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**The influence of antimicrobial additives in toothpastes and toothbrushes on toothbrush-associated microbes**

Final Report for

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## Abstract

*Background:* Antimicrobials used in hygiene products are supporting the dissemination of multi-resistant pathogens and are one of the main causes for an imbalanced human microbiome.

*Purpose:* The purpose of this study was to determine how antimicrobials like Zinc and Silver on toothbrushes, or like Triclosan in toothpaste, will affect the growth and diversity of toothbrush-associated microbes.

Material and Methods: To investigate that, a mock community out of 5 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 5 different conditions on a built set up, that simulates toothbrushing. In 2 conditions the effect on the mock community of a nanoparticle and a standard toothbrush were compared. In another two, both types of toothbrushes with toothpaste in absence and presence of TCS were tested. The fifth condition was a standard toothbrush with toothpaste without TCS. At 7 different time points, the head of toothbrushes were cut, cultivated and processed for non-selective culturing techniques, followed by identification and quantification of the 5 isolates. Additionally, qPCR was used to quantify the total bacterial mass.

*Results:* Cultivation and molecular approach showed that toothpaste significantly inhibits the microbial growth, regardless of whether it contains triclosan. Culture results suggest that nanoparticles on antimicrobials toothbrushes are only active in the beginning of the usage. Both approaches showed that antimicrobial toothbrushes accumulate a higher number of bacteria than standard toothbrushes. Comparing the presence of all isolates in the culture dependent approach *M. paraoxydans* and *K. rhizophila* showed a higher survival rate compared to the other isolates.

*Discussion:* Further studies are needed to proof the said speculations. The results suggest that toothpaste has a greater inhibiting effect on microbes surviving on toothbrushes than antimicrobial coated toothbrushes.

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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, microbes are an essential part of Earth´s biota (Karoum *et al.*, 1980). Humans, as a 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, referred to as the human microbiota, outnumber our eukaryotic cells by a ratio of around 1.3:1 . Due to differences in nutrition, age, body size, and environment the human commensal microbiota varies from person to person. (Weiman and Torres, 2014; Sender, Fuchs and Milo, 2016).

The human microbiota is abundant and very diverse in species and strains. However, this was not always the case, over several thousand years of coevolution, strong selective stimuli have decreased the microbial diversity in the human body. For example, of more than 50 identified bacterial phyla, only 4 (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria) are found on human mucosal and cutaneous tissues.

The microbial composition of the microbiota can change in response to several variables such as lifestyle, nutrition, host behavior, use of drugs and hygiene. These microbial communities can also be influenced by human genetic and other environmental factors (Blaser and Falkow, 2009) (Blaser, 2018) (Hadrich, 2018). For example, in industrialized countries people will usually spend 90% of their time inside of buildings. Through increased urbanization and packed offices, 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 microbial ecosystem (Berg, Mahnert and Moissl-Eichinger, 2014). To support the diversity in an indoor environment and to influence the composition of the human commensal microbiota we can also open our windows instead of turning on the air conditioning (Hanski *et al.*, 2012).

A high beneficial microbial diversity is of high importance because it can prevent infections (Pham and Lawley, 2014). The alteration to the microbiota might be a reason for the spread of

pathogenic strains and thus the rise of several common diseases that are linked to inflammation is promoted.

Currently, hygiene, cleaning methods and medicine are some of the main causes for an imbalanced microbial consortium in humans. Current research suggests that these might be the primary factors that lead to global microbial loss, which might also explain the deficiency of the ancestral microbes. Chemicals used in modern cleaning and hygiene products are supporting the spread of multi-resistant pathogens while at the same time they cause a decrease in the beneficial microbes in the indoor microbiome (Berg, Mahnert and Moissl-Eichinger, 2014) (Blaser, 2018) (Von Hertzen, Hanski and Haahtela, 2011) (Blaser and Falkow, 2009). Microbes confronted with the previously mentioned 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 emerge.

The spread of antibiotic resistance genes (ARG's) is enhanced by frequent exposure to antimicrobials in hygiene products (Mora *et al.*, 2016; Han *et al.*, 2017). Especially toothbrushes are known to be not only a source and but also a sink for antimicrobial chemicals (Han *et al.*, 2017). Common antimicrobials used in oral hygiene products include triclosan and stannous fluoride in toothpastes, chlorhexidine in mouthwashes, and silver and sometimes zinc nanoparticles on toothbrushes (bristle coating and elastomer infusion). 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. Microbial culture-dependent analyses has demonstrated that the use of triclosan-containing toothpaste lessened 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 silver nanoparticles have been reported to decontaminate toothbrushes and reduce total bacterial genome counts and bacterial species 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 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). Undoubtedly, routine tooth-brushing and other oral care practices (e.g., flossing, mouthwash) are essential for maintaining oral hygiene and a healthy microbiota.

Microbial interactions have important implications for ARG dissemination. Mobile genetic element (MGE)-mediated horizontal gene transfer (HGT) is largely responsible for the spreading 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.

Although 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 and how the presence or absence of antimicrobial additives like triclosan (TCS), in toothpaste and silver (Ag) and zinc (Zn) on toothbrushes may influence the diversity of toothbrush associated

microbes. TCS a lipid synthesis-inhibiting compound, is one of these antimicrobials widely applied in oral care products. It is used as an antimicrobial additive 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 that, it is an imperative to determine the impact of antimicrobials on the diversity of the microbiota that is found on used toothbrushes. It is expected that the absence of antimicrobials will preserve the natural microbial diversity. Furthermore, we will show that the use of antimicrobials will lead to a decrease of healthy oral biomass as well as a reduction of general microbial diversity.

This project is based on 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 out of 5 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. As a toothpaste we used a TCS-free mint fluoride gel toothpaste also from the same brand. Five different toothpaste-slurry-conditions were tested in triplicates and were established to test the given hypothesis. All of them included artificial saliva and the mixture of 5 isolates representing the mock community. Within the first two conditions, the effects of a standard and a toothpaste with nanoparticle coated bristles are compared to each other. The third condition show 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 is examined. As controls a set of standard and antimicrobial toothbrushes were included into the simulation experiment without exposing them to anything. These controls show that there is no risk of contamination while performing

the experiment. Another control group consisting out of both types of toothbrushes were treated the same way as the experimental ones just inoculated with natural toothpaste (Himalaya, Texas, USA). 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 toothbrushes were inoculated 29 times. The toothbrushes were sampled, cultivated and analysed at 8 different timepoints.

This study used whole genome sequencing to identify 5 selected members of the mock community, culture-based approaches to characterize the microbial diversity at the sampling points and a real-time quantitative PCR (qPCR) to quantify abundance of the total bacterial concentrations.

# 2 Material and Methods

## 2.1 Design of mock community

Microbial biomass recovered from used toothbrushes was cultivated. Using the resulting isolates, a mock community of 5 isolates was selected. Taxonomy was identified by amplifying and sequencing the 16S rRNA gene of each isolate. Also, whole genome sequencing was performed to elucidate the presence of potential antimicrobial genes of the mock community associated with the toothbrush microbiome. The mock community was combined with artificial saliva and was exposed to unused toothbrushes in the absence and presence of antimicrobial additives in toothpaste and on toothbrushes within a simulated brushing experiment.

### 2.1.1 Collection and cultivation of toothbrush samples

The mock community was established by culturing 19 used electric or manual toothbrushes. These were collected from and with the permission of faculty, staff, and students associated with the department of Civil and Environmental Engineering in the McCormick School of Engineering and Applied Science at Northwestern University.

Toothbrushes were cultivated within 1 week after they were last used. Toothbrushes were stored in a zip 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. 2 toothbrushes were handled

differently. For those 2, 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. Because of practical reasons and because microbes stuck on the head itself, it was decided to use whole toothbrush-head for detaching remaining microbes.

The head of the toothbrush was cut off with a pair of sheers, 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 the toothbrush consisted of a metal component inside, which was often 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 saline buffer solution (PBS buffer) containing filter-sterilized (Whatman 0.2u µm nylon syringe filters) 0.01% Tween (Tween 80, Fisher Scientific, USA) (PBS+Tween). 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.

### 2.1.2 Microbial culture analysis

30.0 µl of the PBS buffer solution containing eluted microbes which were dedicated for culture were plated on tryptic soy agar (TSA). The undiluted sample and 3 dilutions ($10^{-2}$, $10^{-4}$, $10^{-5}$) were plated to ensure isolated growth on TSA plates. Plates were incubated at 25°C and 37°C for at least 48h until growing colonies were observed. Plates were stored in the fridge at 4°C until colony morphology was characterized.

### 2.1.3 Bacterial colony morphology characterization of isolates

Isolated colonies were classified based on their morphotype. Characterization properties are shape, margin, elevation, size, texture, appearance, pigmentation, optical property and effect on growth medium (figure 1).

Colony shape includes form, margin, elevation and the size of the bacterial colony. Form of the bacterial colony refers to the shape of the colony. These forms represent the most common colony shapes, which are found e.g. circular, rhizoid, irregular, filamentous or spindle. The margin of a bacterial colony may be an important characteristic in identifying an organism. Common margins are entire, undulate, lobate, curled, rhizoid or filamentous. The elevation describes a side view of a colony and the most common descriptions can be flat, raised, convex, pulvinate or umbonate (having a knobby protuberance). The size can also be a very useful characteristic for identification. The diameter of the colony can be measured or described in relative terms such as punctiform, small, moderate or large. The texture and consistency of the colony can be described with smooth or rough. The appearance of the colony surface is either glistening (shiny) or dull (the opposite of glistening). Pigmentation or color of the colonies can either be described with nonpigmented or pigmented. Nonpigmented means e.g. cream, tan or white. Some bacteria produce pigment when they grow on the agar plates e.g. *Pseudomonas aeruginosa* produces green pigment. The color of colonies can also be purple, red and yellow depending on the media colonies are growing on. The last characterization point is the optical property which describes the opacity of the bacterial colony. The colony can be observed as opaque (not transparent or clear), translucent (almost clear, you can see light shining distortedly through the colony-like looking through frosted glass) or transparent (clear).

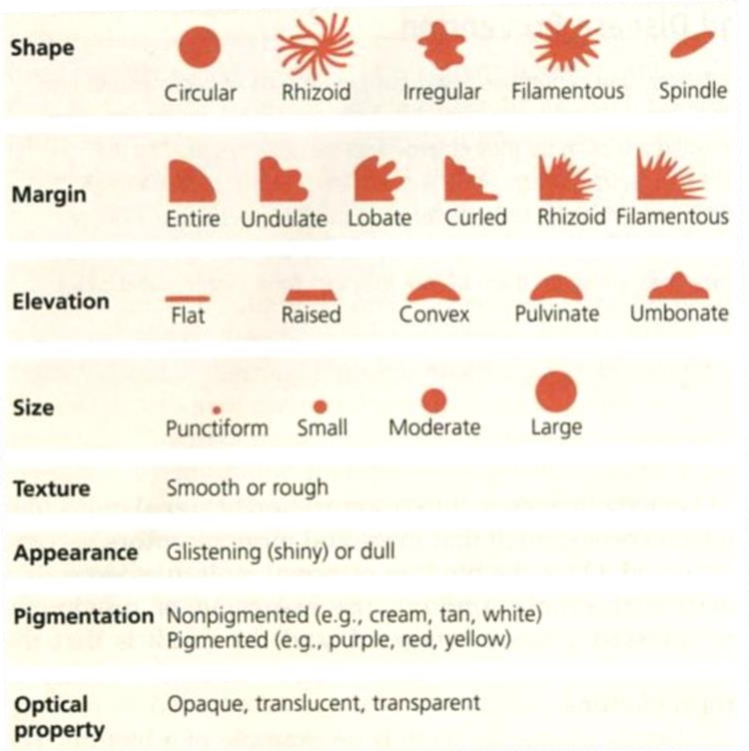


**Figure 1: Characteristics of bacterial colonies.** Shape, margin, elevation, size, texture, appearance, pigmentation and optical properties are described by several terms.

### 2.1.4 Picking isolates

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

Every picked isolates was streaked out on a sterile TSA agar plate to assure 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 were growing before on TSA agar plates. If the solution was turbid on the following day, 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. Final concentration of glycerol stock solution was 25% (Fisher scientific, USA). Samples were gently vortexed and stored at -80°C.

### 2.1.5 Selection of mock community strains

In total 102 isolates were found on 19 examined toothbrushes. First of all, a preselection of 10 isolates was made. Therefore, isolates were clustered into the 5 most common groups showing the same morphotype within one group using R (Coghlan, 2015). The most common group contains 7 isolates with the same observed morphotype and the less common one shows 4 isolates with the same morphotype. 1 isolate of each of those groups was selected. 5 more were chosen randomly from the other isolates which did not belong to a group. This selection was made aiming a community representing the toothbrush microbiome as diverse as possible.

## 2.2 Identification of the mock community

### 2.2.1 DNA extraction of the mock isolates

To obtain genomic DNA from test toothbrushes, QIAGEN DNAeasy Kit (Quiagen, Venlo, Netherlands) was used. Therefore 6 mL TSB broth was inoculated with each isolate and incubated overnight in a shaking incubator. Optimal growth temperature can either be 25°C or 37°C, depending on each individual isolate. 1 mL of the active growing culture was used for imaging

the sample in order to avoid yeast in samples. 5 mL of the ONC was centrifuged for 3 min at 4200 rpm. The supernatant was discarded, and the resulting pellet was resuspended in 250 µL bead-tube solution. Following steps were performed according to the manufacturer´s protocol. DNA quality was determined using Synergy HTX Multi-Mode Reader (Biotek, Winooski, VT). DNA concentration was measured by QuantiT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA).

