

**EX VIVO MODELLING OF THE MICROBIOME
OF DENTAL CARIES**

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ABSTRACT

The complexity of the microbiome associated with dental caries necessitates the need for dental-carries-specific in vitro/ex vivo microbial models to enable a better understanding of the microbial interactions, drivers of dysbiosis, and response to various treatments. Several such models have been described but none include a reference healthy (normobiotic) microbiome along with the dental-carries associated (dysbiotic) microbiome. There is also a gap in the literature with respect to the optimal medium and growth conditions required to maximize similarity to the in vivo microbiomes. The aim of this study is to (i) develop an ex vivo model that recaptures health- and dental caries-associated microbiomes in parallel, and (ii) directly compare nutrient-rich and nutrient-limited media to assess their ability to support the growth of microbiomes similar to the in vivo microbiomes.

Saliva samples were collected from 5 caries-free individuals and 4 individuals with dental caries. The levels of *Streptococcus mutans* were quantified by qPCR in each sample to exclude caries-free samples with high *S. mutans* and caries samples with low *S. mutans*. Subsequently, samples from caries-free and caries subjects were separately pooled to form healthy and caries inocula. An additional inoculum was prepared by spiking the healthy inoculum with *S. mutans*. Ex vivo microbiomes were grown from each inoculum type in MBEC devices using two types of media: saliva with 0.1% sucrose and SHI with 0.1% sucrose. The microbiomes were harvested after 24 hours, 48 hours without changing the media, or 48 hours with a media change. The generated biofilms were subjected to biomass quantification (DNA yield) and microbiome analysis using 16S rRNA gene sequencing.

Biomass significantly increased as a function of growth time, medium change, and medium type (SHI vs. saliva). However, SHI, but not saliva, was associated with a significant drop in viability after 48 h. Beta diversity analysis revealed separate clustering of health- and caries-derived microbiomes with their respective inocula regardless of medium type (PERMANOVA, p : 0.002). Spiking with *S. mutans* didn't result in significant microbial shifts. Caries-derived microbiomes were enriched in *Prevotella* and *Selenomonas* spp., while health-derived microbiomes were enriched in *Streptococcus* and *Veillonella* spp. No significant differences in alpha diversity were found among microbiomes grown in the different conditions. Based on a similarity index, microbiomes grown in SHI for 24 h had the highest similarity to their respective inocula, followed by those grown in saliva for 24 h. However, changes in similarity were not statistically significant.

This study successfully developed an ex vivo model that replicates differences between health- and dental caries-associated microbiomes. While SHI maximized the similarity to the clinical inocula, the use of saliva is recommended where the generated microbiomes are used for further experiments that require biofilm viability.

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CHAPTER 1

INTRODUCTION

1.1. Dental caries

Dental caries is a chronic, multifactorial disease of dental hard tissues affecting millions worldwide (Selwitz et al., 2007). It primarily arises from a microbial biofilm-mediated imbalance driven by sugar intake, leading to the demineralization of the tooth structure (Pitts et al., 2017; Selwitz et al., 2007). While salivary calcium and phosphate ions continually aid in remineralizing the enamel, prolonged exposure to acids can lead to subsurface demineralization or early softening of the tooth structure. This initial mineral loss manifests as a white spot or streak on the enamel (Mathur & Dhillon, 2018). If the process continues unchecked, the demineralization progresses, resulting in a break in the enamel's continuity and eventual cavitation of the tooth as the minerals diffuse outward (Featherstone, 2004).

The dental pellicle is primarily a protein-rich layer in which proteins bind with calcium ions of the dental hard tissue, and acts as a natural lubricant for the teeth and helps prevent the demineralization of hard tissue (Chawhuaveang et al., 2021; Siqueira et al., 2012). The pellicle forms in a stepwise, specific manner (Siqueira et al., 2012). Its selectively permeable nature allows it to protect the tooth from acidic erosion (Lubarsky et al., 2012). However, this protective mechanism is functional for only 10 minutes (Hara et al., 2006). The pellicle also contains minute amounts of carbohydrates, such as glucose and lactose, which are mainly derived from the diet and act as an energy source for cariogenic bacteria (Enax et al., 2022; Meyer & Enax, 2018).

