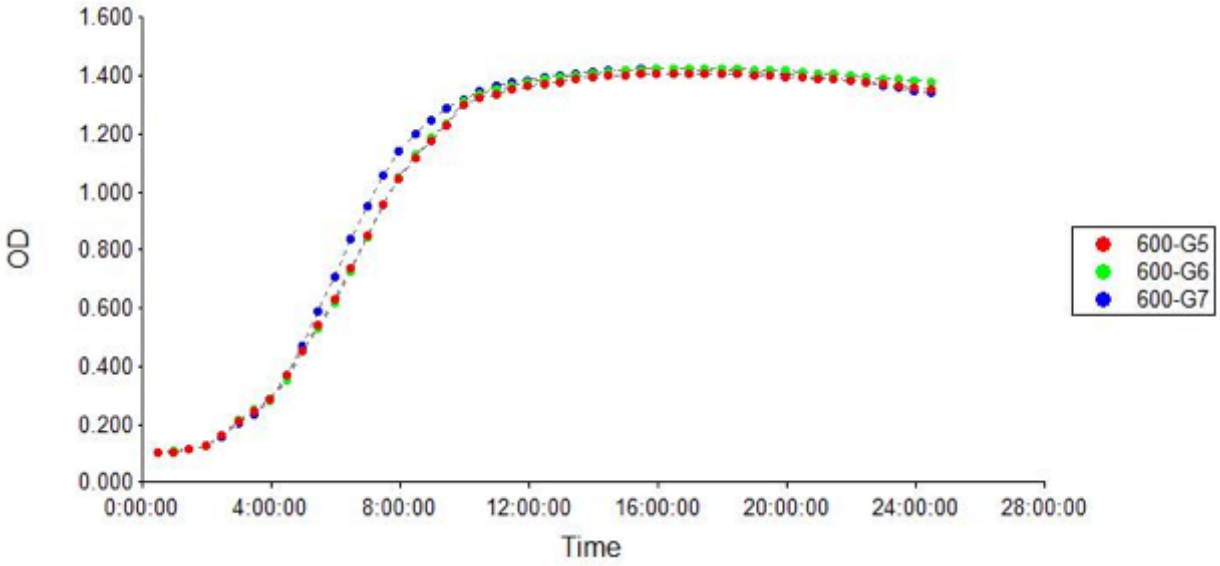


Figure 9: Growth curve for *S. epidermidis* (TB 17/04). OD_{600} as a function of time in TSB incubated at 37°C

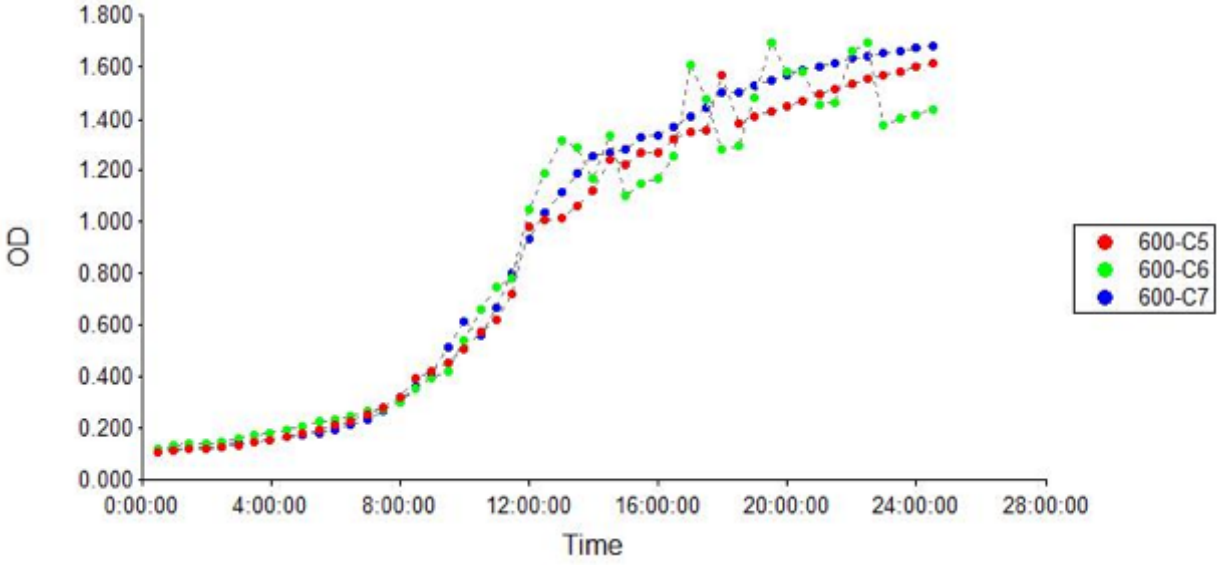


Figure 10: Growth curve for *K. rhizophila* (TB 18/05). OD_{600} as a function of time in TSB incubated at 37°C

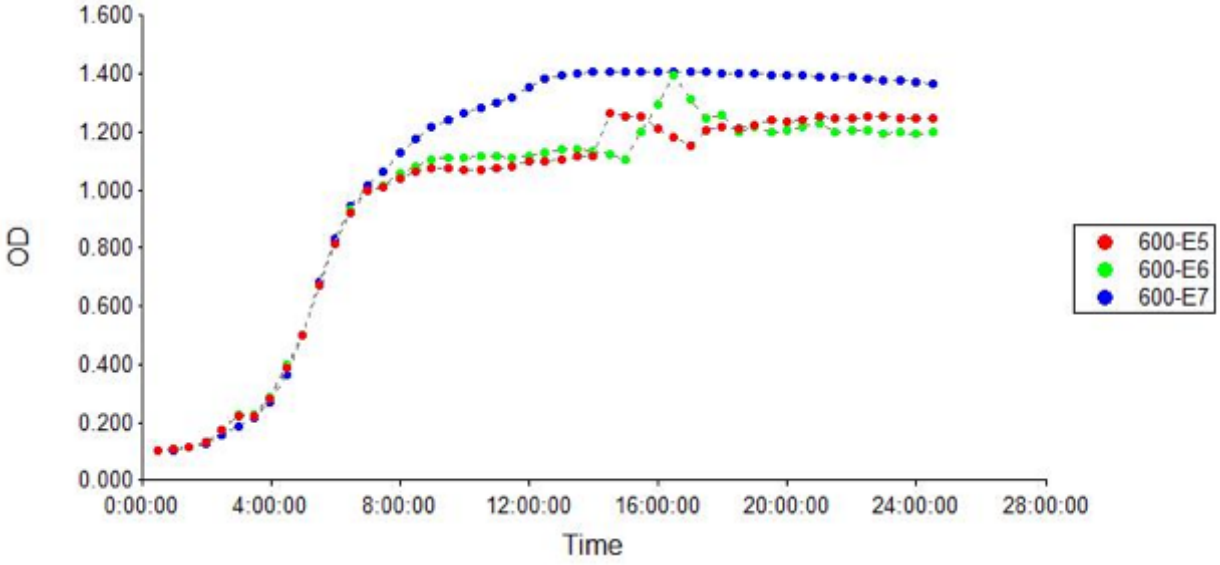


Figure 11: Growth curve for *S. warneri* (TB 21/06). OD_{600} as a function of time in TSB incubated at 37°C

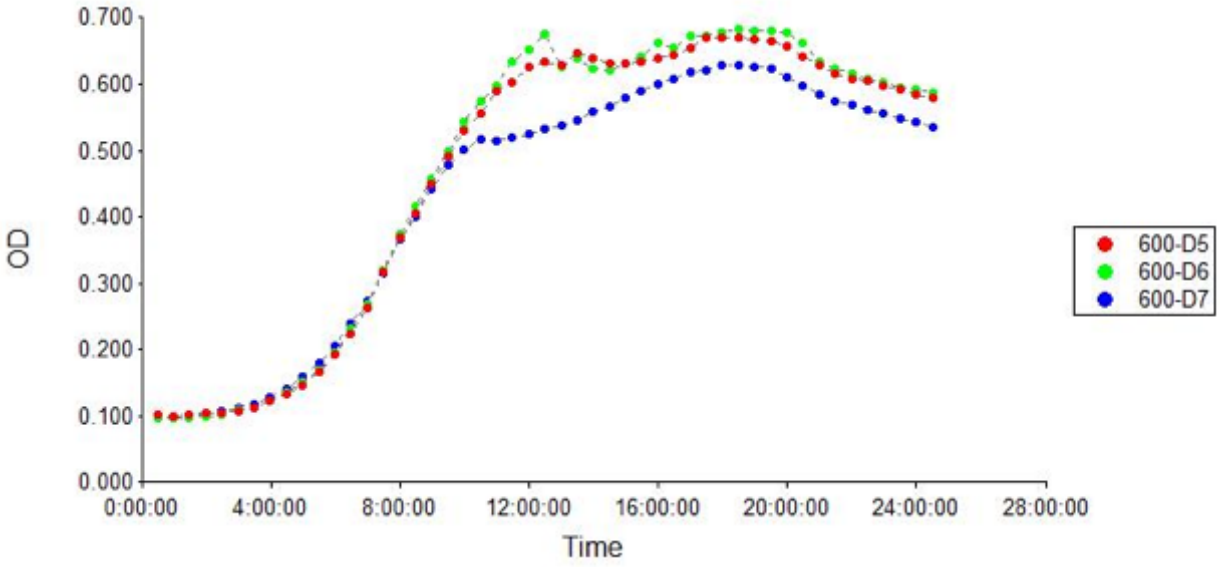


Figure 12: Growth curve for *R. dentocariosa* (TB 22/02). OD_{600} as a function of time in TSB incubated at 37°C

Growth curve experiments show that *S. epidermidis* is the fastest growing bacterium, followed by *S. warneri* (Figure 9, 11). *M. paraoxydans*, *R. dentocariosa* and *K. rhizophila* show slower growth rates compared to the other two isolates (Figure 8, 10, 12). This observation is a reflection of colony growth on agar plates. While colonies of *S. epidermidis* and *S. warneri* were clearly visible and countable colonies after 24 h incubation, the other three needed longer incubation to develop fully grown colonies.