### 2.2.2 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 experiment (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 or 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. Quiagen DNAeasy Kit, Phenol/Ethanol extraction (Köchl, Niederstätter and Parson, 2005), Fecal DNA Extraction (Evans *et al.*, 2014), MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI), were the DNA extraction methods, which were tested out and compared to each other. 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.

### 2.2.3 DNA quantification

Biotek Synergy™ HTX Multi-Mode Microplate Reader and Take3™ Multi-Volume Plate (Biotek, Winooski, VT) were used for quantifying DNA concentrations of the samples. Take3™ Multi-Volume Plate was cleaned with ethanol and DI-water on a Kimwipe (Kimtech Science, USA). The plate has 16 microspots arranged as 8 rows and 2 columns. Samples were always pipetted in duplicates. 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 the bottom-side through the spots. Then absorbance measurements were performed and recorded with the specific program for measuring Nucleic Acid concentrations on the Biotek Synergy™ HTX Multi-Mode Microplate Reader.

**DNA quantification with Quant-iT™ PicoGreen® Assay**

DNA concentration determined by fluorimetry, was assured by using Quant-iT™ 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 due to low concentrated samples. Assay was performed according to the manufacturer´s manual.

### 2.2.4 16S rRNA PCR

To identify the unknown bacterial isolates, Sanger sequencing of the 16S rRNA gene was processed. Therefore, PCR was performed to amplify 16S rRNA gene by using universal bacterial primer.

The PCR was performed using the thermocycler (Mastercycler nexus, Eppendorf, Hamburg, Germany).

**Table 1: PCR reaction mix** (reaction volume 50 µL)

| Volume [µL] | Reagent |
|---|---|
| **30.75** | $H_2O$ (nuclease free PCR water) |
| **10.0** | 5x buffer |
| **4.0** | MgCl2 |
| **1.0** | dNTP |
| **1.0** | 27f-Primer [10µM] |
| **1.0** | 1391r-Primer [10µM] |
| **0.25** | Go-Tag Flexi DNA (Promega) |
| **2.0** | DNA |

**Table 2: List of universal primers**

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

**Table 3: Thermocycler program for PCR**

| | Temperature [°C] | Time | |
|---|---|---|---|
| Initial DNA denaturation | 95 | 2:00 | |
| DNA denaturation | 95 | 1:00 | |
| Primer annealing | 55 | 0:45 | x25 |
| 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).

### 2.2.5 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. Samples were packed in an envelope and sent to collaborating laboratory for performing 16S Sanger Sequencing.

### 2.2.6 DNA preparation for Whole genome Sequencing

Out of 10 isolates, 5 were finally selected for representing the mock community. Whole genome sequencing (WGS) was performed with 5 chosen isolates to ensure the results of 16S Sanger Sequencing.

Isolates were grown for 24-48 hours in TSB media at 25°C while continuous shaking. Cells were then pelleted by centrifugation at 10,000 g for 3 minutes. DNA was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI), following manufacturer's instructions. DNA quality was measured by 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 MiSeq run. Prepared samples were sent to collaborating lab for MiSeq run.

### 2.2.7 Growth curve

TSA medium was inoculated with the isolates and culture was grown overnight at 37°C to saturation. The inoculums were diluted 1:100 so that the initial $OD_{600}$ was between 0.05 and 0.1. 200 µL of each dilution was filled into a 96-well plate in triplicates. The plate reader manufacturer´s instructions for programming were followed. Temperature was set at 37°C. The plate was shaken continuously to keep the culture aerated, to ensure adequate oxygen for growth and that possibly resulted sediments on the bottom of the plate were mixed before each reading. Reading interval was set on 15 min, which means $OD_{600}$ was measured every 15 min. The total run time of the growth curve assay was 24h that all cultures were able to reach a plateau in OD. 200 µL TSA 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, 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.

### 2.2.8 Kirby-Bauer disk diffusion susceptibility test

For the disk diffusion test the Kirby Bauer method was applied (A. W. BAUER , M.D. , W. M . M . KIRBY , M.D. , J . C . SHERRIS , M.D. , AXU M. TURCK, 1966). Tetracycline, Colistin, Gentamicin, Chloramphenicol, Vancomycin and Ampicillin (BD BBL™ Sensi-Disc™, USA) was tested out.

### 2.2.9 Screening on different agar plates

All 5 isolates were screened on agar like 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). Therefore, a sterile wooden stick was soaked into each ONC. 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.

## 2.3 Simulated brushing experiment

### 2.3.1 Toothbrush and toothpaste

It was decided to use toothbrushes and toothpaste from the brand PRO-SYS, which were purchased on Amazon. Two types of manual toothbrushes were used in the experiment. One type is a standard toothbrush with a compact-size head and white soft DuPont™ Tynex bristles. As a reason for comparison and to explore the impact of chemicals on bristles an antimicrobial toothbrush 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. Said nanoparticles are known to eliminate bacterial growth on the toothbrush itself. Both toothbrushes have a rubber-free handle to keep the toothbrush clean and gunk-free. 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. This one 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.

### 2.3.2 Design of the experimental brushing setup

The goal was to design an apparatus in order to simulate brushing realistic yet practical under a laboratory setting. Most important conditions for designing the set up was that the bristles were soaked in the slurries completely, that the bristles were touching the glas petri dishes evenly during the simulation experiment and that the 2 different types of toothbrushes were located apart from each other.

On square cutted plates out of ¼” clear acrylic sheets (6.35 mm - Mc Master-Carr Supply Company, Illinois, USA) 5 sets of 3 toothbrushes are fixed on the plate with Velcro™ tape. The 5 sets of toothbrushes, representing the 5 different conditions in the experiment, will be explained later in more detail (figure 5, 7).

As a basis for the set up a rotating shaker (Thermo Scientific, USA) was used for simulating the brushing movement/rotation. A box out of the same beforementioned material was built around the shaker, so that plates with the toothbrushes on it, can be fixed on the box with bolts and nuts. Brushing was simulated using the aid of the rotation of the shaker (figure 2, 3).

Parts of the set-up 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. Set up parts were stick together with an appropriate glue for acrylic plates (Scigrip, smarter adhesive solutions, North Carolina, USA).

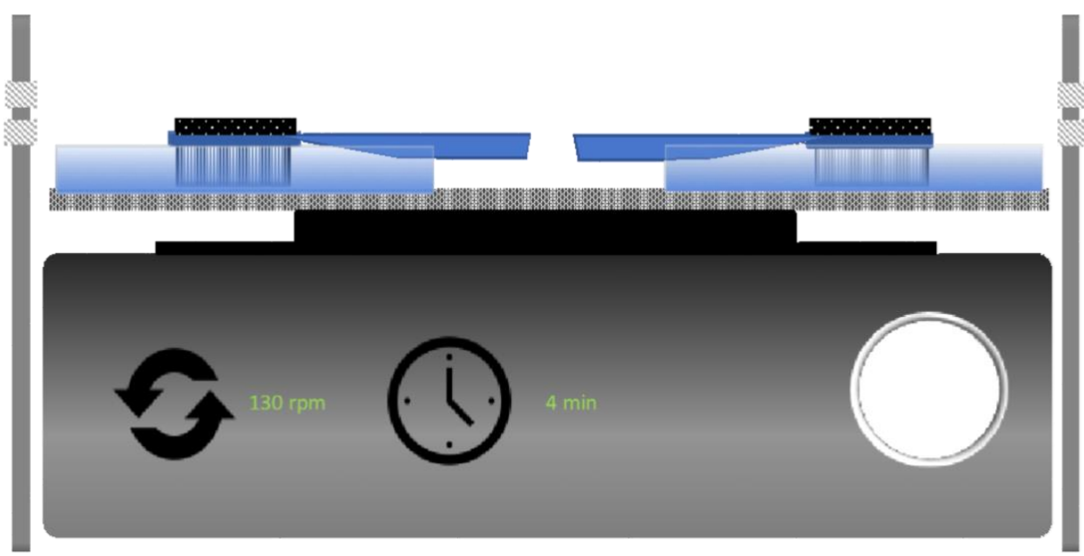


**Figure 2 Simulation set up** with toothbrush plate connected through bolts and screwed on the top of the box. Bristles soaked in the slurries filled in the petri dishes.

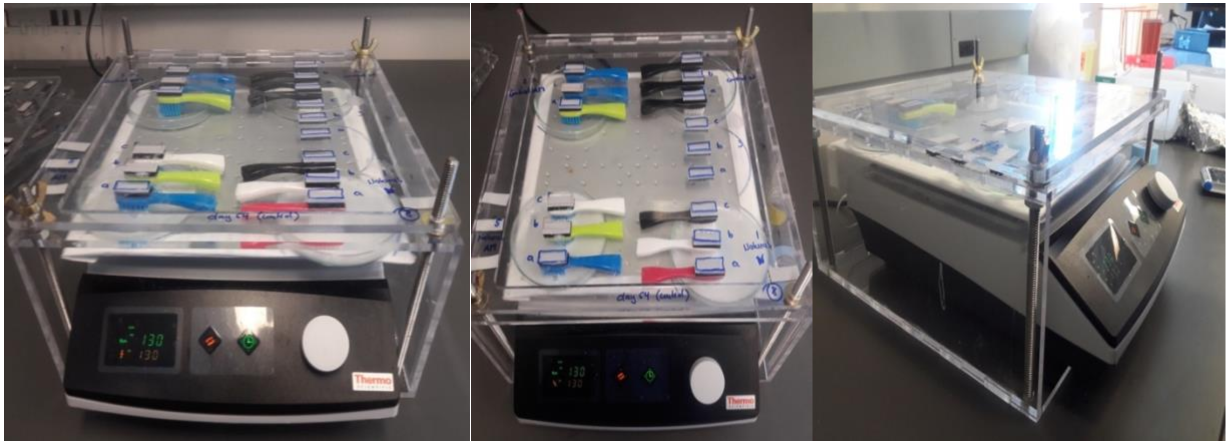

**Figure 3: Pictures of the experimental brushing set up**

### 2.3.3 Preparing toothbrush plates

First the autoclave pans, which were used as a cover for the toothbrush plates, and a scissor, a role of Velcro tape (Velcro, United Kingdom) and a plier, 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 unpacked in the biosafety cabinet and the toothbrush handle was cut off with a plier. The plier was wiped clean with 70% ethanol between every new toothbrush. Antimicrobial toothbrushes and standard toothbrushes without nanoparticles were collected separately in 2 autoclave pans and sterilized for 45 min as well. The Velcro tape was cut into 1 cm pieces. The protective foil was carefully removed from the sticky part, and the Velcro was taped on the back of the toothbrush head and on

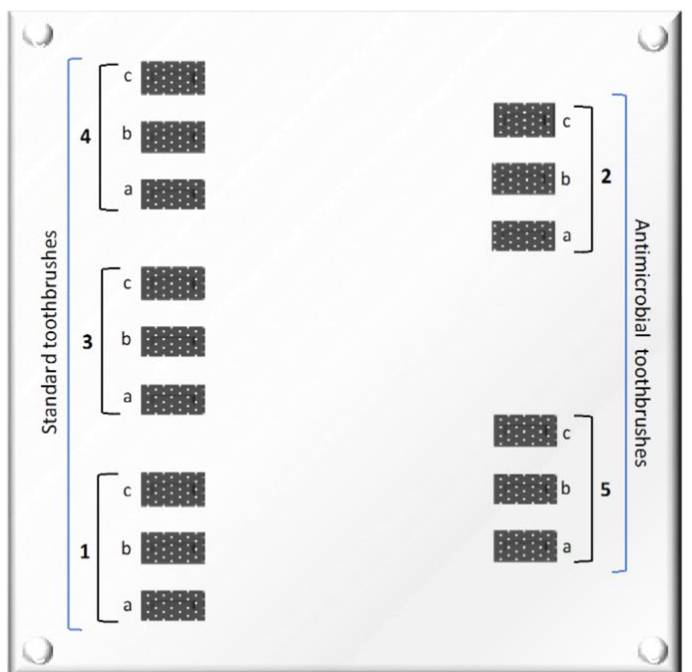


**Figure 4: Acrylic plate** without toothbrushes but with Velcro tape, it will be upside down on the shaker (mirrored on the left side).

the predefined position on the acrylic plate (figure 4). Toothbrushes were finally taped on the Velcro stick on the acrylic plate.

Toothbrushes were inoculated in triplicates in one specific slurry. Therefore 3 toothbrushes were placed next to each other with a distance of 1 cm. Next set of 3 was placed next to it with a distance of 2 cm to the other set of 3. On one side of the acrylic plate 3 standard toothbrush sets were positioned. The other side consisted of 2 sets of 3 toothbrushes, which represented the antimicrobial toothbrushes. The goal was to keep the 2 different types of toothbrushes apart, therefore they were separately placed on the acrylic plates (figure 5). Eventually one experimental toothbrush plate consisted of 9 standard and 6 antimicrobial toothbrushes. One of this toothbrush plates was prepared for each sample point (0, 1, 2, 7, 13, 19, 23, 29). Control plates consisted of 6 standard and 6 antimicrobial toothbrushes (figure 6, table 5).