A biofilm is characterized by microbial communities in which cells adhere to a specific surface or to one another, enveloped in a matrix of extracellular polymeric substances (Donlan & Costerton, 2002). Dental plaque is a biofilm on the surface of teeth, which is composed of a polymeric matrix and bacteria (Socransky & Haffajee, 2002). Dental plaque is formed sequentially and contains a complex structure of microbial community with increased pathogenicity (Socransky & Haffajee, 2002). This plaque primarily mediates dental caries through the enrichment of its microflora, which has strong acid-producing capabilities (Mathur & Dhillon, 2018).

1.2. Prevalence of dental caries

The prevalence of dental caries has been studied extensively worldwide among all age groups. As reported by the 2016 Global Burden of Disease (GBD) study, dental caries affecting permanent teeth ranks as the 11th most common disease worldwide (Roth, 2018; Wen et al., 2022). On average, approximately 15 new cases of dental decay in primary teeth and 27 permanent teeth develop annually per 100 individuals. The disease burden is noted to peak around the age of 25 (Kassebaum et al., 2015). In 2015, the economic burden of dental caries worldwide was estimated to be \$544.41 billion. Regions like North America, Australia, and Western Europe spent the most on dental treatment procedures and loss of dental goods. Furthermore, the study found that dental productivity loss is mainly attributed to factors such as severe periodontitis and dental caries. Additionally, there was a 21% rise in expenditure from 2010 to 2015 (Righolt et al., 2018).

1.3. Etiology and risk factors of dental caries

Dental caries is multifactorial. Its development is primarily associated with three main factors: oral bacteria in dental plaque, the presence of fermentable carbohydrates, and a susceptible tooth surface (Rathee & Sapra, 2019). Additionally, various other factors can influence the rate of tooth decay, such as oral hygiene practices, tooth shape and morphology, surface characteristics, dietary habits, and the qualitative and quantitative characteristics of saliva, such as the flow rate, buffering capacity, and the presence of protective enzymes and molecules (Mathur & Dhillon, 2018). Early genome-wide association studies (GWAS) have linked certain genes of the *WNT* signalling pathway to an increased risk of dental caries (Wang et al., 2012). Additionally, genes such as *HLA*, *CA12*, and *PITXI* have also been identified in caries development (Wang et al., 2012). Mutations in genes such as *AMELX*, which produces amelogenin, and *ENAM*, which produces enamel, can also affect the enamel tissue, increasing the risk of caries. A variation in the genetic makeup of genes like *HLA-DRB1* can alter the host's immune response to cariogenic bacteria (Wang et al., 2020). Genes affecting the quantity and quality of saliva, such as those encoding carbonic anhydrase 6 (*CA6*) and mucins (*MUC* genes), can affect the oral cavity's protective ability against caries (Slobodnikova et al., 2024).

1.4. Microbial etiology of dental caries – pre-molecular era

Understanding the microbiology of dental caries dates back to the late 19th when work by Milles and Underwood demonstrated that bacteria in saliva were capable of

demineralizing dental hard tissues in the presence of carbohydrates (Searle, 1884). However, the aseptic conditions of their experiments were questioned (Ismail et al., 2001).

In 1890, the chemo-parasitic theory of Willoughby Dayton Miller linked lactic acid production from bacteria such as *Lactobacillus* and *Bifidobacterium* from carbohydrate fermentation to demineralization of enamel (Miller, 1890; Spatafora et al., 2024). These bacteria were later cultured from dental caries in 1910 by Percy Howe (Howe, 1917).