3.2 Culture-dependent work

During the simulated brushing experiment, which lasted for six weeks, samples were collected at seven timepoints (Table 6). Samples were cultivated on TSA plates and analyzed as described in sections 2.2.1 Collection and cultivation of toothbrush samples and 2.2.2 Microbial culture analysis. Samples were plated undiluted, diluted 1:100 and 1:1000. The reason for plating dilutions was to get a number of 30-300 colonies on a plate. Of those plated dilutions, the one with 30-300 colonies was selected for representing that timepoint and was used for calculating the colony forming units (CFU) per mL. CFU/mL were calculated as described in section 2.4.2 (Table 12-14). These values were used to visualize the data and to create graphs. Error bars on

all figures denote + or – one standard deviation. In every graph CFU values were plotted on the *y*-axis. The limit of quantitation is indicated by 30 colonies, resulting as 1000 or 1.0E+03 CFU/mL. A dashed line at 1000 CFU/ml shows the limit quantitation in the graphs. Any averages less than 30 but greater than 0 are marked as 15. The meaning is that growth was visible, but it was not possible quantify. These values were included in the graphs to illustrate that isolates were growing to a certain extent. Values below the limit of quantitation are written in grey in the tables.

3.2.1 Effects of the different conditions

First, each condition was shown in one separate graph (Figure 13-15), therefore the number of inoculations of the toothbrushes were plotted on the *x*-axis. Each inoculation represents the set of five isolates next to each other, whereby every isolate is shown in different colors. An inoculation is an analog for a brushing simulation event. Conditions 4-5, using both TCS as an antimicrobial additive, are not visualized by a graph because no bacterial growth was observed for those conditions.

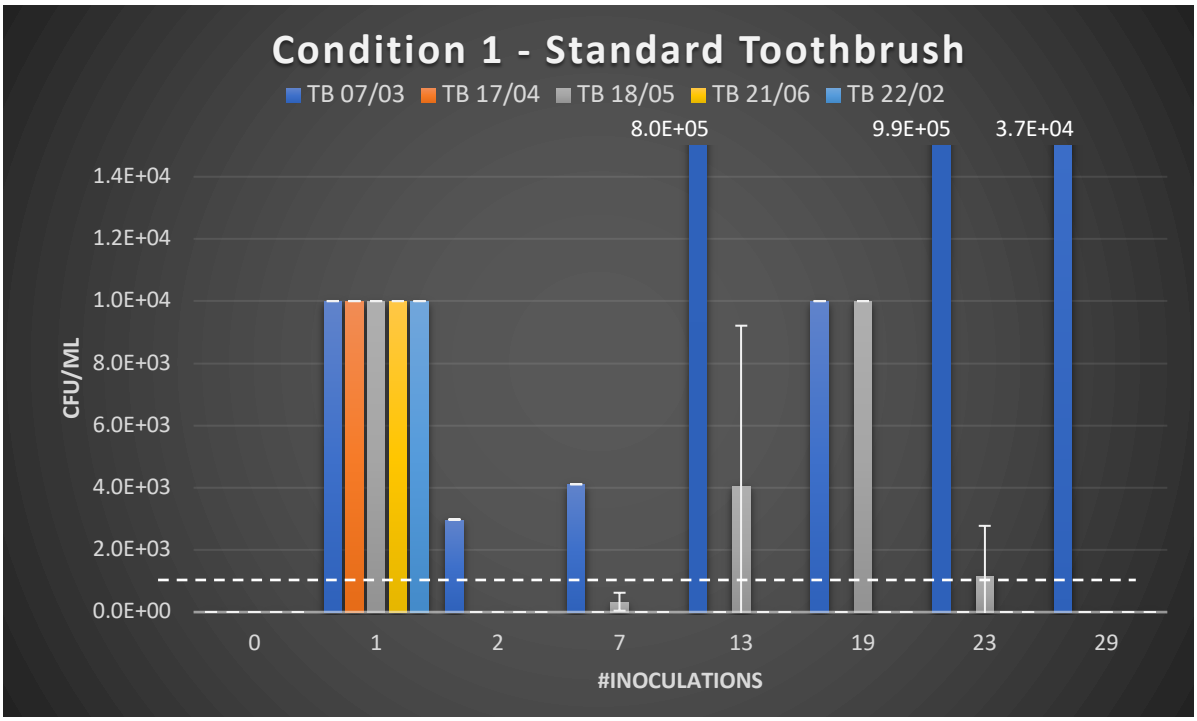


Figure 13: Development of bacterial growth for Condition 1 - standard toothbrush inoculated with mock community. Number of inoculations are shown on the x-axis. Each isolate is represented by a different color.

First, Condition 1 was compared over the whole experiment time period (Figure 13). As expected, no growth was determined on toothbrush heads for timepoint 0 because toothbrushes were sampled without any inoculation. These toothbrushes were used as a blank. For inoculation 1, toothbrushes were sampled and cultivated immediately after the first inoculation. All the other samples for later timepoints (inoculations 2-29) were sampled 24 hours after the previous inoculation and before the next inoculation.

After one inoculation all five isolates grew as a bacterial lawn. Therefore, they are counted as 300 colonies and are shown as 1.0E+04 CFU/mL in the Figure 13. After more inoculations, mainly *M. paraoxydans* (TB 07/03) and *K. rhizophila* (TB 18/05) were recoverable. Biomass concentration of *K. rhizophilia* raises from timepoint 13 to 19 and drops again on timepoint 23 to 1.2E+03 CFU/mL. *M. paraoxydans* shows CFU values above 1.0E+04 CFU/mL at time point 13, 23 and 29. Due to presentation reasons, the complete bar is not visible but instead values are demonstrated on the top of the bars. However, all values can be seen in the Tables 12-14. According to the figures and data presented in the tables, the standard deviations are particularly high, which is common with culture data.

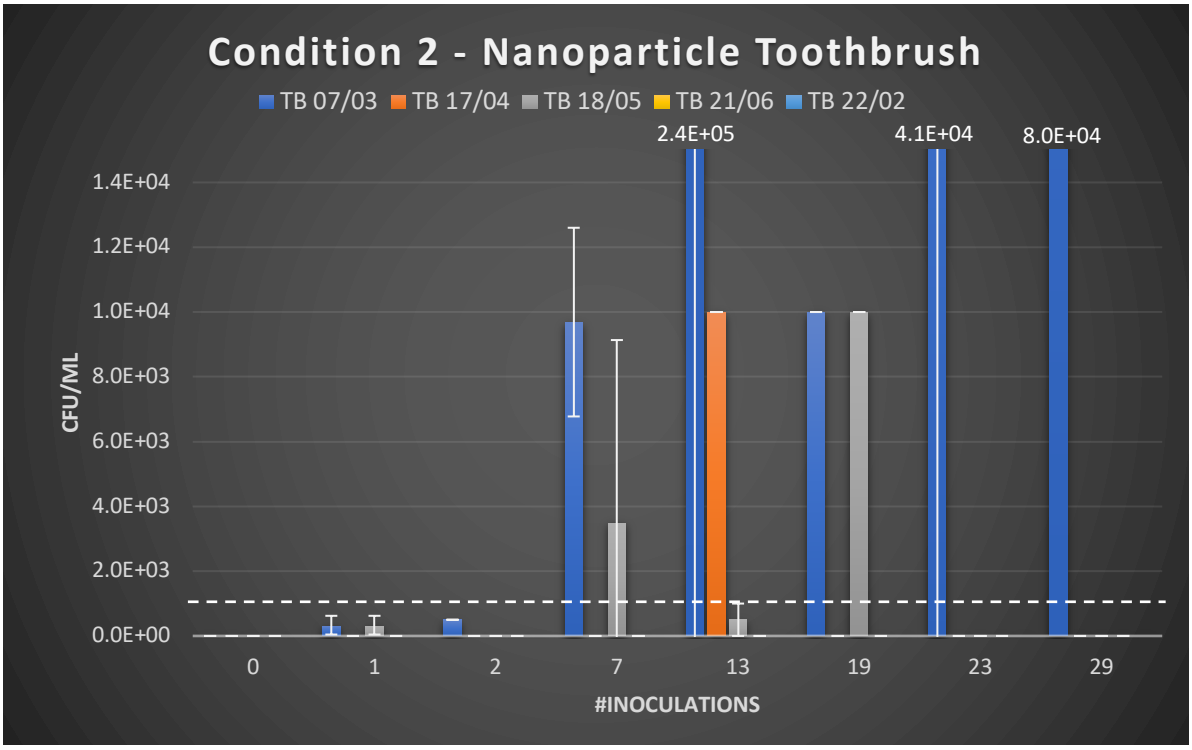


Figure 14: Development of bacterial growth for Condition 2 - nanoparticle toothbrush without toothpaste. Number of inoculations are shown on the x-axis. Each isolate is represented by a different color.

In condition 2 a nanoparticle toothbrush with coated filaments was used to examine the difference in microbial abundance between the coated (Figure 14) and the standard control brushes (Figure 13). In contrast to the standard toothbrushes in condition 1 were all five isolates grew till maximum of quantitation, almost no bacteria were collected from the nanoparticle toothbrush after the first two inoculations. Repeatedly, *M. paraoxydans* (TB 07/03) and *K. rhizophila* (TB 18/05) are primarily present and shows CFU values below the limit of quantitation after one inoculation. After two inoculations only *M. paraoxydans* (TB 07/03) shows little bacterial growth. These small values are almost not visible in the graph, but for comparison data can be seen in the aforementioned tables as well. Similar to condition 1, *M. paraoxydans* (TB 07/03) shows growth till or above maximum of quantitation in all inoculations except 0, 1, and 2. *K. rhizophila* (TB 18/05) is present at timepoint 7, 13, and at 19 to the same extent as *M. paraoxydans* (TB 07/03). As shown in the table *S. epidermidis* (TB 17/04) illustrates maximum growth only at timepoint 13 within testing nanoparticle toothbrushes.