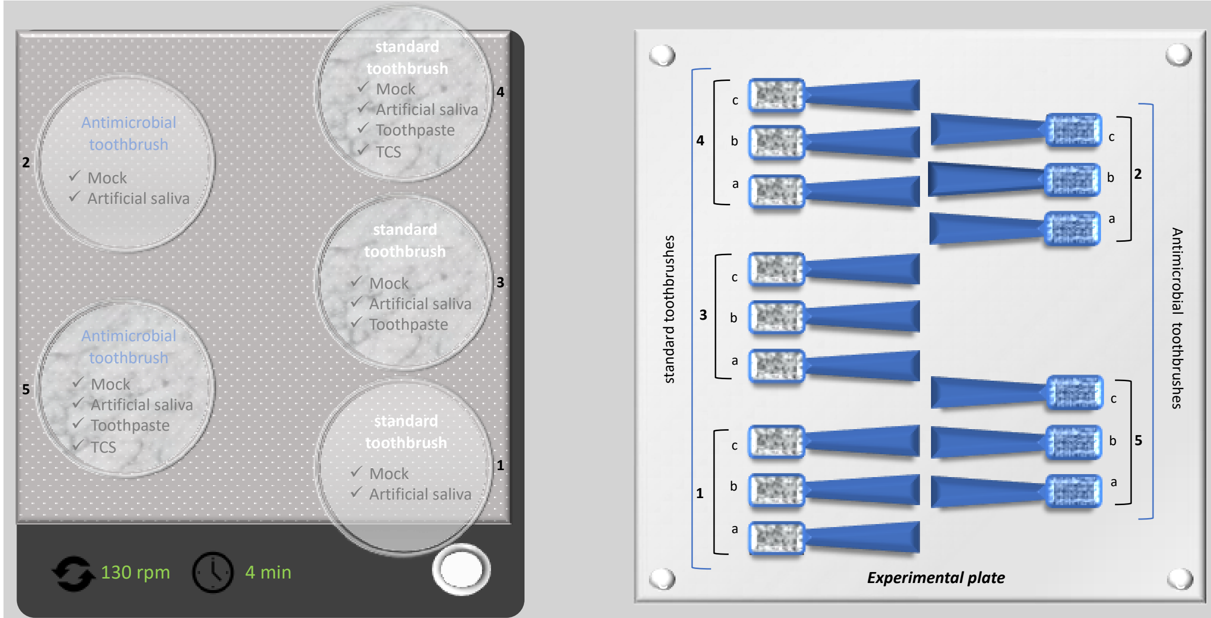


**Figure 5: Experimental plate and slurry composition.** Left: Shaker with glas petri dishes filled with slurries and individual type of toothbrush will be brushing in the slurry. 1= condition 1, standard toothbrush, mock community in artificial saliva; 2= condition 2= antimicrobial toothbrush, mock 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. Left side consists of standard toothbrushes and right side of antimicrobial toothbrushes. During brushing the acrylic plate was upside down on the shaker, which means number of toothbrush set belonged to the same number next to the petri dishes filled with appropriate slurries.

Table 4: Conditions representing slurry compositions for experimental plates

| Experimental plates | Condition 1 | Condition 2 | Condition 3 | Condition 4 | Condition 5 |
|---|---|---|---|---|---|
| Toothbrush | Standard | Antimicrobial | Standard | Standard | Antimicrobial |
| Mock | Yes | Yes | Yes | Yes | Yes |
| Artificial saliva | Yes | Yes | Yes | Yes | Yes |
| Toothpaste | no | no | Yes | Yes | Yes |
| TCS | no | No | no | yes | yes |

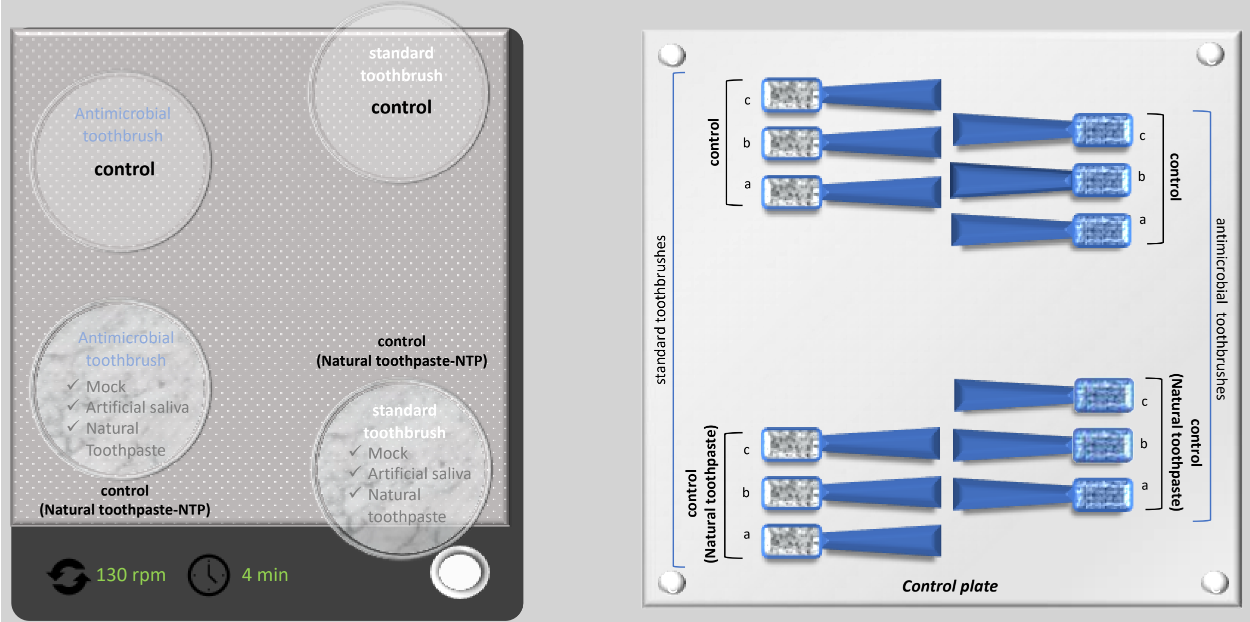


**Figure 6: Control plate and slurry composition**. Left: Shaker with glas petri dishes filled with slurries and which kind of toothbrush will be brushing in the slurry. Top left= antimicrobial toothbrush-control without treatment. Top right= standard toothbrush-control without treatment. For both conditions brushing was performed in a sterile petri dish. Bottom left= Natural toothpaste (NTP) control with antimicrobial toothbrush, mock community in artificial saliva and natural toothpaste. Bottom right= Natural toothpaste control with standard toothbrush, mock community in artificial saliva and natural toothpaste. Right: toothbrushes taped on an acrylic plate using Velcro tape. a, b, c: representing triplicates for each slurry. Left side consists of standard toothbrushes and right side of antimicrobial toothbrushes.

**Table 5: Conditions, representing slurry composition for control plate**

| Control plate | Control | Control | Control NTP | Control NTP |
|---|---|---|---|---|
| Toothbrush | Standard | Antimicrobial | Standard | Antimicrobial |
| Mock | no | no | Yes | Yes |
| Artificial saliva | no | no | Yes | Yes |
| Toothpaste | no | no | Yes | Yes |
| TCS | no | No | no | no |

### 2.3.4 Mix 5 isolates to represent the mock community

From an ONC of each isolate a ½, ¼, 1/8, 1/16, 1/32 dilutions were made (serial dilutions I). $OD_{600}$ of each of those dilutions was measured in duplicates. The particular media, the cells were growing in, was always used as a blank. In this case Tryptic Soy Broth (TSB) was used. Another 4 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 agar plates and incubated at 37°C for at least 48h. 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**

Therefore, 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 blotted 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 (paper). in the mixed mock community. Dividing this number by 5 means each isolate will be represented by a number of 2.00E+06 microbes.

For performing the experiment, the mock community mix was created every day anew. Therefore, a fresh ONC was made every day. $OD_{600}$ of all isolates and blank was measured in duplicates. Average was calculated, and the average blank was subtracted from the average values of the isolates to figure out 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 show 2.00E+06 microbes of each isolate in the total needed volume of the mock community mix. The needed volume was dependent on the amount of the toothbrushes which needed to be inoculated on the particular day in the experiment.

### 2.3.5 Preparation of the Mock community – Artificial Saliva (AS) – Mix

Artificial saliva was prepared following the Klimek method which is commonly used for imitating saliva (Klimek1982). The mock community consists of 5 isolates. The 5 isolates were streaked out in TSA agar plates aiming single colonies once a week using the frozen glycerol stock sample in order to get fresh growing isolates to inoculate ONCs. 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.

$OD_{600}$ of the ONC of all your 5 isolates was measured in duplicates in a plate reader. Therefore, tube containing the culture was shaken manually and vortexed gently in case a sediment was formed on the bottom of the tube. 200 µL of each homogeneous mixed ONC was transferred in a well of a 96 well plate. Measured $OD_{600}$ values were used to calculate the appropriate volume of ONC ($OD_{600}$ value was included into the standard equation), which is needed to obtain the correct CFUs per isolate in order to imitate a total number of 1,00E+07 microbes in the mouth. Calculated volume of each isolate was transferred into a new tube. Samples were centrifuged for 3 min at 10000 rpm in a microcentrifuge. 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 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 all slurries. Solution was mixed by gently shaking manually.

### 2.3.6 Preparation of toothpaste slurries

As a basis, each slurry consisted of 3 mL artificial saliva and 1,00E+07 microbes representing the mock community. 1.0 g toothpaste or 3 mg TCS (0.3% TCS in 1.0 g toothpaste) was added if necessary, for the individual condition (figure 3, 4, 5; table 5, 6). 1.0 g toothpaste per brushing event is a standardized usage amount for adults (Jensen *et al.*, 2012). Toothpaste- artificial saliva ratio (1:3; w:w) was adapted from previous studies (Alshara *et al.*, 2014). For each of the 5 conditions, specific slurries were prepared for a triplicate of toothbrushes individually in 50 mL conical tubes. Therefore, 3 ± 0.02g 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 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 homogeneous slurry solution was obtained. Slurries were preheated to 37°C in a shaking incubator before use, to simulate temperature in the mouth. After the heating step slurry tubes were gently vortexed again.

Condition 1 was a standard toothbrush without toothpaste. Condition 2 is an antimicrobial toothbrush without toothpaste. Condition 3 is a standard toothbrush with toothpaste. Condition 4 is a standard toothbrush with toothpaste and added TCS. Condition 5 is an antimicrobial toothbrush with toothpaste and added TCS. Conditions are tabulated and visualized in figure 7.

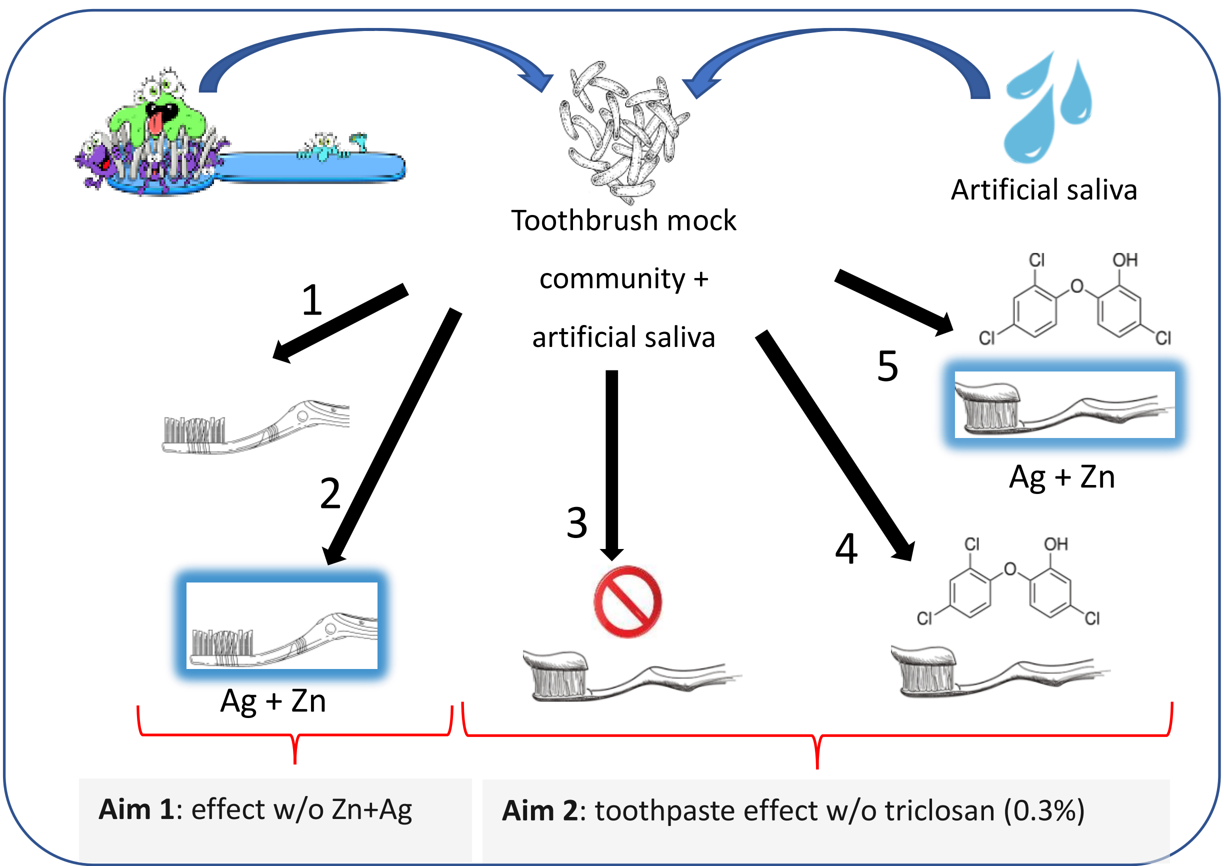


**Figure 7: Overview of experimental conditions.** Toothbrush mock community found on used toothbrushes is exposed to 5 different toothbrush-toothpaste settings together with artificial saliva. 1: standard toothbrush without toothpaste. 2: antimicrobial toothbrush without toothpaste. 3: standard toothbrush 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 6 as well. Ag=Silver; Zn=Zinc.