In 1924, Kilian Clarke cultured *Streptococcus mutans* from early carious lesions, emphasizing its role in initiating dental caries (Howe, 1917). Later, Theodore Rosebury reported the acidogenic properties and acid tolerance of *Lactobacillus acidophilus* in 1930 (Rosebury, 1932). In early 1960, Paul Keyes and Fitzgerald performed experimental animal studies to establish that caries was caused by an acidogenic *Streptococcus* (Fitzgerald & Keyes, 1960). Later, in the same year, Edwardsson discovered that *S. mutans* isolated from humans was similar to the one in the experimental animal studies done by Keyes and confirmed its strong association with dental caries (Fitzgerald & Keyes, 1960)

Around 1994, the concept of the ecological plaque hypothesis took the forefront of caries-associated microbiology (Marsh, 1994). The ecological plaque hypothesis was based on chemostat studies, which grew nine oral bacteria in glucose-rich conditions at different pHs over varied time periods (Marsh, 1994). This study demonstrated that at a stable neutral pH, there was an increase in acid-producing bacteria but not acid-tolerant, such as *Streptococcus gordonii*. However, under uncontrolled pH conditions of 4.5 or below, there was an increase in acid-tolerant species such as *S. mutans*, *Lactobacillus casei*, and *Veillonella dispar* (Marsh, 1994). The inference of this model was that though all the acid-producing species in the model lowered the environmental pH, only the acid-

tolerant bacteria were able to thrive, and the shift in the oral microbial biofilm's ecology is a complex process (Marsh, 1994). Under healthy conditions, commensal micro-organisms belonging to the genera *Streptococcus*, *Actinomyces*, *Haemophilus*, *Neisseria*, and *Veillonella* colonize the tooth surface (Marsh, 1994). They attach to the enamel pellicle through molecular interactions, establishing a consistent and balanced microbial environment (Marsh & Zaura, 2017; Marsh, 1994). However, excessive dietary carbohydrate consumption disrupts this balance, leading to a significant increase in populations of *S. mutans* and *Lactobacilli* and a reduction in other streptococcal species (Bradshaw & Lynch, 2013; Van Loveren & Lingström, 2015).

1.5. The microbiology of dental caries – molecular era

Over the last two decades, more emphasis has been placed on understanding the etiology of dental caries in the context of the entire microbial community. The communities of micro-organisms, their genetic makeup, and the environment with which they interact are collectively termed the microbiome (Cho & Blaser, 2012). The study of the human microbiome and its role in health and disease has been facilitated by the advent of next-generation sequencing technologies (NGS). NGS can be used with a marker gene such as the 16S rRNA gene that is primarily present in all bacteria and archaea for taxonomy profiling (metataxonomic approach), or the whole genome for functional analysis (metagenomic approach), thus enabling a detailed analysis of the microbial community structure and function (Moussa et al., 2022; Zhang et al., 2020). Applying these technologies and other molecular methods to investigate the microbiome associated with dental caries has resulted in a major paradigm shift. Namely, current evidence indicates

that dental caries results from an imbalance in the composition and function of the microbiome (dysbiosis) involving more species beyond *Streptococci* and *Lactobacilli*, including *Propionibacterium* spp., *Bifidobacterium* spp., *Veillonella* spp., *Actinomyces* spp., *Atopobium* spp., *Selenomonas* and *Prevotella* spp. (Teng et al., 2015). Mechanistically, some of these species, e.g., *Selenomonas sputigena*, *Prevotella salivae*, and *Leptotrichia wadei* have shown to synergistically interact with *S. mutans* (H. Cho et al., 2023).

When 16S rRNA reads were obtained from Whole-Metagenome Sequencing data and analysed in healthy and diseased microbial communities, results showed that bacteria such as *Rothia* and *Aggregatibacter* were strongly associated with healthy oral environments, whereas *S. gordonii*, *Leptotrichia buccalis*, and *Veillonella parvula* were more abundant in individuals with dental caries (Belda-Ferre et al., 2012). In 2013, Simón-Soro et al. (2013) conducted the first metagenomic analysis of the bacterial microbiome at different stages of caries development. In the early stage of caries development, *S. mutans* increased significantly in dental plaque and enamel lesions. However, *S. mutans* wasn't the dominant species in the lesions; *Streptococcus mitis* and *Streptococcus sanguinis* were the most abundant *Streptococci* in the early lesions (Simón-Soro et al., 2013). Analysis of the functional profile of caries-associated bacterial communities showed that genes related to acid stress tolerance and dietary sugar fermentation are predominantly overrepresented during the early stage of enamel caries. In contrast, genes involved in osmotic stress tolerance, collagenases, and other proteases that facilitate dentin degradation are significantly overrepresented in dentin cavities (Simón-Soro et al., 2013). A longitudinal study on early childhood caries using 16S rRNA gene sequencing revealed the role