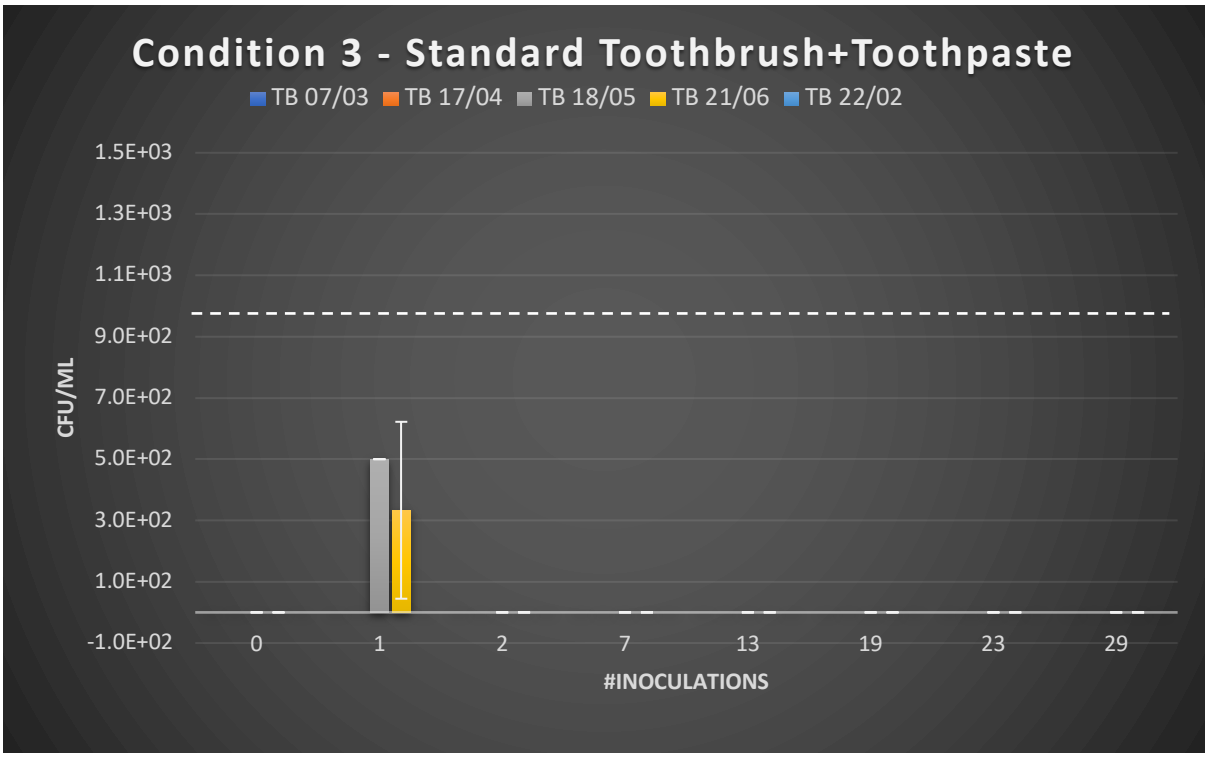


Figure 15: Development of bacterial growth for Condition 3 - standard toothbrush with toothpaste. Number of inoculations are shown on the x-axis. Each isolate is represented by a different color.

The results from condition 3 are quite different from the previous conditions. In condition 3, where a standard toothbrush was used and toothpaste was added to the mock community, *K. rhizophila* (TB 18/05) and *S. warneri* (TB 21/06) were present in a concentration below the limit of quantitation after one inoculation. *M. paraoxydans* (TB 07/03), which was highly abundant in the previous conditions was not able to grow in condition 3 at all (Figure 15). According to Figure 14, no bacteria are growing on toothbrushes after inoculation 1.

Considering all three conditions, on the *x*-axis there is no trend on CFU values as a function of number of inoculations. The blank, shown by inoculation 0, displayed no bacterial growth, which is as expected consistent for all conditions.

All tables (Tables 12-14) include calculated average CFU values and standard deviations shown in the previous graphs. The first number in the sample ID refers to the number of inoculation and the second one refers to the condition used. Conditions 4 and 5 are not included because no bacterial growth was observed for these conditions, which means CFU values are 0. Values below

the limit of quantitation (1.0E+03 CFU/mL) are written in grey and values above the maximum of quantitation are marked with a star*.

Table 12: Overview of CFU/mL values for Condition 1. First number in Sample ID refers to the number (#) of inoculations. Second number refer to the condition. AVG= average value; STD=standard deviation. Grey written values below limit of quantitation. Values labelled with a *, shows values above maximum of quantitation.

Sample ID	TB 07/03 CFU/mL	TB 17/04 CFU/mL	TB 18/05 CFU/mL	TB 21/06 CFU/mL	TB 22/02 CFU/mL	# inoculations
0-1 AVG	0	0	0	0	0	0
1-1 AVG	1.0E+04	1.0E+04	1.0E+04	1.0E+04	1.0E+04	1
2-1 AVG	3.0E+03	0	0	0	0	2
7-1 AVG	4.1E+03	0	3.3E+02	0	0	7
13-1 AVG	8.0E+05	0	4.1E+03	0	0	13
19-1 AVG	1.0E+04	0	1.0E+04	0	0	19
23-1 AVG	9.9E+05	0	1.2E+03	0	0	23
29-1 AVG	3.7E+04	0	0	0	0	29
0-1 STD	0	0	0	0	0	0
1-1 STD	0	0	0	0	0	1
2-1 STD	1.1E+03	0	0	0	0	2
7-1 STD	5.3E+03	0	2.9E+02	0	0	7
13-1 STD	2.1E+05	0	5.1E+03	0	0	13
19-1 STD	0	0	0	0	0	19
23-1 STD	4.3E+05	0	1.6E+03	0	0	23
29-1 STD	4.9E+04	0	0	0	0	29

Table 13: Overview of CFU/mL values for Condition 2.

Sample ID	TB 07/03 CFU/mL	TB 17/04 CFU/mL	TB 18/05 CFU/mL	TB 21/06 CFU/mL	TB 22/02 CFU/mL	# inoculations
0-2 AVG	0	0	0	0	0	0
1-2 AVG	3.3E+02	0	3.3E+02	0	0	1
2-2 AVG	5.0E+02	0	0	0	0	2
7-2 AVG	9.7E+03	0	3.5E+03	0	0	7
13-2 AVG	2.4E+05	1.0E+04	5.0E+02	0	0	13
19-2 AVG	1.0E+04	0	1.0E+04	0	0	19
23-2 AVG	4.1E+04	0	0	0	0	23
29-2 AVG	8.0E+04	0	0	0	0	29
0-2 STD	0	0	0	0	0	0

1-2 STD	2.9E+02	0	2.9E+02	0	0	1
2-2 STD	0	0	0	0	0	2
7-2 STD	2.9E+03	0	5.6E+03	0	0	7
13-2 STD	2.5E+05	0	5.0E+02	0	0	13
19-2 STD	0	0	0	0	0	19
23-2 STD	7.1E+04	0	0	0	0	23
29-2 STD	6.3E+04	0	0	0	0	29

Table 14: Overview of CFU/mL values for Condition 3.

Sample ID	TB 07/03 CFU/mL	TB 17/04 CFU/mL	TB 18/05 CFU/mL	TB 21/06 CFU/mL	TB 22/02 CFU/mL	# inoculations
0-3 AVG	0	0	0	0	0	0
1-3 AVG	0	0	5.00E+02	3.33E+02	0	1
2-3 AVG	0	0	0	0	0	2
7-3 AVG	0	0	0	0	0	7
13-3 AVG	0	0	0	0	0	13
19-3 AVG	0	0	0	0	0	19
23-3 AVG	0	0	0	0	0	23
29-3 AVG	0	0	0	0	0	29
0-3 STD	0	0	0	0	0	0
1-3 STD	0	0	0	2.89E+02	0	1
2-3 STD	0	0	0	0	0	2
7-3 STD	0	0	0	0	0	7
13-3 STD	0	0	0	0	0	13
19-3 STD	0	0	0	0	0	19
23-3 STD	0	0	0	0	0	23
29-3 STD	0	0	0	0	0	29

3.2.2 Effects of the number of inoculations

Following graphs (Figure 16-20) emphasize each individual isolate over time. The abbreviations in the graphs refer to condition 1-5 illustrated in Figure 1 (1=TB=toothbrush; 2=NP TB=nanoparticle toothbrush; 3=TB+TP= toothbrush + toothpaste; 4=TB+TP+TCS=toothbrush + toothpaste + triclosan; 5=NP TB+TP+TCS= nanoparticle toothbrush + toothpaste + triclosan).

According to Figure 16, the highest bacterial concentration of *M. paraoxydans* (TB 07/03) at condition 1 (TB) was observed after 23 inoculations, followed by 13 inoculations. After 19 and 29 inoculations, the number of recoverable cells decreased suddenly after a high peak in the previous sampling point. For *M. paraoxydans* (07/03) there is no trend determined as a function of number of inoculations. All isolates show a peak for TB after one inoculation, which is also conducted in Figure 13. As the variation between the values is very high, the maximum value for the vertical axis is higher in Figure 16 compared to the following graphs in order to see all values. Given that, this beforementioned trend is not visible in this chart. All CFU values visualized in the graphs are traceable in Tables 12-14.

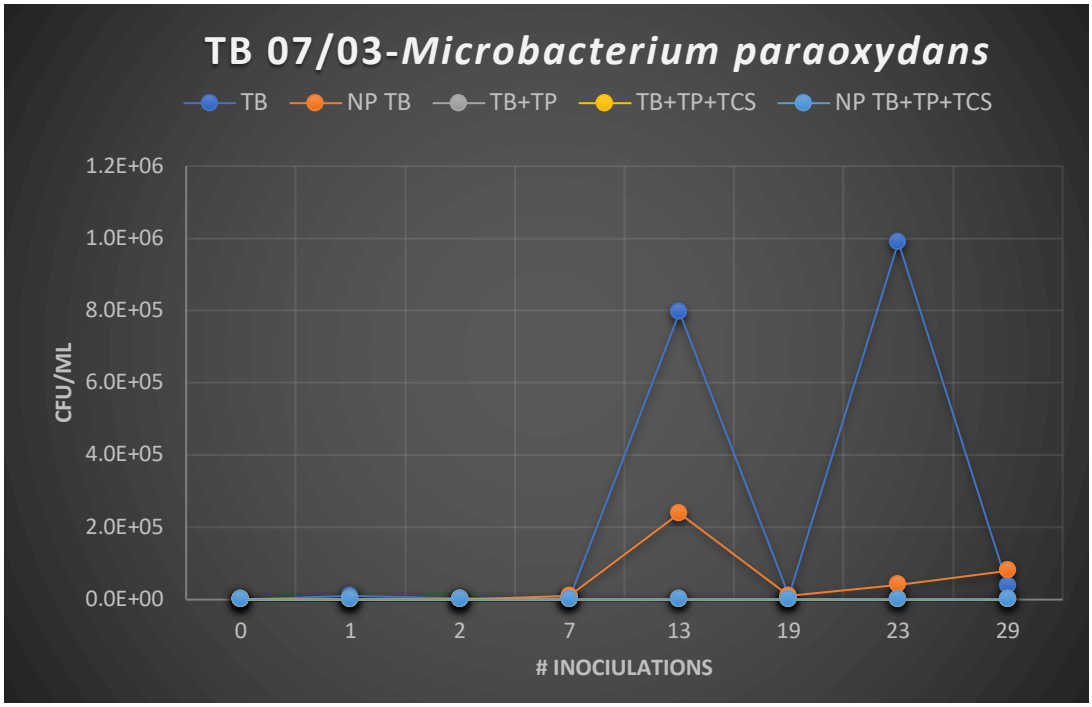


Figure 16: Development of bacterial growth of *M. paraoxydans* (TB 07/03) over time. Abbreviations refer to the five conditions. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS=nanoparticle toothbrush+ toothpaste+ triclosan

TB 17/04, which represents microbial abundance of *Staphylococcus epidermidis*, was cultivated from a standard toothbrush (TB) after one inoculation and from a nanoparticle toothbrush (NP TB) after 13 inoculations. Both peaks yielded 1.00E+04 CFU/ml (Figure 17).