### 2.3.7 Simulated Brushing experiment – experimental protocol

The autoclaved glas petri dishes were stick on the marked locations on the shaker plate with a double-sided adhesive tape. The preheated slurries were manually shaken a last time and filled 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. Shaker was switched on for 4 min at 130 rpm in order to simulate brushing twice a day (2x2min). 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 twice rinsed with deionized water. After drying overnight, they were wiped again with 70% ethanol, wrapped into aluminum foil and autoclaved.

### 2.3.8 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-tip to avoid contaminations 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. Therefore, water was sprinkled very gently directly on the bristles. The distance between toothbrush head and tip of the syringe was 10 cm. The washing step was performed as steady 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. 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. Cool syringes were filled with water for further use.

### 2.3.9 Sampling and Cultivation

Toothbrush sampling was performed in the biosafety cabinet (table 6). First of all, toothbrushes were removed from the plate and the Velcro tape was removed from the back of the toothbrush-head. 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 part 2.1.1 Collection and cultivation of toothbrush samples and 2.1.2 Microbial culture analysis.

Aberrations are that undiluted, 10-2 and 10-3 dilutions were plated and incubated only at 37°C. Colonies were classified as one of the 5 isolates, counted and recorded.

**Toothpaste Slurry Sampling**

Immediately after the simulated brushing event was finished, slurries were absorbed with an automatic pipette out of the glas petri dishes and filled in the conical tubes the slurries were prepared in. Samples were labelled properly and frozen at -20°C for further analysis.

**Table 6: Experimental Workflow** 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 exposure times at the moment of sampling the particular plates.

| Week | Day | Date | Day# | Exposure# | Washing | Sampling plates* |
|---|---|---|---|---|---|---|
| Week 1 | Mon | 8/27/18 | Day 0 | 1 | | 0 ; 1 |
| | Tue | 8/28/18 | Day 1 | 2 | | |
| | Wed | 8/29/18 | Day 2 | 3 | | 2 |
| | Thu | 8/30/18 | Day 3 | Break | Break | |
| | Fri | 8/31/18 | 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 | 9/13/18 | Day 17 | 13 | | |
| | Fri | 9/14/18 | Day 18 | 14 | | 13 |
| | Sat | 9/15/18 | Day 19 | 15 | | |
| | Sun | 9/16/18 | Day 20 | 16 | | |
| Week 4 | Mon | 9/17/18 | Day 21 | 17 | | |
| | Tue | 9/18/18 | Day 22 | 18 | | |
| | Wed | 9/19/18 | Day 23 | 19 | | |
| | Thu | 9/20/18 | Day 24 | 20 | | 19 |
| | Fri | 9/21/18 | Day 25 | 21 | | |
| | Sat | 9/22/18 | Day 26 | Break | Break | |
| | Sun | 9/23/18 | Day 27 | Beak | Break | |
| Week 5 | Mon | 9/24/18 | Day 28 | 22 | | |
| | Tue | 9/25/18 | Day 29 | 23 | | |
| | Wed | 9/26/18 | Day 30 | 24 | | 23 |
| | Thu | 9/27/18 | Day 31 | 25 | | |
| | Fri | 9/28/18 | Day 32 | 26 | | |
| | Sat | 9/29/18 | Day 33 | Break | Break | |
| | Sun | 9/30/18 | 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 |

### 2.3.10 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 EtOH and DI-water. Then the tip of the sonotrode was carefully plunged into the sample without touching the tube itself. After everyone in the lab protected their ears properly the sonicator program was started. The total time for sonication was 2 min, where the progress alternates 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% EtOH.

**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 proceeded according to the manual of MasterPureTM Complete DNA and RNA Purification Kit.

### 2.3.11 Real time quantitative PCR (qPCR)

In addition to the cultivation-based approach to analyse microbial diversity on toothbrushes, a qPCR was performed to determine 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.

Consequently, a primer set that targets a 16S rRNA gene, that is 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 replicates of three.

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 [100µM] |
| **0.5** | 534r primer [100µM] |
| **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** | x40 |
| | **55.0** | **15 sec** | |
| | **72.0** | **1 min** | |
| **Melt curve** | **95.0** | **15 sec** | |
| | **60.0** | **1 min** | |
| | **95.0** | **15 sec** | |

The resulting data was analysed using Quant Studio software v1.4.1.

# 3 Results

## 3.1 Mock community

As described in 2.1.3 isolated colonies, were characterized based on their morphotype. Characterization properties are shape, margin, elevation, size, texture, appearance, pigmentation and optical property. 5 isolates, which represent the artificial mock community, were selected out of 102 characterized isolates cultivated from 19 cultivated toothbrushes (figure 8). 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.

**Figure 8: Colony morphology characterization of 5 isolates forming the mock community.** **S**hape, **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 16 S rRNA Sanger Sequencing and Whole Genome Sequencing the 5 isolates were identified. The mock community consists of a *microbacterium paraoxydans*, *staphylococcus epidermidis*, *kocuria rhizophila*, *staphylococcus warneri* and *rothia dentocariosa.*

*Microbacterium paraoxydans,* a gram-positive bacterium, which is able to cause disease in fish, was first isolated from the fish Nile tilapia in Mexico (Komen and Culture, 1995). *Kocuria rhizophilia* belongs to the family *Micrococcaceae*. They are common skin commensals in mammals and they also occur in soil, chicken meat, fresh water or food (Pękala *et al.*, 2018). *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 but can also cause bacteremia, infective endocarditis and other infectious diseases (Buttery *et al.*, 1997) (Schleifer and Kloos, 1975). An anaerobic gram-positive *coccobacillus*, named *Rothia dentocariosa* is part of the oral cavity (Broeren and Peel, 1984). More details about each isolate are summarized in table 10. Although a big part of the oral microbial community also consists of a large number of anaerobic bacteria, that possibly may occur also on toothbrushes. However, because of practical reasons the focus for this project was set at anaerobic bacteria.

**Table 10: 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; oxygen: refers to the 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 |

## 3.2 Culture work

During the simulated brushing experiment, which lasted for 6 weeks, samples were collected on 7 sample points. Samples were cultivated on TSA agar plates and analyzed as described in section 2.1.1 Collection and cultivation of toothbrush samples and 2.1.2 Microbial culture analysis. Samples were plated undiluted, diluted 1:100 and 1:1000. The reason for plating dilutions was trying to get a number between 30-300 colonies on a plate. The plate, which showed 30-300 colonies, was selected for representing one sample as well as for calculating the CFU/mL. CFU/mL were calculated as described in 2.3.4. 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. First each condition was shown in one graph, therefore the number of inoculations of the toothbrushes were plotted on the x-axis. Each inoculation includes the set of 5 isolates next to each other. An inoculation is an analog for the brushing 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.

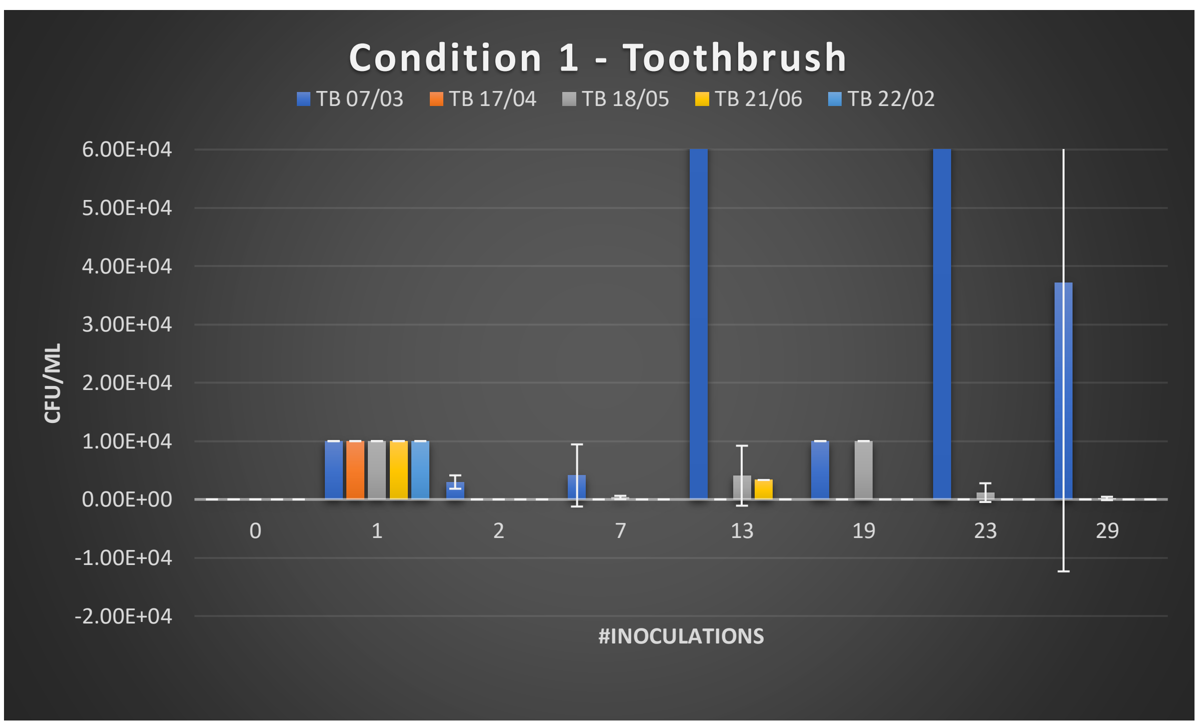


**Figure 9: Development of bacterial growth for Condition 1 -** standard toothbrush inoculated with mock community. Number of inoculations are shown on the x-axis. Each isolate is represented by a color.

First Condition 1 was compared over the whole experiment time period (figure 9). 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 inoculation. All the other samples for later timepoints (inoculations 2-29) were sampled 24h hours after the inoculation and before the next inoculation. All 5 isolates grow to the same intensity after 1 inoculation. After more inoculations mainly, TB 07/03 and TB 18/5 were growing. It is speculated that this might be because *Microbacterium paraoxydans* and *Kocuria rhizophila* are capable to handle the conditions of the brushing experiment better than the other strains. Besides them, only TB 21/06-*Staphylococcus warneri* was also growing after inoculation 13. After 13 and 23 inoculations *Kocuria rhizophila* shows the highest CFU values with 7.96E+05 and 9.90E+05 CFU/mL. Due to presentation reasons, these values are not visible in the graph. If the maximum vertical value would be higher the smaller columns would not be apparent. However, all values can be seen in the tables 11-13. The standard deviations are quite large, which is occurring often with culture data.

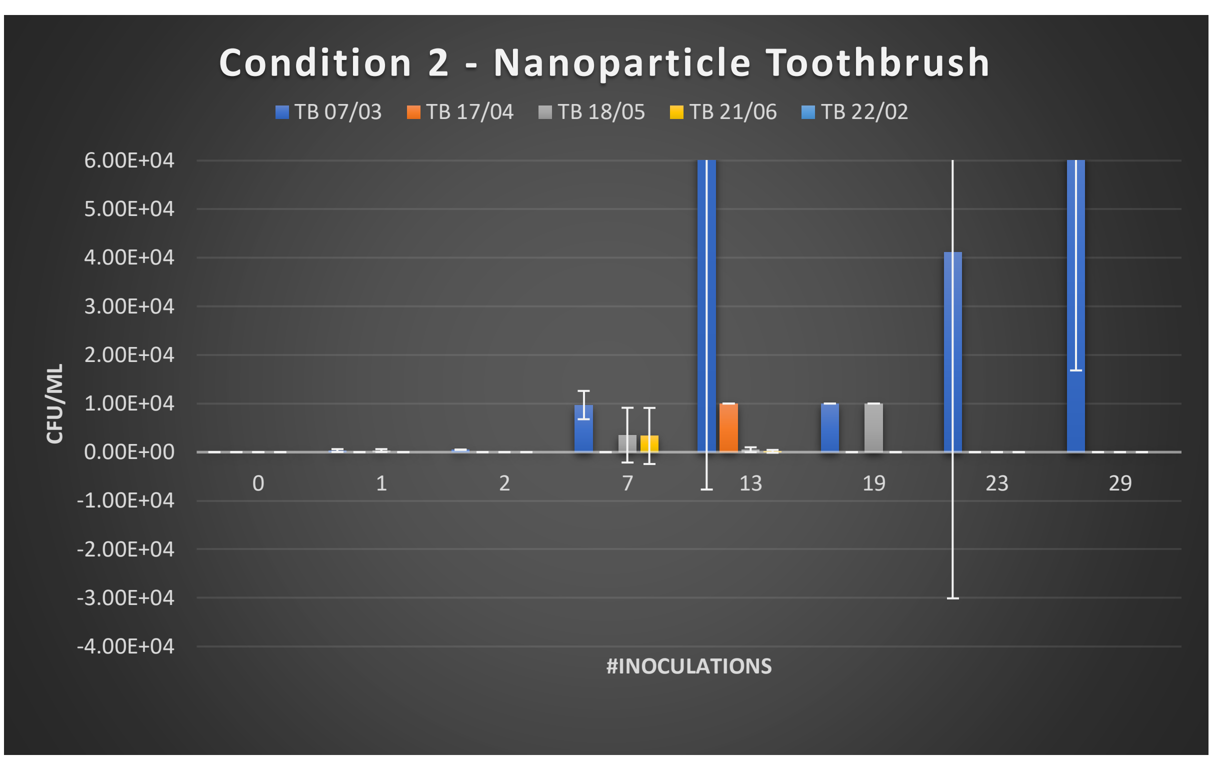


**Figure 10: Development of bacterial growth for Condition 2 -** nanoparticle toothbrush without toothpaste. Number of inoculations are shown on the x-axis. Each isolate is represented by a color.