of *Prevotella* spp. as a significant predictor of early childhood caries by playing a substantial role in carbohydrate-derived acid production (Teng et al., 2015). In 2019, a study that used whole metagenome shotgun sequencing examined supragingival microbiomes from children with and without dental caries and found that *Prevotella*, *Veillonella*, certain *Actinomyces*, and *Atopobium* species were associated with caries, while *Streptococcus* sp. *AS14* and *Leptotrichia* sp. oral taxon 225 were more abundant in caries-free children (Al-Hebshi et al., 2019).

1.6. Modelling the microbiome of dental caries:

The increasing complexity of the dental caries microbiome necessitates microbial models. In vitro or ex vivo models are valuable tools for studying microbial biofilms' establishment, development, and structure (Fernández et al., 2017). They can also be used to study microbial interactions, virulence, drivers of dysbiosis, and response to various agents (antimicrobials, prebiotics, probiotics, etc.) (Ceri et al., 1999; Van Holm et al., 2023). In vitro/ex vivo microbial models related to dental caries can be classified into closed-batch culture models and open continuous culture models (Salli & Ouwehand, 2015). Typically, the former models grow biofilms from a handful of species (up to ten) in a microtiter plate along the plastic surface of the wells themselves or on the surface of hydroxyapatite or bovine enamel discs inserted in the wells (Arthur et al., 2014; van de Sande et al., 2011). Alternatively, biofilms are grown on hydroxyapatite-coated pegs attached to the plate's lid as in the Calgary Biofilm Device (or MBEC device) (Ceri et al., 1999). The main advantages of closed-batch models are simplicity, affordability, the usage of smaller amounts of reagents, and the ability to test several compounds and conditions

simultaneously (reproducibility) (Salli & Ouwehand, 2015; Yu et al., 2017). However, these models are static, i.e., they lack the flow of fluids and shear forces found in in-vivo, creating an artificial environment that may not accurately mimic the complexity of the biofilm development and function (Salli & Ouwehand, 2015; Yu et al., 2017).

The continuous biofilm models use plaque or saliva samples as inoculum and thus support the growth of complex biofilms/microbiomes. They include artificial mouth models and flow cells (Amaechi et al., 2023; Salli & Ouwehand, 2015; Schlafer et al., 2011). The artificial mouth models have a continuous flow of fluid on an open surface, which keeps nutrient concentration constant (Shu et al., 2000; Tang et al., 2003). The flow cell models contain a reactor where the liquid phase moves in a specific direction, and the conditions differ at different sites (Coenye & Nelis, 2010). It helps assess the biofilm formation in real-time. The models grown under such conditions are more likely to mimic the true in vivo interactions of the biofilm as the physical and chemical properties and the nutrients are studied under more natural conditions (Luo et al., 2022). Another advantage of these microbiome models is the replacement of bacteriological culture medium with artificial or human saliva. On the other hand, continuous biofilm models have several disadvantages: they are sophisticated, require large volumes of reagents, are expensive, and of low throughput, i.e., a very limited number of conditions and replicates can be tested simultaneously (Crusz et al., 2012; Salli & Ouwehand, 2015; Yu et al., 2017).

In the context of dental caries, the simplest model involves growing a biofilm from a single species (e.g., *S. mutans*) (Prasanth, 2011; Salli et al., 2016; Zhou et al., 2016). Dual-species biofilm models have also been described as a combination of two cariogenic organisms, *S. mitis* and *S. mutans* and *S. mutans* and *Candida albicans* (Ellepola et al.,

2017). While these models are useful for studying the properties and interactions of individual species, they are an oversimplification of the microbiome associated with dental caries. More complex models employing 6 species have been described, such as the Zurich Biofilm Model (Guggenheim et al., 2004). This model describes different bacterial species' complex spatial arrangement and interactions within the biofilm (Guggenheim et al., 2004). This model is more realistic for the oral cavity than the single and dual-species model. Yet, it does not fully capture the saliva flow and immune response of the host and lacks continuity in the nutrient flow (Guggenheim et al., 2004).