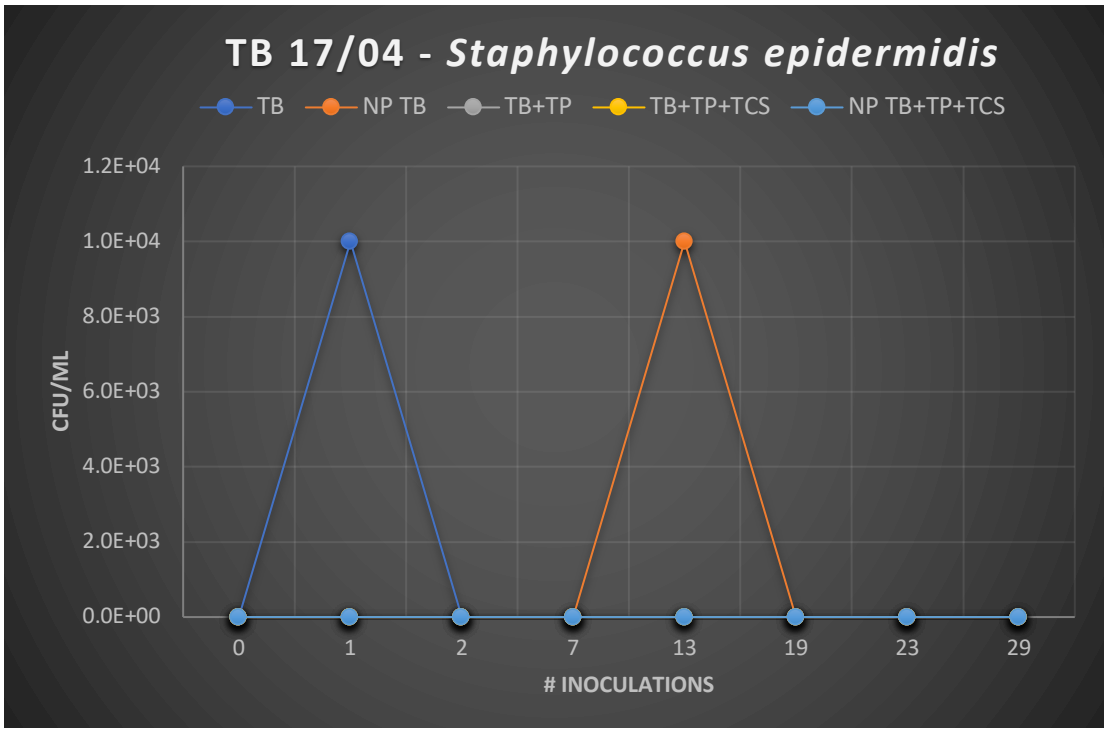


Figure 17: Development of bacterial growth of *S. epidermidis* (TB 17/04) over time. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush + toothpaste; TB+TP+TCS=toothbrush + toothpaste+ triclosan; NP TB+TP+TCS=nanoparticle toothbrush + toothpaste + triclosan.

K. rhizophila (TB 18/05) was present in the first 3 conditions (TB, NP TB, TB+TP) after the first inoculation. From Figure 17 it can be estimated that the growth of *K. rhizophila* (TB 18/05) for condition 1 (TB, represented by the dark blue line) increased from inoculation 2 to 19 and declined again from inoculation 19 to 29. This strain was identifiable after 19 inoculations both on standard (TB) and nanoparticle toothbrushes (TB NP) (Figure 18).

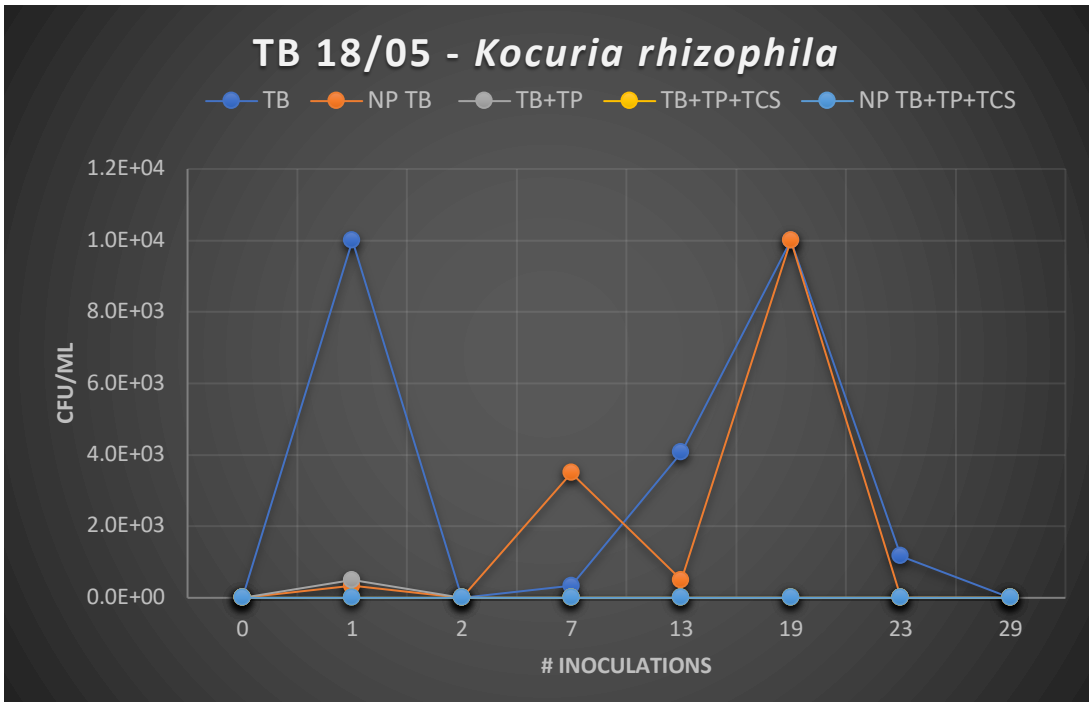


Figure 18: Development of bacterial growth of *K. rhizophila* (TB 18/05) over time. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush + toothpaste; TB+TP+TCS=toothbrush + toothpaste + triclosan; NP TB+TP+TCS=nanoparticle toothbrush + toothpaste + triclosan.

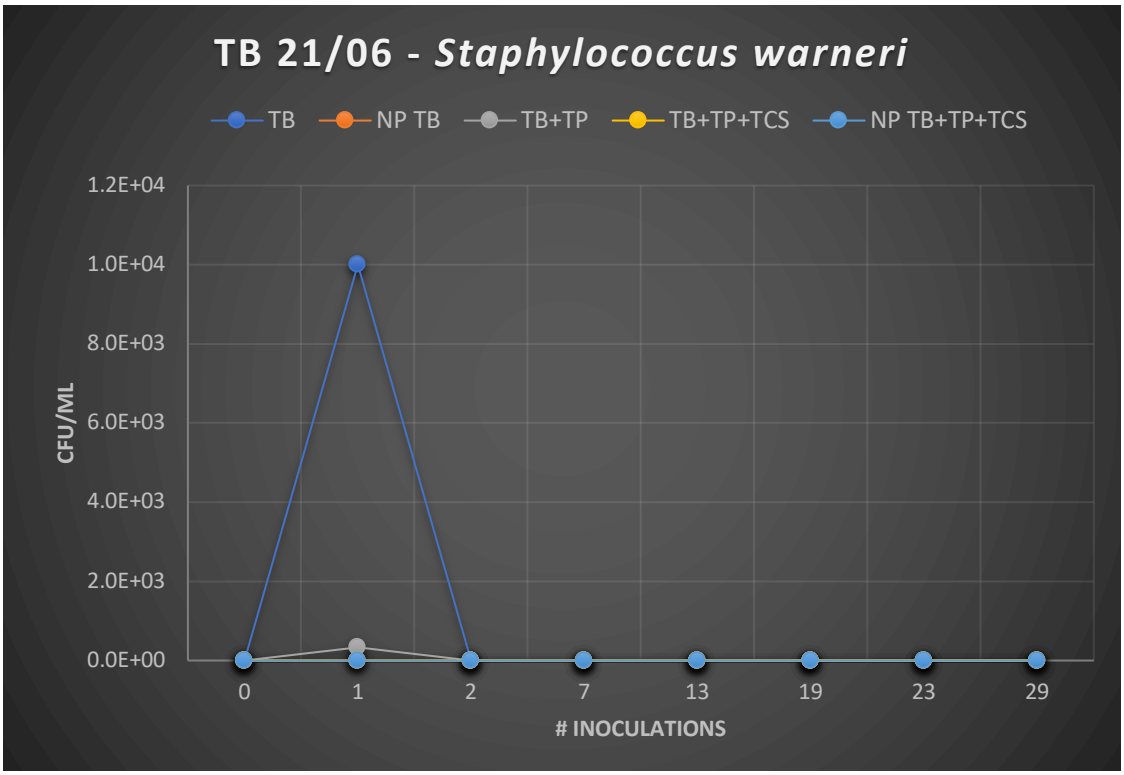


Figure 19: Development of bacterial growth of *S. warneri* (TB 21/06) over time. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP=toothbrush + toothpaste; TB+TP+TCS=toothbrush + toothpaste + triclosan; NP TB+TP+TCS=nanoparticle toothbrush + toothpaste + triclosan.