In condition 2 an antimicrobial toothbrush with coated filaments was used to show the difference in the contamination between the coated and the normal standard control brushes (figure 10). In contrast to the standard toothbrush almost no bacteria were collected from the nanoparticle toothbrush after the first 2 inoculations. TB 07/03 and TB 18/05 show very low CFU values after 1 inoculation and TB 07/03 grows after 2 inoculations. These small values are not visible in the graph, but data is shown again in the beforementioned tables.

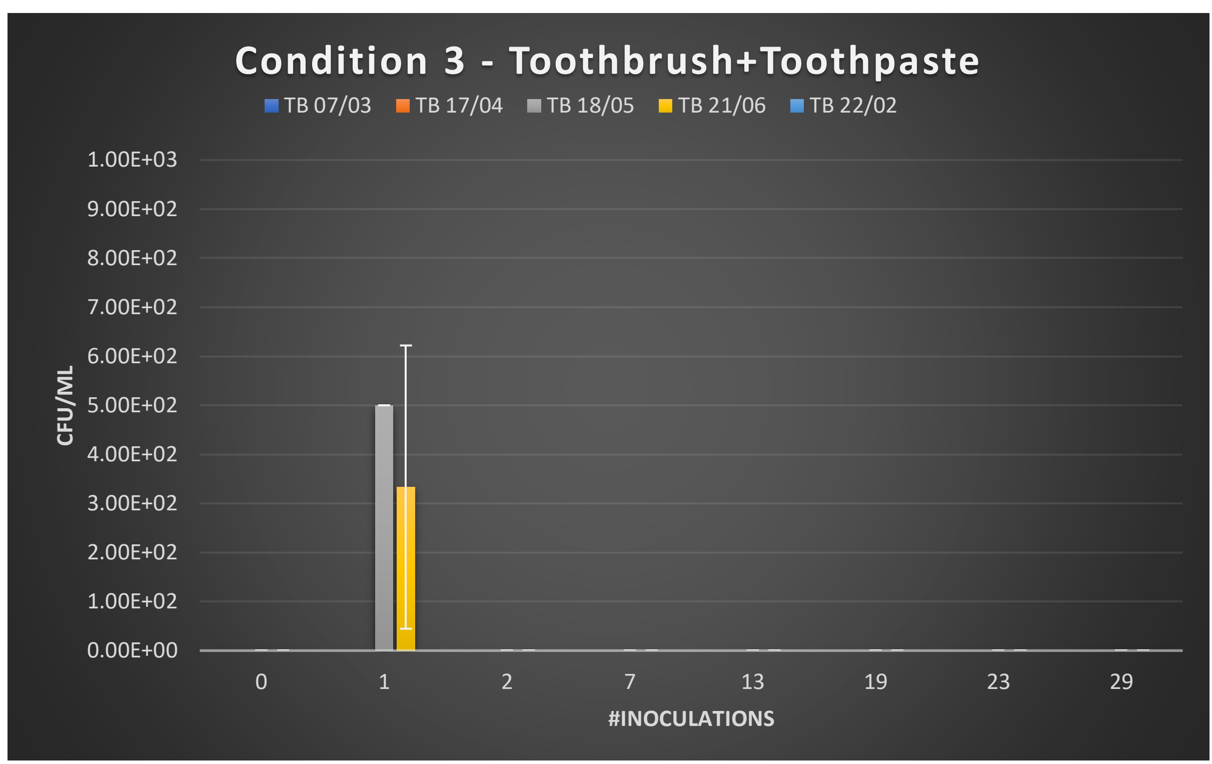


**Figure 11: Development of bacterial growth for Condition 3** - standard toothbrush with toothpaste. Number of inoculations are shown on the x-axis. Each isolate is represented by a color.

In condition 3, where toothpaste was added to the mock community, TB 18/05 and TB 21/06 were present after 1 inoculation. TB 07/03, which was mainly growing in the previous conditions, and the other isolates, were not able to grow in this condition containing toothpaste (figure 11). Considering all 3 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 consistent for all conditions.

The high variations within the CFU values made the visualization through graphs quite challenging. Due to that and for better comprehension the tabulated CFU data was added (table 11-13). As already mentioned above, condition 4 and 5 are not included because no bacterial growth was observed for these conditions, which means CFU values are 0 as well.
All tables include calculated average CFU values and standard deviations. The first number in the sample ID refer to the number of inoculation and the second one refers to the condition used.

**Table 11: Overview of CFU/mL values for Condition 1.** First numbers refer to the number (#) of inoculations. Second number refer to the condition. AVG= average value; STD=standard deviation

| 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 | 1.00E+04 | 1.00E+04 | 1.00E+04 | 1.00E+04 | 1.00E+04 | 1 |
| 2-1 AVG | 2.98E+03 | 0 | 0 | 0 | 0 | 2 |
| 7-1 AVG | 4.11E+03 | 0 | 3.33E+02 | 0 | 0 | 7 |
| 13-1 AVG | 7.96E+05 | 0 | 4.07E+03 | 3.33E+03 | 0 | 13 |
| 19-1 AVG | 1.00E+04 | 0 | 1.00E+04 | 0 | 0 | 19 |
| 23-1 AVG | 9.90E+05 | 0 | 1.17E+03 | 0 | 0 | 23 |
| 29-1 AVG | 3.71E+04 | 0 | 1.67E+02 | 0 | 0 | 29 |
| 0-1 STD | 0 | 0 | 0 | 0 | 0 | 0 |
| 1-1 STD | 0 | 0 | 0 | 0 | 0 | 1 |
| 2-1 STD | 1.15E+03 | 0 | 0 | 0 | 0 | 2 |
| 7-1 STD | 5.33E+03 | 0 | 2.89E+02 | 0 | 0 | 7 |
| 13-1 STD | 2.10E+05 | 0 | 5.14E+03 | 5.77E+03 | 0 | 13 |
| 19-1 STD | 0 | 0 | 0 | 0 | 0 | 19 |
| 23-1 STD | 4.32E+05 | 0 | 1.61E+03 | 0 | 0 | 23 |
| 29-1 STD | 4.95E+04 | 0 | 2.89E+02 | 0 | 0 | 29 |

**Table 12: 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 | 3.33E+02 | 0 | 3.33E+02 | 0 | 0 | 1 |
| 2-2 AVG | 5.00E+02 | 0 | 0 | 0 | 0 | 2 |
| 7-2 AVG | 9.69E+03 | 0 | 3.50E+03 | 3.33E+03 | 0 | 7 |
| 13-2 AVG | 2.39E+05 | 1.00E+04 | 5.00E+02 | 1.67E+02 | 0 | 13 |
| 19-2 AVG | 1.00E+04 | 0 | 1.00E+04 | 0 | 0 | 19 |
| 23-2 AVG | 4.11E+04 | 0 | 0 | 0 | 0 | 23 |
| 29-2 AVG | 8.00E+04 | 0 | 0 | 0 | 0 | 29 |
| 0-2 STD | 0 | 0 | 0 | 0 | 0 | 0 |
| 1-2 STD | 2.89E+02 | 0 | 2.89E+02 | 0 | 0 | 1 |
| 2-2 STD | 0 | 0 | 0 | 0 | 0 | 2 |
| 7-2 STD | 2.91E+03 | 0 | 5.63E+03 | 5.77E+03 | 0 | 7 |
| 13-2 STD | 2.47E+05 | 0 | 5.00E+02 | 2.89E+02 | 0 | 13 |
| 19-2 STD | 0 | 0 | 0 | 0 | 0 | 19 |

| 23-2 STD | 7.12E+04 | 0 | 0 | 0 | 0 | 23 |
|---|---|---|---|---|---|---|
| 29-2 STD | 6.32E+04 | 0 | 0 | 0 | 0 | 29 |

**Table 13: 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 |

Graphs (figure 12-17) below include basically the same tabulated values as used above for the previous graphs (figure 9-11). The only modification made is that the emphasis is on each individual isolate and therefore a different chart type was used in order to observe the growth over time. The abbreviations in the graphs refer to condition 1-5 (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 highest bacterial concentration of TB 07/03 at condition 1 (TB) is determined after 23 inoculations followed by 13 inoculations. After 19 and 29 inoculations the growth decreased suddenly after a high peak in the previous sampling point. For 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 visible in figure 9. As the variation between the values are very high and to make all

values visible, the maximum value for the vertical axis is higher in figure 12 compared to the other graphs. Given that, this beforementioned trend is not visible in this graph. The CFU values visualized in the graphs are traceable in tables 11-13.

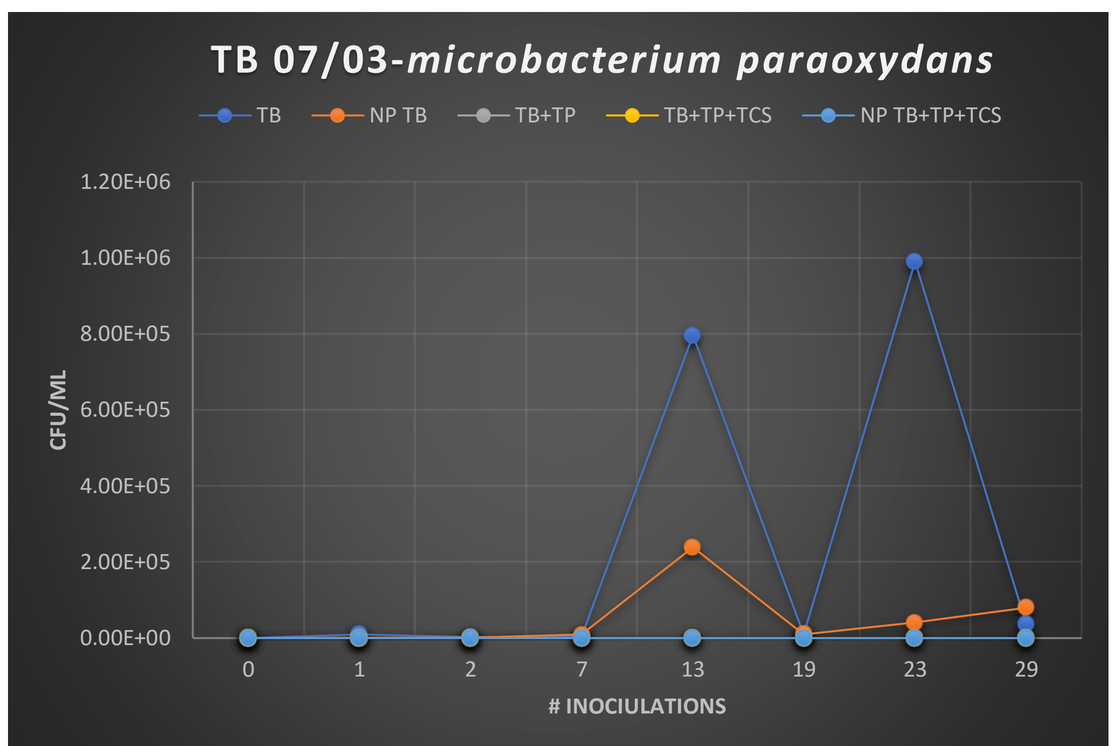


**Figure 12: Development of bacterial growth of TB 07/03 over time.** Abbreviations refer to the 5 conditions. TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush+ toothpaste+ triclosan

TB 17/04, which represents staphylococcus epidermidis, was cultivated from a standard toothbrush (condition 1) after 1 inoculation and from a nanoparticle toothbrush (condition 2) after 13 inoculations. Both peaks in graph yielded 1.00E+04 CFU/ml (figure 13).

TB 18/05 was present in the first 3 conditions after 1 inoculation. After 2 to 19 inoculations the growth of TB 18/05 for condition 1 (represented by the blue line) increased and decreased again from 19 to 29 inoculations. This strain was identifiable after 19 inoculations both on standard and nanoparticle toothbrushes (figure 14).