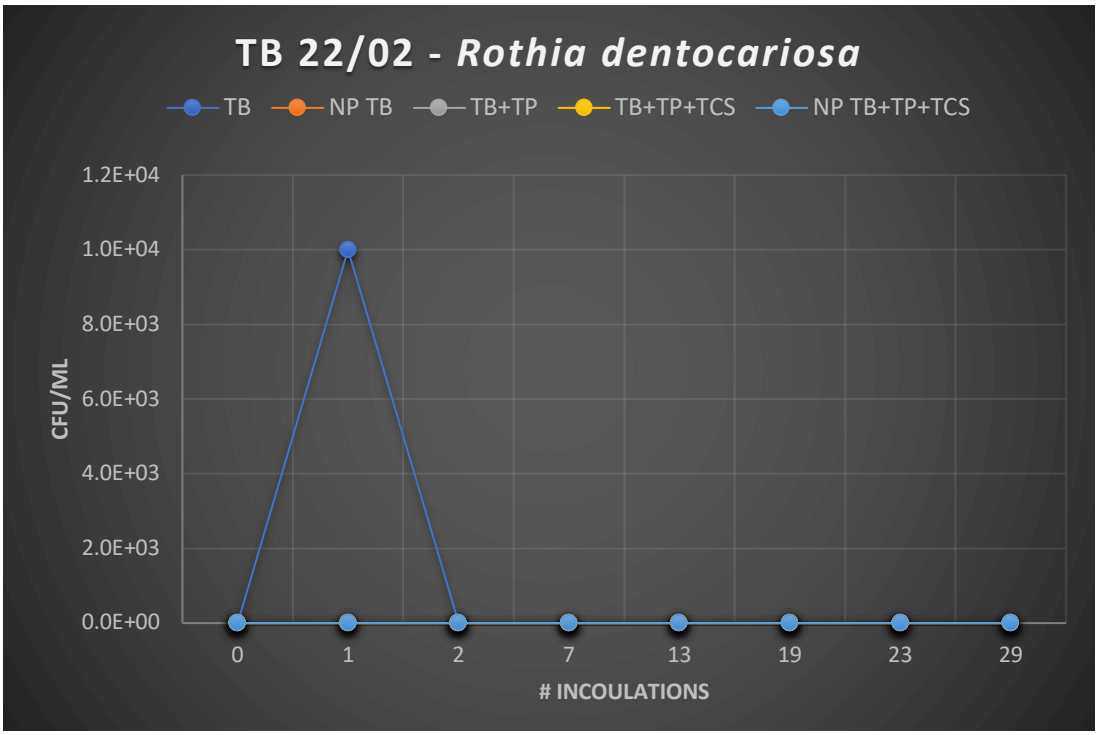


Figure 20: Development of bacterial growth of *R. dentocariosa* (TB 22/02) over time. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush + toothpaste; TB+TP+TCS=toothbrush + toothpaste + triclosan; NP TB+TP+TCS=nanoparticle toothbrush + toothpaste + triclosan.

Staphylococcus warneri (TB 21/06) was cultivable from toothbrushes, which were part of condition 1 (TB) and condition 3 (TB+TP) after one inoculation, whereas with very low abundance from toothbrushes in condition 3 (Figure 19).

As shown in Figure 20, *R. dentocariosa* (TB 22/02), the only isolate primarily associated with the oral cavity, was only cultivable immediately after the first inoculation.

In general, from the figures it is apparent that there is no steady increase or decrease in the number of CFU/mL. From all figures it may be concluded that isolates are present in condition 1 after the first inoculation. TB 07/03 and TB 18/05 are by far the most abundant isolates. Comparing standard and nanoparticle toothbrushes, nanoparticle toothbrushes show less abundance after the first two inoculations compared to standard toothbrushes.

Lastly, looking at the total biomass for each individual condition, CFU values for each condition were summed. Nanoparticle toothbrushes accumulate almost twice as many bacteria than standard toothbrushes. Condition 3 yielded 833.33 CFU/mL in total (Figure 21).

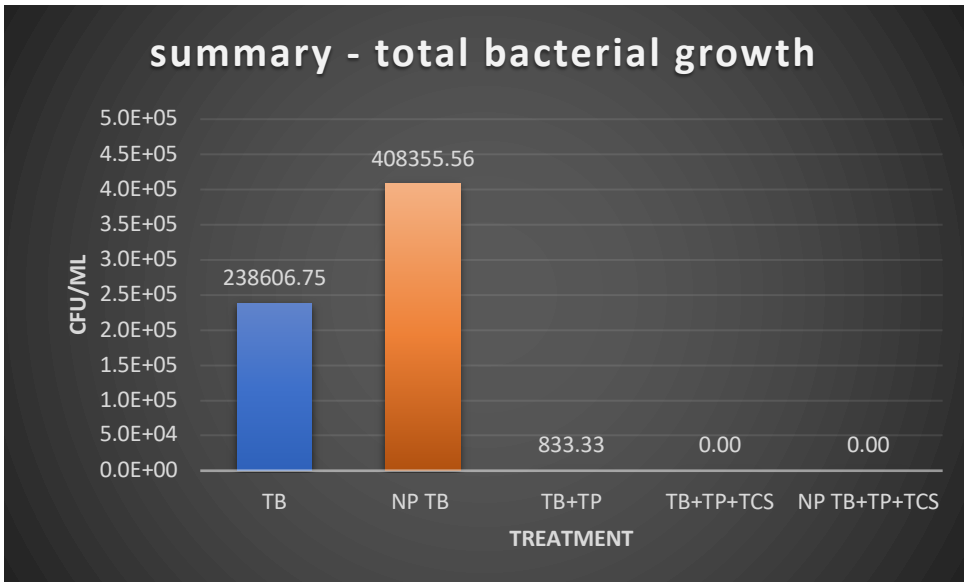


Figure 21: Summarized data for each condition. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP=toothbrush + toothpaste; TB+TP+TCS=toothbrush + toothpaste + triclosan; NP TB+TP+TCS=nanoparticle toothbrush + toothpaste + triclosan.

3.3 qPCR results

In addition to the culture dependent approach, a molecular technique was used to examine the quantity of total bacterial DNA in the samples. For cost and time reasons, only the controls and five of seven timepoints were tested (0, 1, 2, 7, 19, 29). Concentration determinations were made by comparing sample´s Ct-values to Ct-values of a standard curve. The concentration range of the standard curve covered the full range of sample 16S rRNA gene concentrations.

3.3.1 Effects of the different conditions

Data was separated in two ways, first by the number of inoculations and second by treatment/condition. 16S rRNA gene copies in ng DNA/well can be seen on the *y*-axis and the treatments, featured in different colors, are shown on the x-axis.

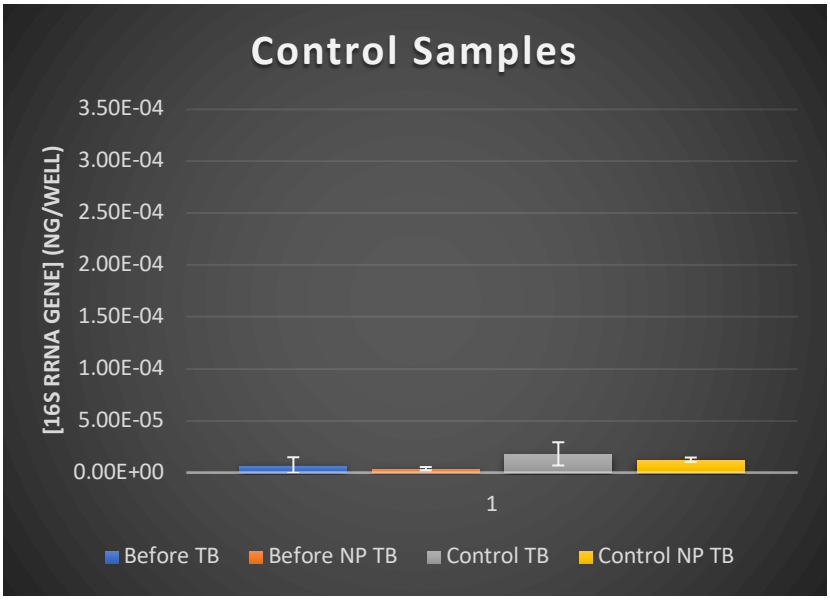


Figure 22: Total bacterial biomass of control samples. Before TB and Before NP TB refer to the blank samples, which were not inoculated but sampled right after the sterilization step; Control TB and Control NP TB show the toothbrush samples, which were not inoculated within the brushing experiment.

According to the graph, the control samples accumulated some bacteria over the course of the experiment, but still less than any of the experimental toothbrushes. As predicted sterilized blank samples, shown as “Before TB” and “Before NP TB” in Figure 22, accumulate little to no bacteria.

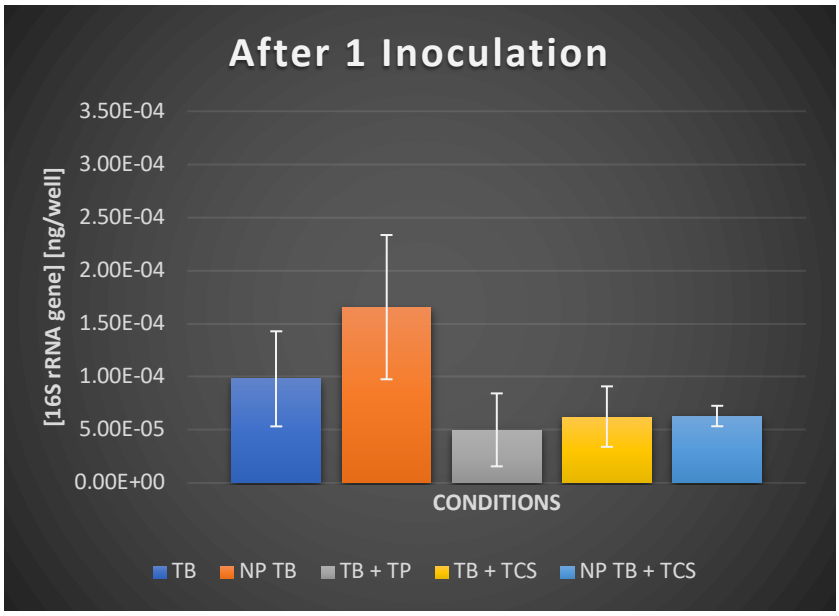


Figure 23: Total bacterial biomass after 1 inoculation. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

In general, the biomass recoverable from toothbrushes was extremely low. After one inoculation one can clearly see a higher number of bacteria stays on antimicrobial (NP TB) than on standard toothbrush (TB). This fact is determined for all other graphs, where treatments are compared to each other. In condition 3, standard toothbrushes with toothpaste showed less bacterial DNA compared to the two treatments including triclosan. As can be seen from Figure 23, the same trend occurs after two inoculations. The concentration of biomass surviving on standard toothbrushes (TB) is the smallest compared to the other treatments after 2 inoculations (Figure 24).