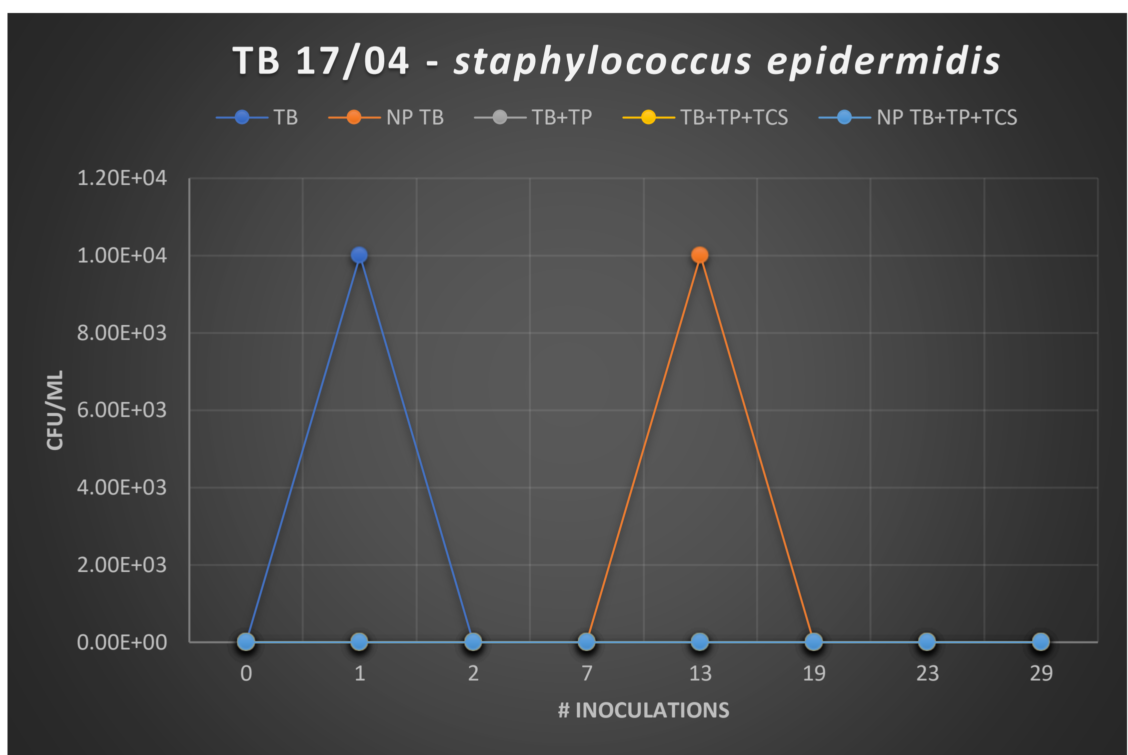


**Figure 13: Development of bacterial growth of TB 17/04 over time.** TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

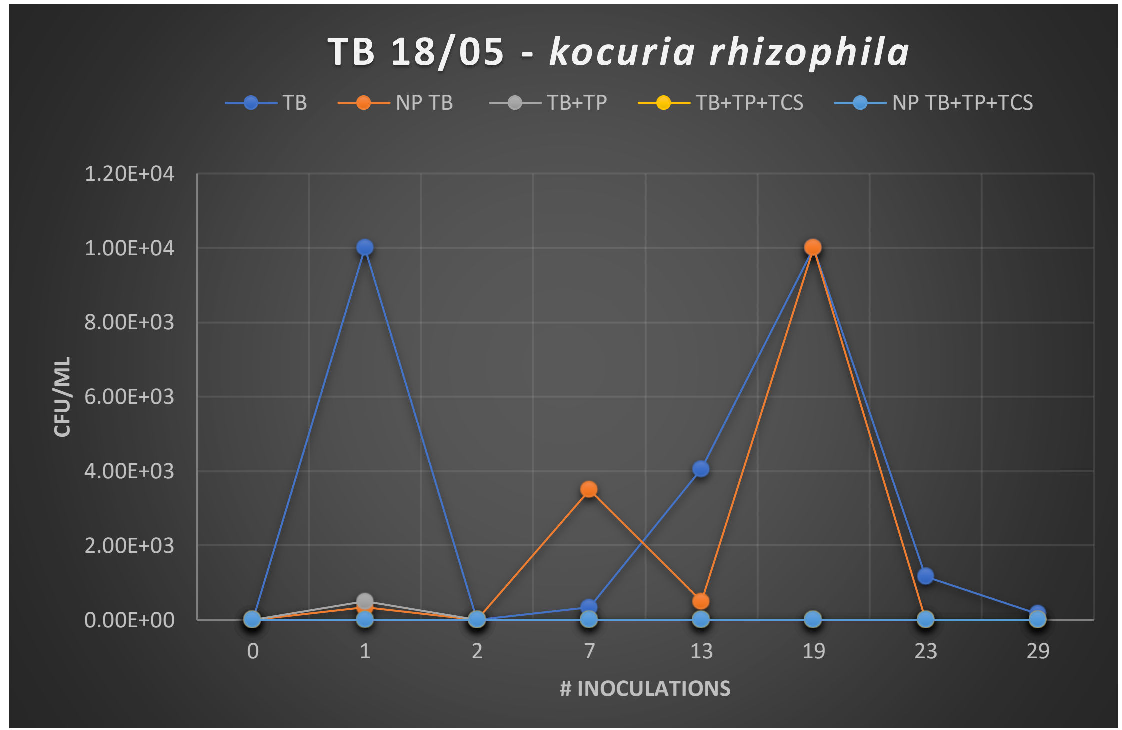


**Figure 14: Development of bacterial growth of TB 18/05 over time**. TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

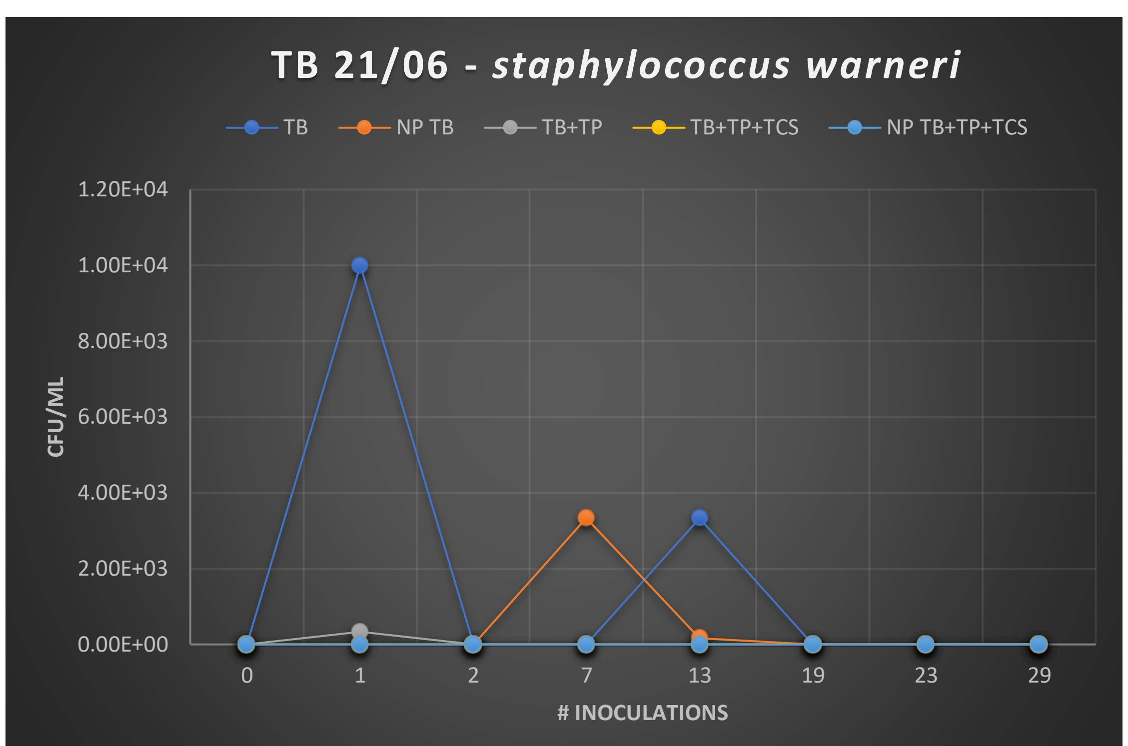


**Figure 15: Development of bacterial growth of TB 21/06 over time.** TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

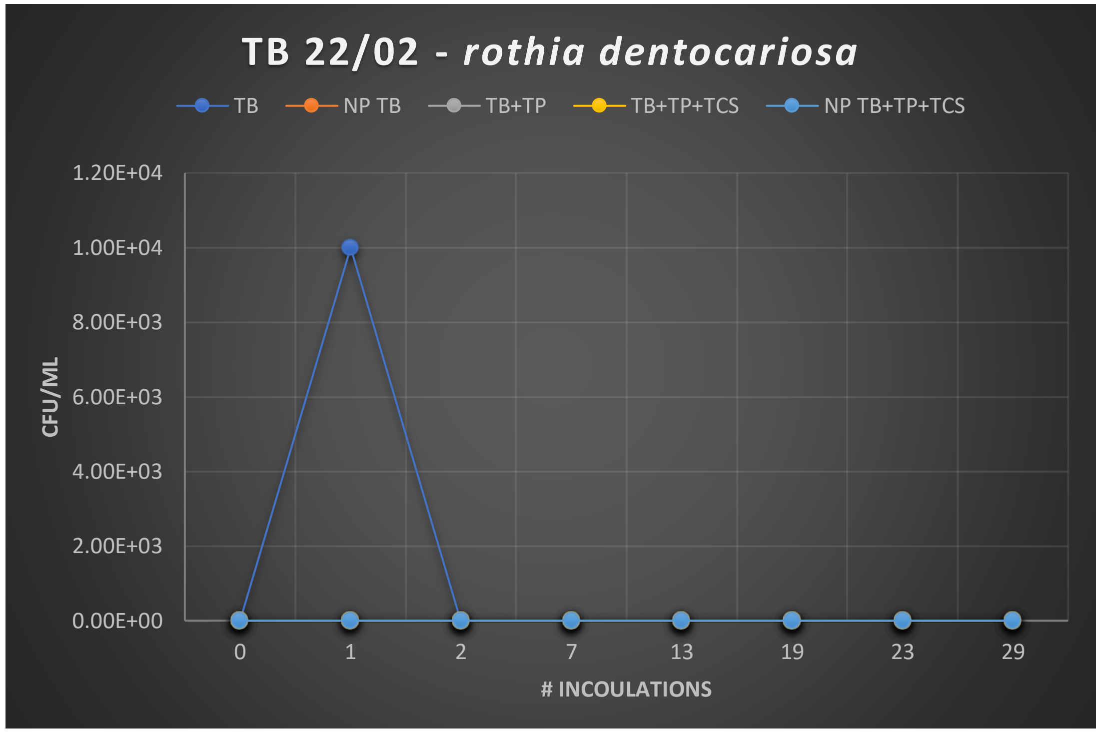


**Figure 16: Development of bacterial growth of TB 22/02 over time.** TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

TB 21/06 (*Staphylococcus warneri*) was cultivable from toothbrushes, representing condition 1 and condition 3 after 1 inoculation. In condition 1, *Staphylococcus warneri* was present after 1 and 13 inoculation. No growth was visible for the other samples. In condition 2 this isolate was present after 7 and 13 treatments, whereby more colonies were found after 7 inoculations than after 13 (figure 15). As seen from figure 15, 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 (trend) in the number of CFU/mL.

For summarizing the total number of bacteria for each individual condition, CFU values for each condition were summed up. Looking at the total biomass, nanoparticle toothbrushes accumulate almost twice as much bacteria than standard toothbrushes. Condition 3 yielded 833.33 CFU/mL in total (figure 17).

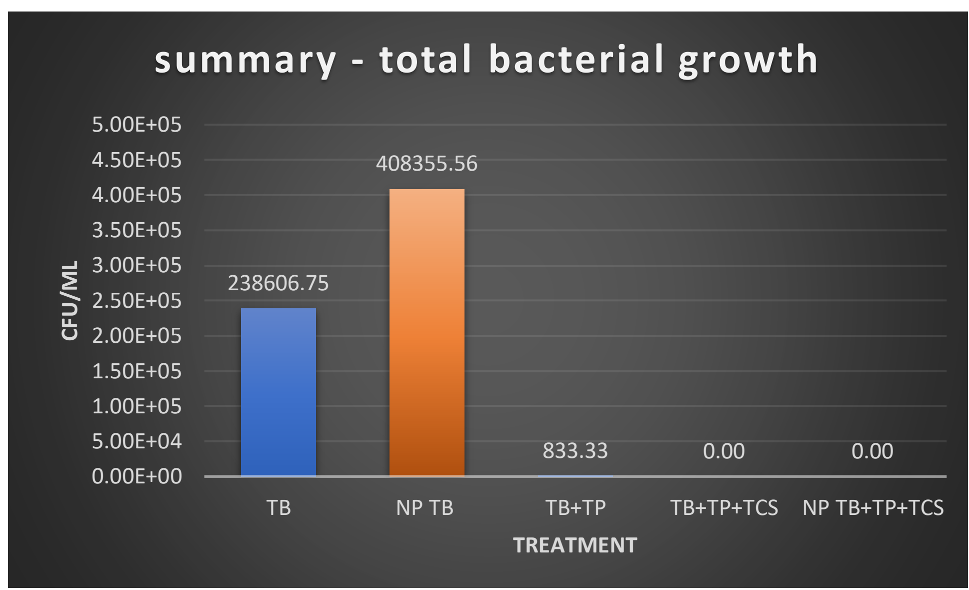


**Figure 17: Summarized data for each condition.** TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

## 3.3 qPCR results

Furthermore, and 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 5 of 7 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. Therefore, 16S rRNA gene in ng of DNA/well can be seen on the y-axis and the treatments, featured in different colors shown on the x-axis.

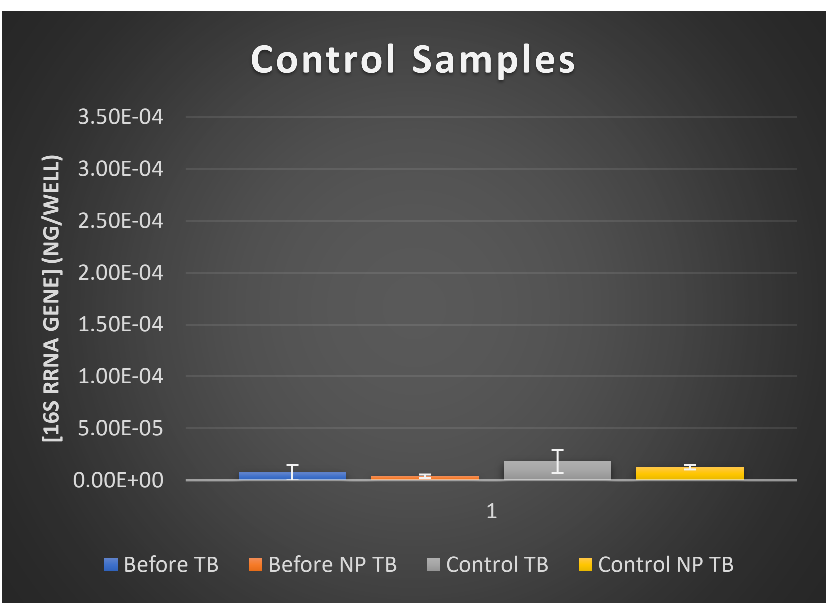


**Figure 18: Total bacterial biomass of control samples.** Before TB and Before NP TB refer to the blank samples, which were not inoculated and sampled right after the sterilization step; Control TB and Control NP TB show the toothbrush samples, which were not inoculated but the brushing event was conducted 29 times on the simulation set up.

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, that had been inoculated. As predicted the blank samples, shown as “Before TB” and “Before NP TB” in the graph, accumulate little to no bacteria as well.

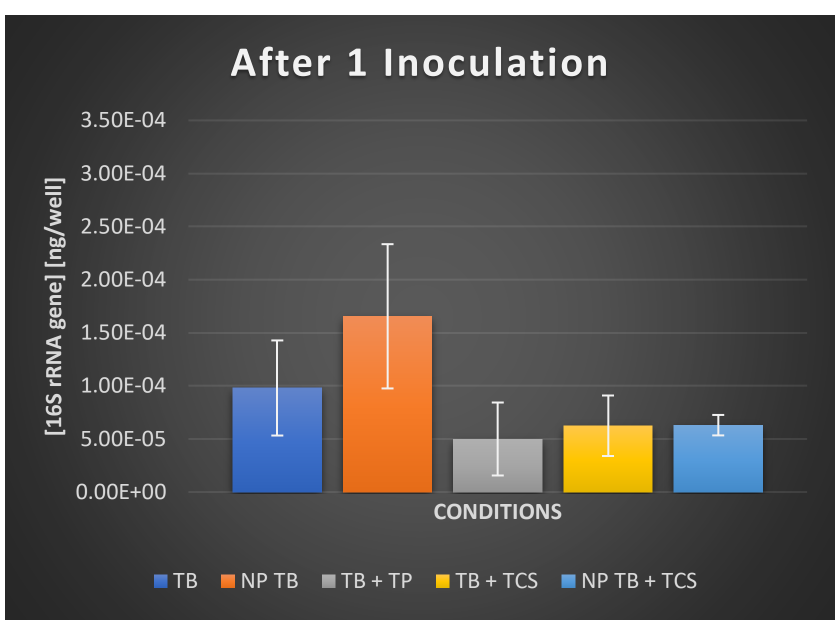


**Figure 19: Total bacterial biomass after 1 inoculation.** TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

In general, the biomass recoverable from toothbrushes was extremely low.

After 1 inoculation one can clearly see a higher number of bacteria stays on an antimicrobial (NP TB) than on a 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. Same trend occurs after 2 inoculations. Biomass surviving on standard toothbrushes is the smallest compared to the other treatments after 2 inoculations (figure 20).

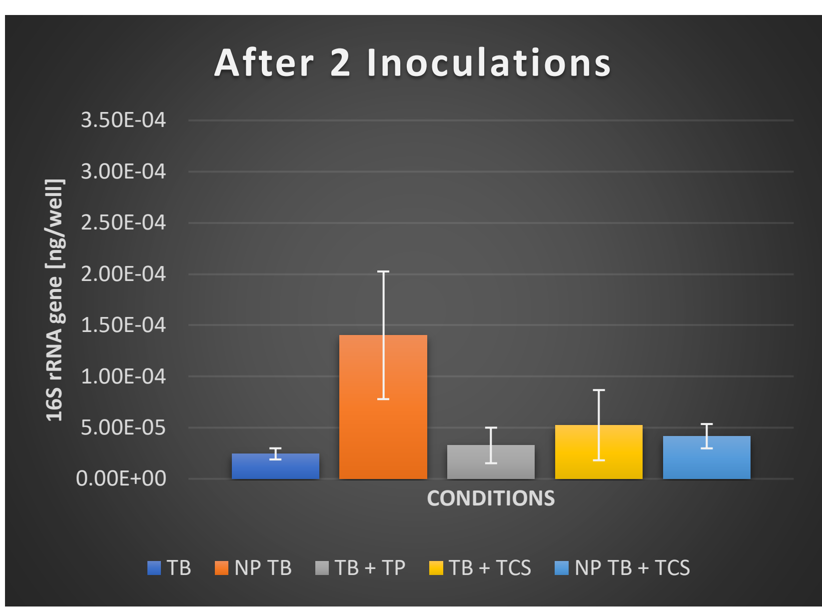


**Figure 20: Total bacterial biomass after 2 inoculations.** TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

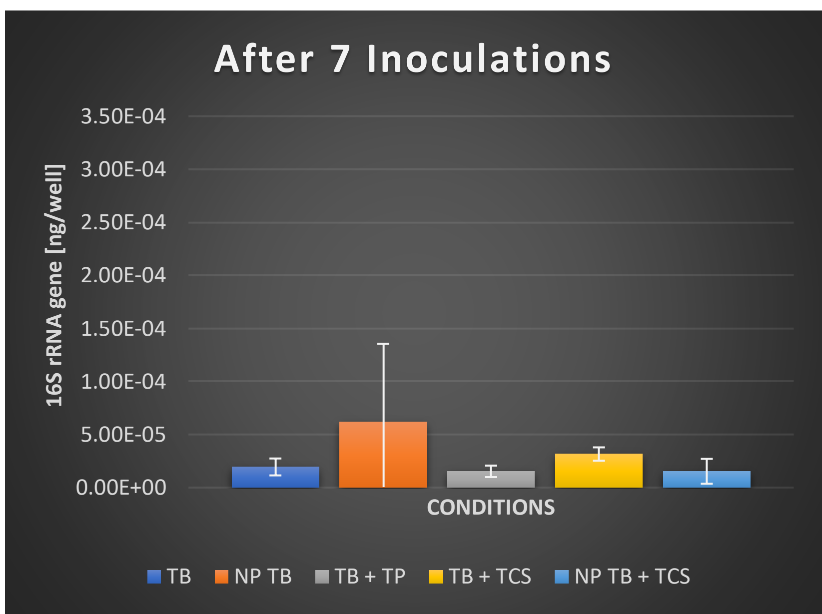


**Figure 21: Total bacterial biomass after 7 inoculations.** TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

Basically, 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, then it increases on toothbrushes with toothpaste and triclosan (TB+TP+TCS) and decreases again on antimicrobial toothbrushes with toothpaste and triclosan (NP TB+TP+TCS) (figure 20, 21).

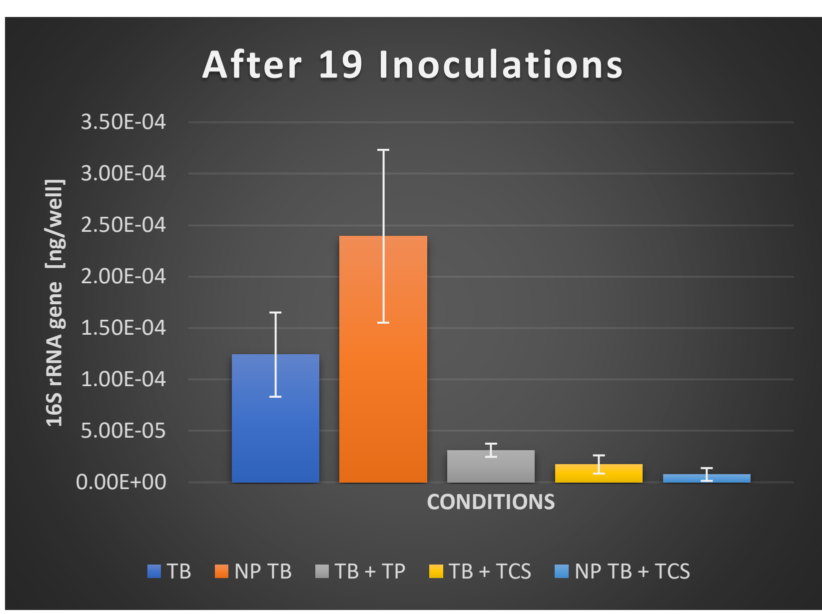


**Figure 22: Total bacterial biomass after 19 inoculations.** TB=Toothbrush; NP TB=Nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

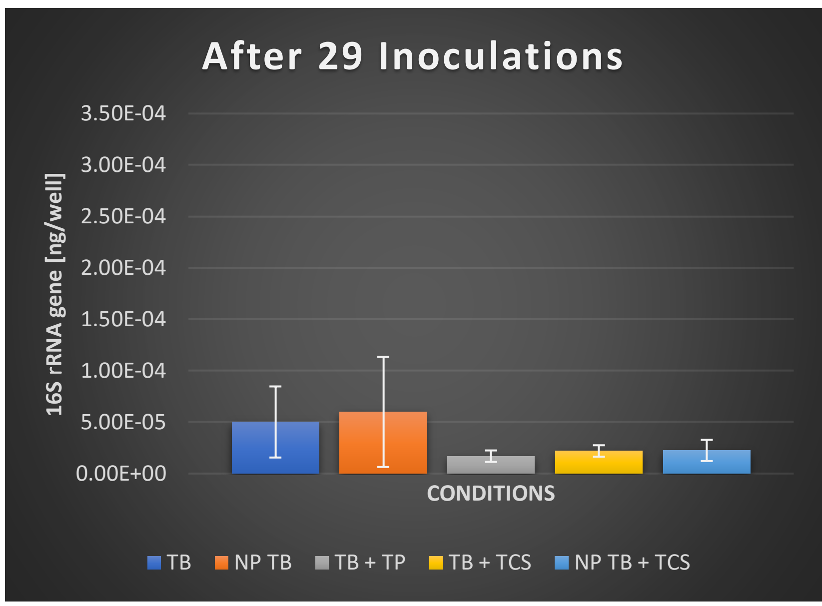


**Figure 23: Total bacterial biomass after 29 inoculations.** TB=toothbrush; NP TB=nanoparticle toothbrush; TB+TP= toothbrush+ toothpaste; TB+TP+TCS=toothbrush+ toothpaste+ triclosan; NP TB+TP+TCS= nanoparticle toothbrush + toothpaste+ triclosan.

After 19 and 29 inoculations, more bacteria survived on toothbrushes without added toothpaste and triclosan (figure 22, 23). Comparing all treatments, highest number of bacteria survive on nanoparticle toothbrushes, which is consistent with the summary graph (figure 17) 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