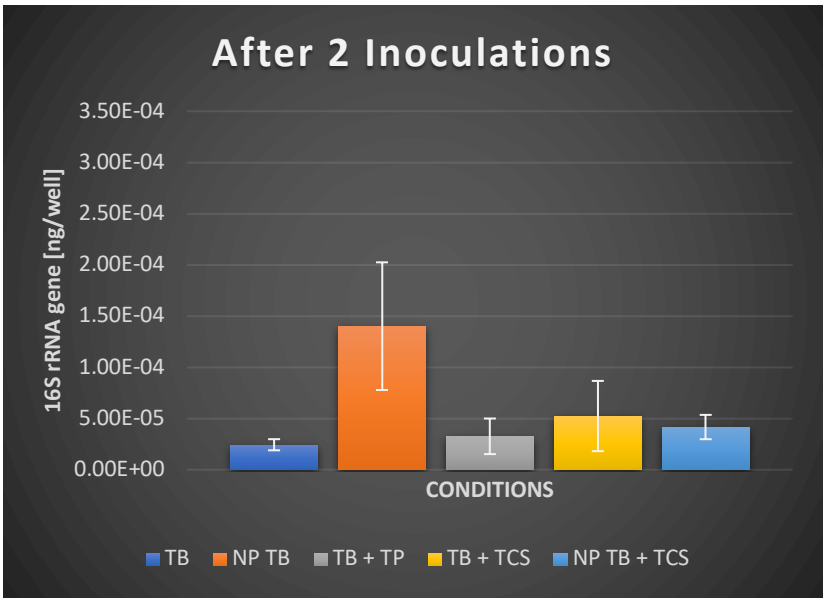


Figure 24: Total bacterial biomass after 2 inoculations. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

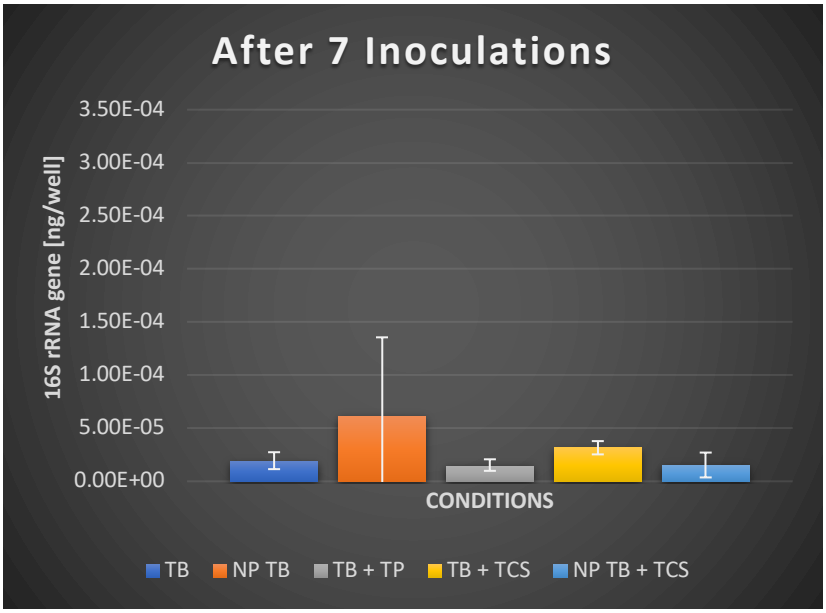


Figure 25: Total bacterial biomass after 7 inoculations. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

The graphs for inoculation 2 and 7 depict the highest bacterial accumulation on antimicrobial toothbrushes (NP TP) compared to the other conditions, very low growth on toothbrushes with toothpaste, less on toothbrushes with toothpaste and triclosan (TB+TP+TCS) and even less on antimicrobial toothbrushes with toothpaste and triclosan (NP TB+TP+TCS) (Figure 24, 25).

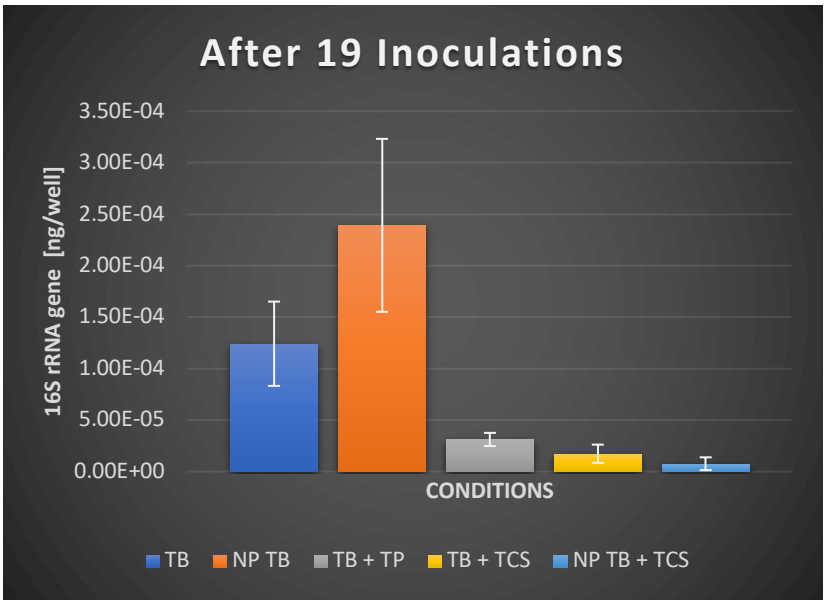


Figure 26: Total bacterial biomass after 19 inoculations. TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

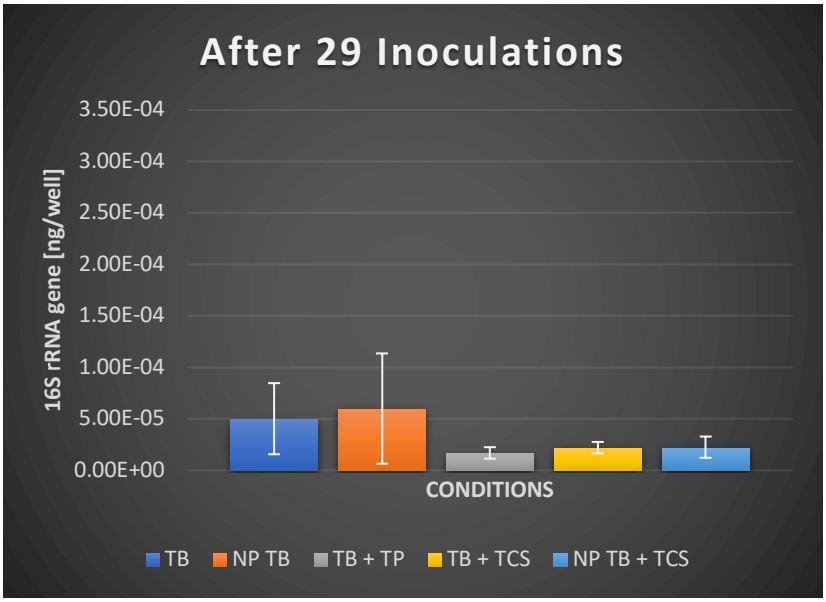


Figure 27: Total bacterial biomass after 29 inoculations. TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

After 19 and 29 inoculations, more bacteria survived on toothbrushes without added toothpaste and triclosan (TB, NP TB) than on those where toothpaste was included (Figure 26, 27). Comparing all treatments, highest number of bacteria survive on nanoparticle toothbrushes, which is consistent with the summary graph (Figure 21) for the culture work.

But as visible in all graphs the bacterial concentration is very low and the error bars are very high, which implies there is no statistical difference as a function of treatment.

3.3.2 Effects of the number of inoculations

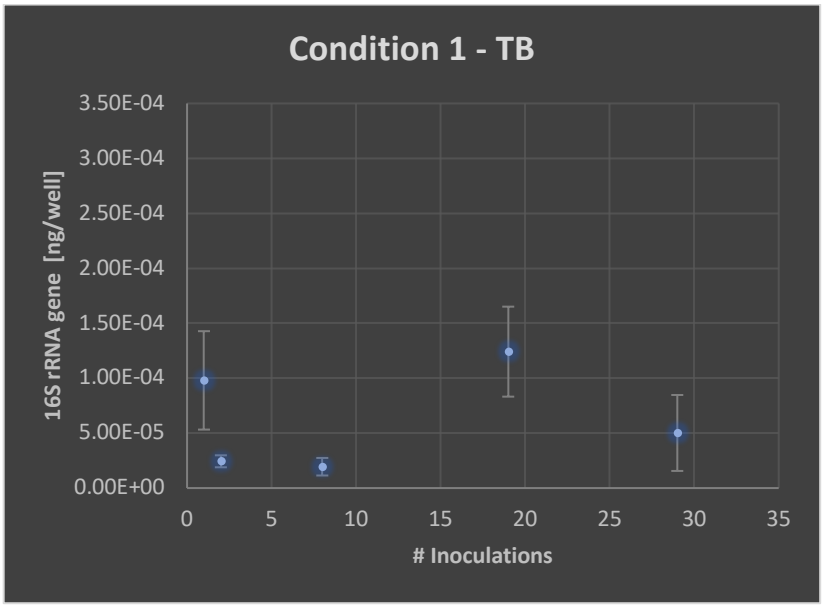


Figure 28: Total bacterial growth on a standard toothbrush over time.