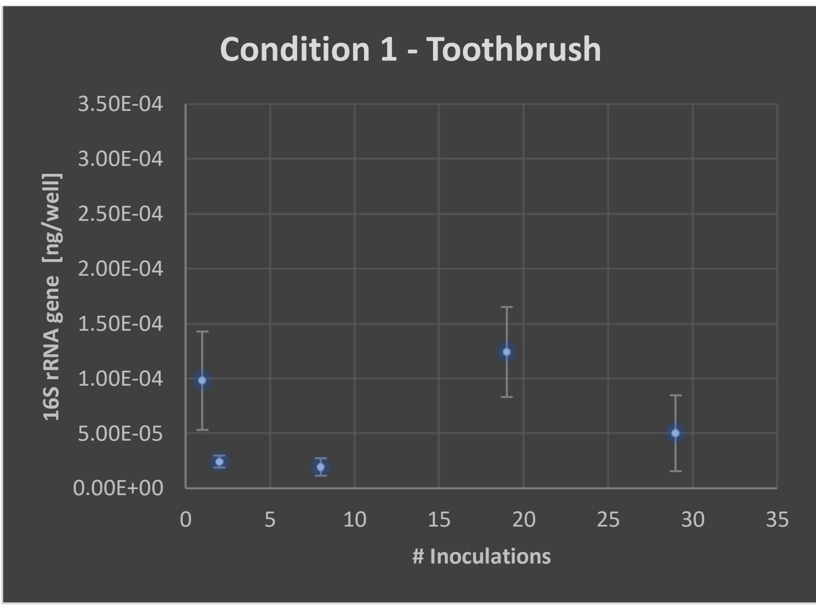


**Figure 24: Total bacterial growth on a standard toothbrush over time.**

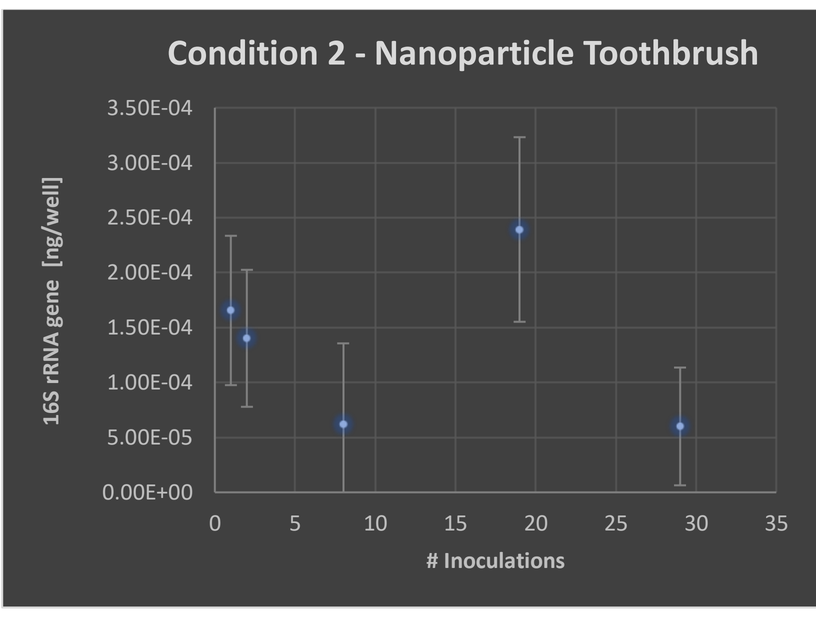


**Figure 25: Total bacterial growth on a nanoparticle toothbrush over time.**

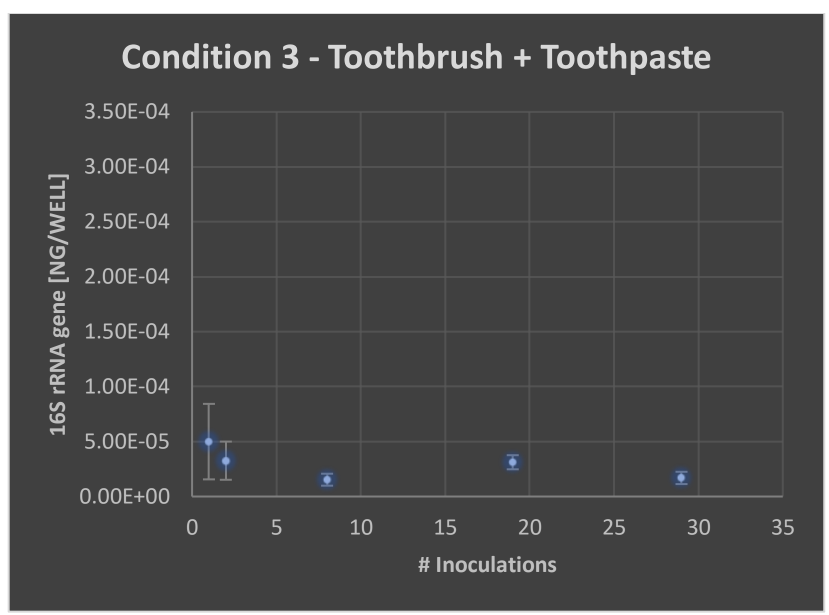


**Figure 26: Total bacterial growth on a standard toothbrush with toothpaste over time.**

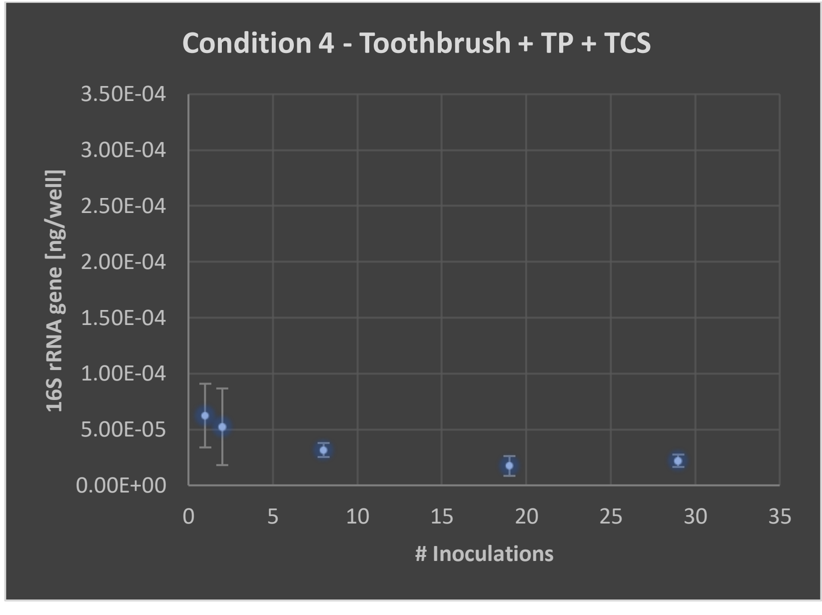


**Figure 27: Total bacterial growth on a standard toothbrush with toothpaste and triclosan over time.**

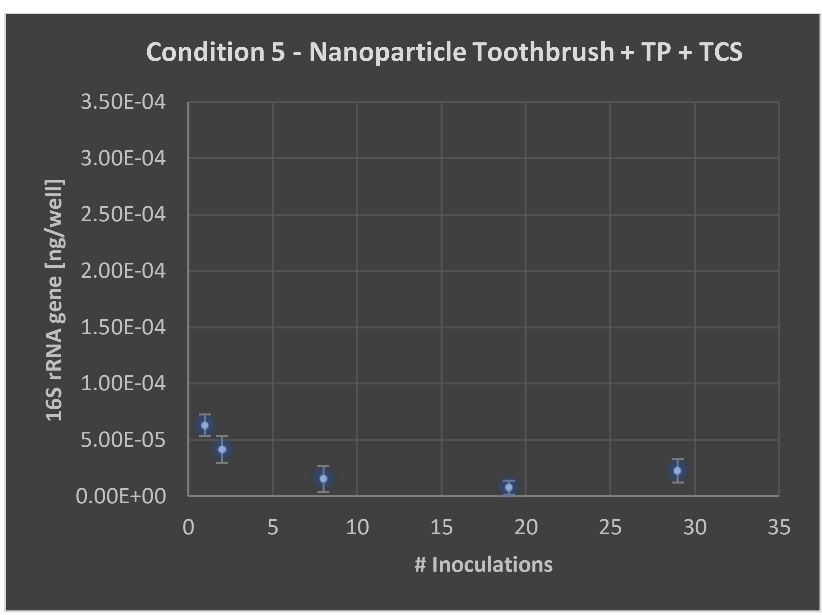


**Figure 28: Total bacterial growth on a nanoparticle toothbrush with toothpaste and triclosan over time.**

The same applies to the next graphs, which are comparing the number of inoculations (figure 24 – 28). 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 following graphs a trend can be observed. The graph show that the bacterial concentration started decreasing steadily from the beginning to inoculation 7, but then suddenly peaking at inoculation 19 (condition 1 and 2) and flattened out. Additionally, in conditions with added toothpaste, regardless of whether it has TCS, lower bacterial concentrations were examined compared to the conditions without toothpaste. The same trend was already shown within the culture work.

# 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 surviving 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, that will be exposed

to unused toothbrushes within a simulated brushing experiment. A very diverse mix of 5 isolates, namely *Microbacterium paraoxydans, Staphylococcus epidermidis, Kocuria rhizophila, Staphylococcus warneri* and *Rothia dentocariosa*, was found. The figures reveal that two bacteria, *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 2 strains 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*. paraoxidans* is also known as a plant-growth-promoting bacteria, which shows a broadly based environment they are able to live in . (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 membrane of human and animals, were also isolated from different environments. Although *Kocuria spp* are non-pathogenic human commensals, they should be considered as potential pathogens in immunocompromised patients because they are also present in the environment (M.K., S.H. and D.W., 2013; Kandi et al., 2016).

*S. epidermidis, S. warneri* and *R. dentocariosa* are mainly associated to live in contact with humans. *S. epidermidis* is permanently colonizing human skin but is also surviving on catheters. *Staphylococci* in general are present in the oral cavity. The most frequent species in the oral enviroment is *S. aureus* with 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 residing in the oral cavity and respiratory tract. It was first isolated from dental plaque and dental caries but is largely non-pathogenic. (Broeren and Peel, 1984).

Comparing our speculations with findings from older studies this might be an explanation for the increased presence of *M. paraoxydans* and *K. rhizophila.* As they were already found in several environments, they are probably more adaptable to unusual conditions. Toothbrush plates were stored at room temperature for around 24h between the brushing events. Due to, issues with

the air conditioner the temperature in the room, where the toothbrush plates were stored, varied from 23-27°C. Additionally, to the unsteady room temperature, dryness might be another stress factor for the isolates. Regarding the harsh conditions of the simulation experiment this might be an indicative why the 3 other strains were not or hardly able to survive on toothbrushes under these conditions because their usual environment is the human body with defined and steady conditions. Nonetheless this is just an attempt to explain our findings. To confirm results the experiment would need to be repeated.

### 4.1.2 The impact of antimicrobial additives on toothbrushes

Another purpose of this study is to get information about how the exposure of antimicrobials in toothpastes and on toothbrushes will influence the microbial diversity on standard and nanoparticle toothbrushes. The impact was tested in 5 different conditions and samples were analyzed at 7 different timepoints.

The toothbrushes handled as a blank, which were sampled before any inoculations showed as expected no bacterial accumulations on the toothbrush heads. Antimicrobial toothbrushes, with silver and zinc-coated filaments, display less to no bacteria after first and second inoculation. Bacterial concentration visibly increased after the first two inoculations, which possibly implies that nanoparticles are probably washed off during the brushing event and the washing step. One study examined the release of silver nanoparticles from toothbrushes in a 24h experiment and a continuous release was shown up to 16h of testing, which became lower over the final 8h (Mackevica, Olsson and Hansen, 2017). This might suggest that nanoparticles were probably washed of after several washing steps within our brushing experiment.

Given that the bacterial number surviving on nanoparticle toothbrushes was higher than on standard toothbrushes, might also emphasis that nanoparticle toothbrushes are not that effective as expected. To improve future experiments nanoparticle release should be tested in used slurries and water used for washing.

Since contamination of toothbrushes is a real concern, several approaches have been made to decrease the microbial biomass on toothbrushes (Frazelle and Munro, 2012). One study tested the efficacy of coated filaments. The nanoparticles included in the coating were zinc and silver

ions and in contrast to our results they did not examined any significant difference in the bacterial number between coated and normal control brushes (Quirynen *et al.*, 2003).

### 4.1.3 The effect of toothpaste and TCS on microbial abundance

A study investigates the antibacterial activity of toothbrushes with TCS-coated filaments. TCS is known for its antibacterial impacts in toothpaste, however their results show 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 get rid of microbes surviving on toothbrushes is using toothpaste. They used toothbrushes in periodontitis patients and tracked the number of microbes at different time levels. Significantly less bacteria survived combined with the use of toothpaste with strong surfactants (Quirynen *et al.*, 2001). The results of our study agree with these studies. No growth was determined in condition including toothpaste and TCS.

*K. rhizophila* and *S. warneri* only grow after the first inoculation in condition 3 containing toothpaste, which may suggest that they are maybe 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 significantly greater effects than TCS (Jenkins, 1991). This might show why almost no bacteria accumulated on toothbrushes where toothpaste was used. It remains unclear if mock community was killed already while slurries were prepared, during the experiment in the toothpaste slurries, or on the toothbrushes together with possible toothpaste residuals. Maybe SLS breaks up the cell membranes and prevent them from adhering to the bristle surface. Next step would be testing isolates viability in toothpaste slurries.

### 4.1.4 Limitations

From the colony pictures it is apparent that, *Kocuria rhizophila* also called *Micrococcus luteus*, is morphologically similar to both *Staphylococci* and *Micrococci*. These results are in agreement with data in the literature (Kandi et al., 2016). However, having 4 very similar isolates together growing on agar plates, made it challenging to distinguish them.

## 4.2 qPCR

qPCR was performed to determine if the treatments and number of treatments affect total cell count 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 if there was any DNA from any of the isolates, it was in extremely low quantity because Ct-values for all of the samples were similar to the Ct-values of the negative controls. Given that, it was 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 concentration was still extremely low. In addition to that also the error bar was very high. Basically, the data reveals that bacterial concentration does not vary as a function of number of inoculations or by treatment.

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

## 4.3 Comparison of cultivation and molecular techniques

Both techniques are rarely used as complimentary approaches because their results can disagree for a number of reasons. However, in this study, 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 examined compared to the conditions without added toothpaste. Comparing number of inoculations, the bacterial number on toothbrushes after the first inoculations starts “high” and was decreasing then to inoculation 7. The peak after 19 inoculations in figure 23 and 24 is consistent to figure 13, where TB and NP TB are peaking after the same number of inoculations.

# 5 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. Also, the washing step was not conducted under sterile conditions, which means toothbrushes were exposed to environmental microbes during this time. Additionally, because of limited space in the incubator, plates were stored at RT. Due to some issues with the air-conditioner in the laboratory, temperature fluctuated between 23 and 27°C, which was not ideal. 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. Due to limited time it was not possible to repeat the experiment with improved conditions but 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, also 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).

# 6 Conclusion and Outlook

The purpose of this study was to examine which kind of microbes are surviving on used toothbrushes and how the presence or absence 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 5 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 is a standard toothbrush with toothpaste and added TCS. Condition 5 is an antimicrobial toothbrush with toothpaste and added TCS.

Although a cultivation and molecular study are different and therefore not necessarily complimentary approaches, both techniques showed some similar results. First the overall story seems to be that toothpaste, regardless of whether it contains triclosan, inhibits bacterial growth. Another result, which was demonstrated in both methods, was that nanoparticle toothbrushes accumulate a higher number of bacteria than the standard toothbrushes. The data from the cultivation data also reveals that the antimicrobials on coated toothbrushes were only active after the first two inoculations until they were possibly washed off from the toothbrushes.

*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 just performed once for a period of 6 weeks. Since results did not show significant differences between the different conditions and between number of treatments, beforementioned causes are just possible speculations.

Nevertheless, we were able to develop a very innovative brushing apparatus and we learned a lot from the results. Due to that, results can be used to improve and to better design future brushing experiments.

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