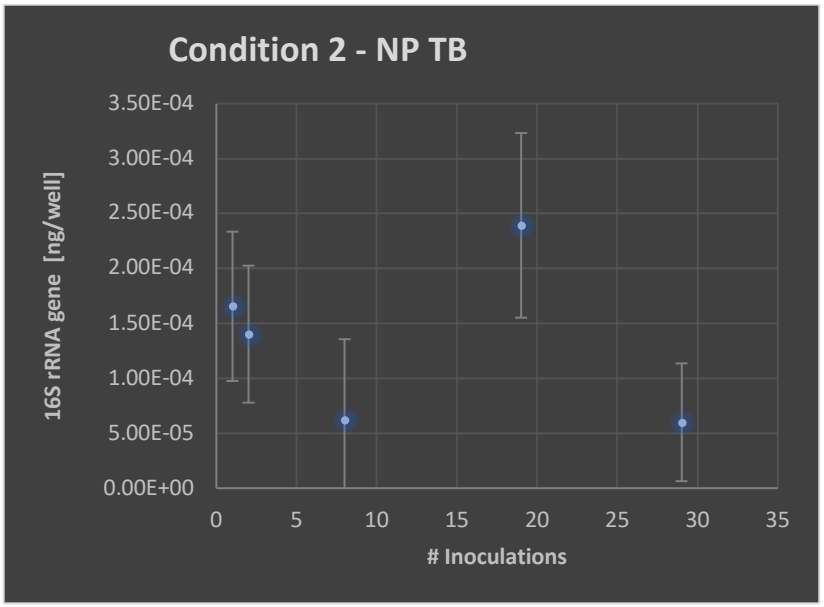


Figure 29: Total bacterial growth on a nanoparticle toothbrush over time.

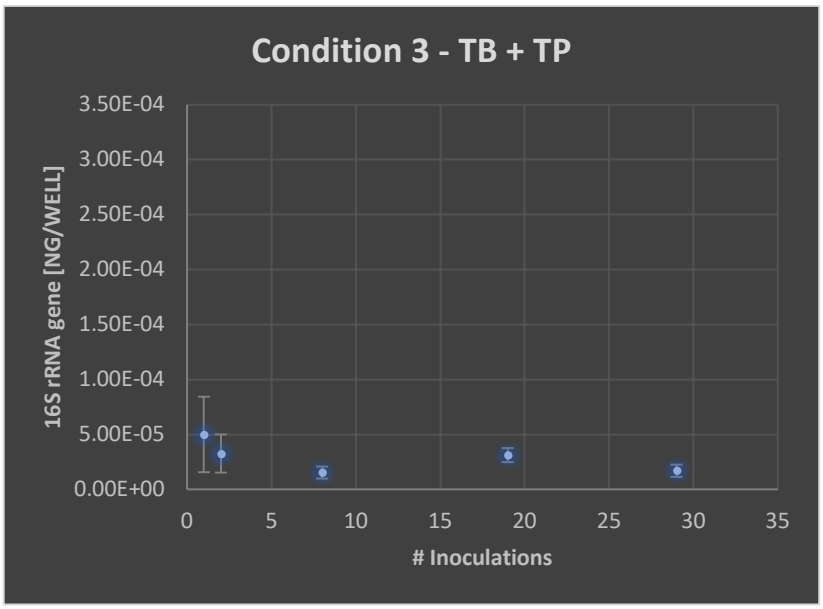


Figure 30: Total bacterial growth on a standard toothbrush with toothpaste over time.

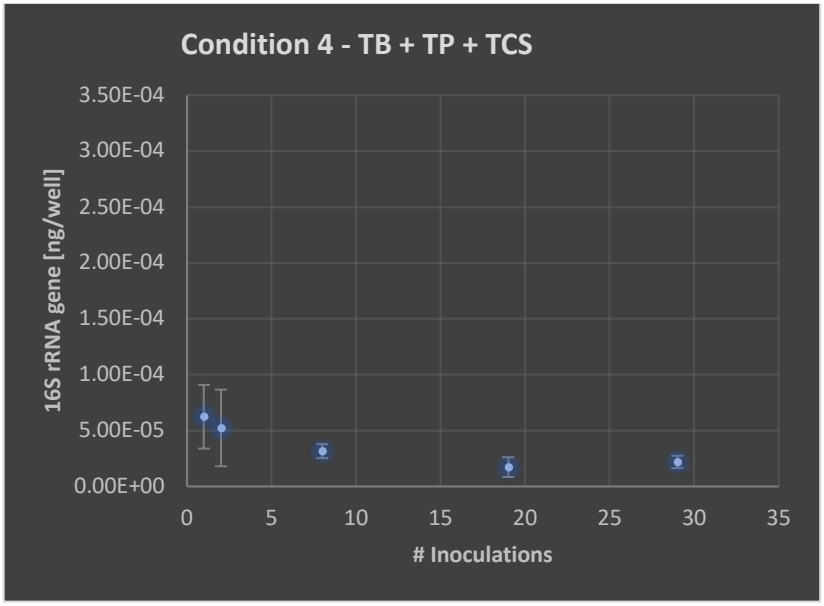


Figure 31: Total bacterial growth on a standard toothbrush with toothpaste and triclosan over time.

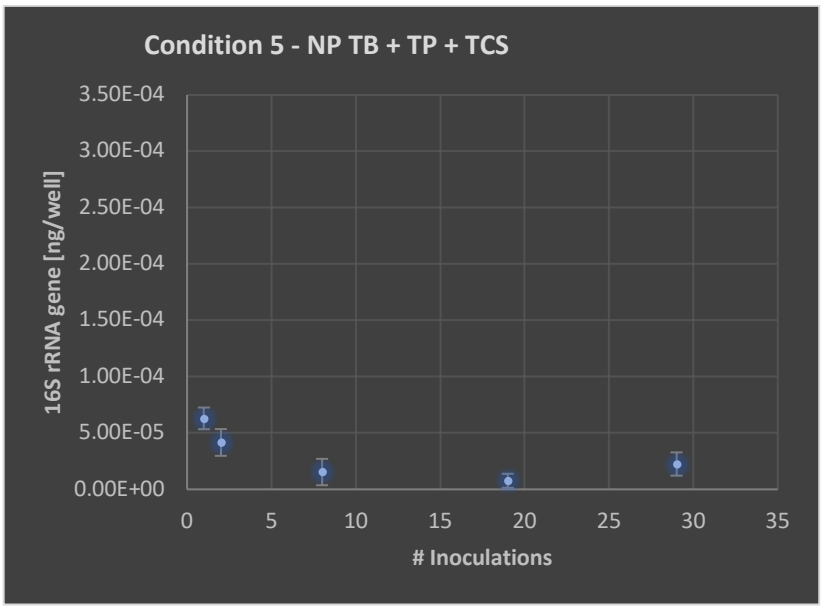


Figure 32: Total bacterial growth on a nanoparticle toothbrush with toothpaste and triclosan over time.

The same applies to the next graphs, which are comparing the number of inoculations (Figure 28 – 32). Since the standard deviations are very high and at the same time the bacterial DNA amount in the samples is so low, no statistical difference as a function of inoculation can be observed. Although comparing all graphs a trend can be observed. The bacterial concentration started decreasing steadily from the beginning to inoculation 7, but then suddenly peaking at inoculation 19 (condition 1 and 2) and then flattened out. Additionally, in conditions containing toothpaste (condition 3-5), regardless of whether it has TCS, lower bacterial concentrations were examined compared to the conditions without toothpaste (condition 1-2). According to Figure 20, the same trend can also be observed within culture work. Conversely, NP toothbrushes harbor more bacterial cells after the first two inoculations than after inoculation 7 (Figure 29), which is different compared to culture work results (Figure 14).

4 Discussion

4.1 Cultivation-based technique

4.1.1 Which isolates are surviving on toothbrushes?

One objective of this study was to examine which type of microbes survive on toothbrushes when they are exposed to different treatments. Therefore, first we had to explore which microbes are surviving on used toothbrushes to design a mock community for exposure to unused toothbrushes within a simulated brushing experiment. A diverse mix of five isolates, namely *Microbacterium paraoxydans, Staphylococcus epidermidis, Kocuria rhizophila, Staphylococcus warneri* and *Rothia dentocariosa*, was established. Results reveal that two strains, *M. paraoxydans* and *K. rhizophila* are visibly more likely to survive on toothbrushes compared to the other three. A possible cause for the higher survival rate on toothbrushes of these two isolates might be that those are more versatile and ubiquitously existing compared to the others which are on the contrary human commensals and therefore used to more steady conditions on humans. For instance, the genus *Microbacterium* has not only been detected in human oral samples but it was also isolated from several other sources like dairy products, soil and water samples. Additionally *M. paraoxydans* is known as a plant growth-promoting bacteria, which shows a wide range of environments in which they are able to live (Grandlic et al., 2008; Tsuzukibashi et al., 2015; Chorost et al., 2018).

Kocuria rhizophila also known as *Micrococcus luteus*, usually found on normal skin and mucous membranes of human and animals, also have been isolated from different environments. Although *Kocuria* spp. are non-pathogenic human commensals, they are considered potential pathogens in immunocompromised patients (M.K., S.H. and D.W., 2013; Kandi et al., 2016).

S. epidermidis, S. warneri and *R. dentocariosa* are mainly associated with humans, which suggests that they are used to constant living parameters. *S. epidermidis* is permanently colonizing human skin but also survives on catheters. The genus *Staphylococci* in general is very present in the oral cavity. A study from 2008 found that the most frequent isolated species in saliva was *S. aureus* at 46.4%, followed by *S. epidermidis* (41.1%) and other *Staphylococci* strains including *S. warneri* (Ohara-Nemoto et al., 2008; Otto, 2009).

Rothia dentocariosa is part of the normal microbiome living in the oral cavity and respiratory tract. It was first isolated from dental plaque and dental caries but it is largely non-pathogenic (Broeren and Peel, 1984).

Comparing our speculations with findings from older studies reveal a potential explanation for the increased presence of *M. paraoxydans* and *K. rhizophila.* As they have been found in several environments, they are probably more adaptable to unusual conditions like in our simulation experiment. Toothbrush plates were stored at room temperature for around 24 h between the brushing events. The temperature in the storage room varied from 23-27°C. Additionally, dryness and nutrition deficiencies while storing might be another stress factor for the isolates. The conditions of the simulation experiment and storage might explain why *S. epidermidis, S. warneri* and *R. dentocariosa* were not as able to survive on toothbrushes. These primarily human-associated strains are likely more adapted to the human body with defined and steady conditions.

Considering growth curves of the strains, there is no connection between growth behavior and abundance of the strains on bristles. *M. paraoxydans* and *K. rhizophila* cells, both which accumulated more readily than the other 3 strains on bristles, are both slow growing bacteria. However, *R. dentocariosa* grows slowly as well compared to *S. epidermidis* or *S. warneri* but did not accumulate as much biomass on bristles as M. *paraoxydans* and *K. rhizophila*. These results emphasize the variability in responses of different bacteria to the same conditions. Assessment of antimicrobial effects on a broad spectrum of species is important to better understand correlations. On top of that, five isolates are mixed together when exposing to toothbrushes which adds another aspect of complexity. Further experiments would be needed to confirm these hypothetical explanations for our findings.

4.1.2 The impact of antimicrobial additives on toothbrushes

Another purpose of this study was to get information about how the exposure of antimicrobials on toothbrushes will influence the microbial diversity on standard and nanoparticle-enabled toothbrushes.

The toothbrushes handled as a blank, which were sampled before any inoculations showed as expected no bacterial accumulation on the toothbrush heads. Antimicrobial toothbrushes, with Ag and Zn-coated filaments, display no bacteria after first and second inoculations. Bacterial concentration visibly increased after the first two inoculations, which possibly implies that nanoparticles are washed off during the brushing event and/or the washing step. One study, which reflects our findings, examined the release of silver nanoparticles from antimicrobial toothbrushes in a 24 h simulated brushing experiment. A continuous release was shown over 16h of testing and a decreased release within the final 8 h (Mackevica, Olsson and Hansen, 2017). Another study showed that antimicrobial brushes inhibit growth of *A. actinomycetemcomitans* within the first four days, but brushes lose their antimicrobial property over the time (Cadena, 2017). Based on these preliminary results compared to prior studies, we hypothesize that nanoparticles are washed off after several washing steps within our brushing experiment. Our results indicate that through using NP toothbrushes, Ag and Zn NPs are exposed to consumer as well as environment. Measuring Ag and Zn release in used slurries or in water used for washing like in our experiment would be a future step to provide better understanding of the effect of antimicrobial additives on human health and the environment.

The number of bacterial cells surviving on used nanoparticle toothbrushes over time was higher than on standard toothbrushes. This observation suggests that nanoparticle toothbrushes are not as effective against bacteria as expected. We propose that the antimicrobial bristle´s surface topography may affect bacterial cell accumulation and adhesion. The increase of accumulated cells by the used antimicrobial bristles is probably a result of surface property change due to Ag and Zn release during the experiment. Antimicrobial bristles release nanoparticles, and thus probably more surface area, where nanoparticles used to be embedded, is available for bacterial cells to adhere (Mackevica, Olsson and Hansen, 2017). This claim is further supported by the fact that standard toothbrushes without NPs accumulate bacterial cells from the beginning. Scanning Electron Microscopy of new and used nanoparticle bristles would help to understand how bristle´s surface topography influences bacterial accumulation.

4.1.3 The effect of toothpaste and TCS on microbial abundance

An additional focus was drawn to the influence of TCS in toothpaste on microbes. A previous study investigated the antibacterial activity of toothbrushes with TCS-coated filaments. TCS is known for its antibacterial impacts in toothpaste, however their results showed no difference between the bacterial concentration surviving on two different types of brushes (Efstratiou *et al.*, 2007). Another study from Quirynen et al. tells us a more effective way to eliminate microbes surviving on toothbrushes is using toothpaste. Significantly less bacteria survived when the use of toothpaste was combined with strong surfactants (Quirynen *et al.*, 2001). The results of our study agree with this study. In our experiment no growth was determined in the condition combining toothpaste and TCS.

K. rhizophila and *S. warneri* only grow after the first inoculation in condition 3 containing toothpaste without TCS, which may suggest that they are probably more robust to toothpaste´s ingredients. Toothpaste that was used in our experiment contained sodium fluoride and sodium lauryl sulfate (SLS), as a strong detergent. One study compared the effects of TCS, SLS and chlorhexidine on the salivary bacterial counts and they found that the effects of chlorhexidine were greater than SLS and TCS. Additionally SLS had even significantly greater effects than TCS (Jenkins et al., 1991). This might reflect why almost no bacteria accumulated on toothbrushes where toothpaste without TCS was used. However, it remains unclear in which step the mock community was killed. Possible steps could have been while toothpaste slurries were prepared, during the experiment in the toothpaste slurries, or during storage on the toothbrushes together with possible toothpaste residuals and other environmental stressors. Conceivably SLS breaks up the cell membranes in beforehand and thus prevent them from adhering to the bristle surface. Future experiments could be performed to test the isolates' viability in toothpaste slurries.

4.1.4 Limitations

From the colony pictures it is apparent that *K. rhizophila* is morphologically similar to both *Staphylococci* species and *M. paraoxydans*. These results are in agreement with data in the literature (Kandi et al., 2016). Having four very similar isolates together growing on agar plates made it challenging to distinguish them by eye.

4.2 Real Time Quantitative PCR (qPCR)

qPCR was performed to determine if the treatment variations and number of inoculations affect the total amount of biomass on the toothbrushes. First it was planned to quantify the concentration of each isolate in all of the samples, as it was done for the cultivation-based approach. Isolate specific primer sets were developed and optimized. Some samples were tested with the specific primer sets, but it was determined that the amount of DNA from any of the individual isolates was below the limit of quantitation of the method. It was therefore decided to use the universal 16S rRNA primer set and qPCR to quantify only total bacterial DNA. As expected, results then showed higher concentrations of total bacteria than the isolates alone, but as the figures reveal, the concentrations were still extremely low. In addition to that also the variation in biological replicates was very high.

A possible cause for the low bacterial biomass might be that bacteria never had time or the correct conditions to adhere to the toothbrush bristles. The aqueous solutions combined with the physical agitation from the brushing are likely not favorable for bacterial adsorption to the Dupont Tynex bristles from Pro-Sys.

4.3 Comparison of cultivation and molecular techniques

Although the results were not ideal due to very low bacterial concentration cultivated, we tried to interpret the preliminary data and found some relationships between the culture and molecular work results. Both approaches show that nanoparticle toothbrushes accumulate a higher number of bacteria than the standard toothbrushes. Furthermore, in conditions with added toothpaste, regardless of whether it has TCS, lower bacterial concentrations were observed compared to the conditions without toothpaste. Higher bacterial abundance on standard toothbrushes after inoculation 1 than inoculation 2 reveals another mutual observation between culture and molecular technique. One difference between culture dependent and molecular work is that qPCR results for NP toothbrushes show higher bacterial abundance in the first two timepoints than after the following sample timepoint. Results from cultivation and molecular approaches can often disagree for a number of reasons.

4.4 Limitations of the set up

The approach utilized suffered from the limitation that the Velcro tape which was used to fix the toothbrushes on the experimental plate was not ideal. As the tape got in contact with the slurries during the experiment and water during the washing step the adhesive part got wet and therefore it lost its ability to stick. Therefore, often toothbrushes fell off the toothbrush plate during the brushing event or were not sticky anymore after the washing step. The tape needed to be renewed, which increased the risk of contamination. Due to practical reasons and limited space the brushing event was not performed under sterile conditions. The cover of the plates needed to be removed to transfer the plate on the brushing set up. The washing step was not conducted under sterile conditions, which means toothbrushes were exposed to environmental microbes during this time. Even though a chance of contamination was present, all controls were negative.

The space in the incubator was limited, thus plates were stored at RT. Due to some issues with the air-conditioner in the laboratory, the temperature fluctuated between 23 and 27°C. In order to keep conditions sterile and steady, an improvement would be to perform the whole experiment in a biosafety cabinet and to store the toothbrush plates in an incubator for constant temperature.

Due to limited time it was not possible to repeat the experiment with improved conditions. However, mentioned limitations and results can be used to enhance experiments in the future. For a possible next brushing experiment, also qPCR of specific isolates could be done, to examine the abundance of each isolate. Furthermore, the abundance of antibiotic resistance genes can be quantified to get an idea about the resistome of the bacteria. That would make it possible to explore if frequent exposure of antimicrobials may enhance the dissemination of ARGs between toothbrush-associated bacteria and the oral microbiota over time (Frost *et al.*, 2005).

5 Conclusion

The results of this study help to better understand which kind of microbes are surviving on used toothbrushes and how the presence or absence of antimicrobial additives may influence the diversity of toothbrush associated microbes.

While designing a very diverse mock community representing the toothbrush microbiome, the DNA extraction protocol was optimized to determine the amount of bacterial DNA recoverable from used toothbrushes. For the experiment an apparatus which simulates the brushing effect was designed and built. With the brushing set up the mock community with artificial saliva was exposed to five different conditions. Condition 1 was a standard toothbrush without toothpaste. Condition 2 was an antimicrobial toothbrush without toothpaste. Condition 3 was a standard toothbrush with toothpaste. Condition 4 was a standard toothbrush with toothpaste and added TCS. Condition 5 was an antimicrobial toothbrush with toothpaste and added TCS.

Although a cultivation and molecular study are different and therefore complementary approaches, both techniques showed similar results. First and foremost, toothpaste inhibits bacterial retention on toothbrushes, regardless of whether it contains TCS. Another result, which was demonstrated in both methods, was that nanoparticle toothbrushes accumulate a higher number of bacterial cells than their counterparts. The data from the cultivation also reveal that the antimicrobials on coated toothbrushes were only active after the first two inoculations until they were possibly washed off from the bristles.

M. paraoxydans and *K. rhizophila* showed a higher survival rate on toothbrushes compared to the other isolates. A possible explanation might be that they are more versatile and ubiquitously existing compared to the others, which are on the contrary human commensals and used to more steady conditions on humans.

Due to limited time, the simulated brushing experiment was performed once for a period of six weeks. Since results did not show significant differences between the different conditions and between number of treatments, we were unable to corroborate the above hypotheses.

Nevertheless, we were able to develop an innovative brushing apparatus, which can be used for future experiments. Moreover, we demonstrated that antimicrobial additives on toothbrushes are unnecessary to prevent the buildup of microbial biomass on toothbrushes over time. Toothpaste, instead, has a much greater inhibiting effect on toothbrush associated bacteria. These results can be used to improve and to better design future brushing experiments.

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7 List of figures

8 List of tables

Table 1: Colony morphology characterization of 102 isolates. OP= optical property. Frequency refers to how often this specific colony type occurs, f.e. isolate Nr.1 occurs seven times and represents therefore the most abundant morphotype. Frequency: 1 stands for a unique isolate, which appears just once within 102 characterized isolates. Single letters represent the first letter