As a compromise, several models have been recently developed in which biofilms/microbiomes are generated in vitro from clinical inocula (saliva/plaque) in microtiter plates or MBEC devices, combining the simplicity and high throughput nature of closed-batch models with the complexity of microbiomes generated with continuous culture models. For example, Dame-Teixeira *et al.* (2024) developed an in vitro dysbiotic biofilm model to mimic root caries and assess microbial modulation strategies using natural compounds, such as cranberry, by cultivating mature biofilms in the Calgary Biofilm Device (Dame-Teixeira et al., 2024). A summary of dental caries-related microbial biofilm/microbiome models and their characteristics are provided in Table 1.

Table 1. Overview of dental caries-related microbial biofilm models.

Biofilm model	Characteristics	Microorganisms included in the model	Reference
Zürich Biofilm Model	The biofilm is formed in microplates where bacteria form biofilms on the bottom of the wells or on enamel discs	<i>Streptococcus oralis</i> , <i>Streptococcus sobrinus</i> , <i>Actinomyces naeslundii</i> , <i>Veillonella dispar</i> , <i>Fusobacterium nucleatum</i> , <i>Candida albicans</i>	(Guggenheim et al., 2004)
Flow Cell Biofilm Model	It consists of unidirectional flow of nutrients	<i>Streptococcus gordonii</i> , <i>Actinomyces naeslundii</i> , <i>Veillonella atypica</i> , <i>Fusobacterium nucleatum</i>	(Foster & Kolenbrander, 2004; Palmer, 1999)
Continuous Culture Model	The biofilm is grown on a defined surface with a continuous nutrient flow	<i>Streptococcus oralis</i> , <i>Streptococcus sanguinis</i> , <i>Streptococcus mitis</i> , <i>Streptococcus downei</i> , <i>Actinomyces naeslundii</i>	(Hodgson et al., 2001)
Constant Depth Film Fermenter	It consists of a controlled depth biofilm with a continuous flow of nutrients	<i>Streptococcus oralis</i> , <i>Actinomyces naeslundii</i> , <i>Veillonella parvula</i> , <i>Fusobacterium nucleatum</i> , <i>Aggregatibacter actinomycetemcomitans</i> , <i>Porphyromonas gingivalis</i>	(Pratten, 2007)

Table 1. (continued)

Biofilm model	Characteristics	Microorganisms included in the model	Reference
Artificial Mouth Model (AMM)	There is a continuous flow of nutrients	<i>Streptococcus mutans</i> , <i>Streptococcus sobrinus</i> , <i>Actinomyces naeslundii</i> , <i>Lactobacillus rhamnosus</i>	(Lee et al., 2010)
Calgary Biofilm Device or microtiter plate	Biofilms are grown on the surface of pegs attached to the lid or in the bottom of wells	Salivary inoculum	(Azeredo et al., 2017; Ceri et al., 1999)

1.7. Gap of knowledge and aim of study

Despite an extensive number of models that have been developed, there remain several gaps in the literature. None of the previously described models includes a reference healthy (normobiotic) microbiome along with the dental-caries associated (dysbiotic) microbiome. Furthermore, there have been few attempts to identify the optimal medium and growth conditions for maximizing similarity to the in vivo microbiomes.

The aim of this study is to:

- 1- Develop an ex vivo model that replicates health- and dental caries-associated microbiomes in parallel, and
- 2- Directly compare nutrient-rich and nutrient-limited media to assess their ability to support the growth of microbiomes similar to the in vivo microbiomes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Ethical review

The study was performed in accordance with the Declaration of Helsinki and approved by the school's Institutional Review Board (protocol No. 26456). Informed written consent was obtained from all subjects who donated saliva.

2.2. Study design

The flow chart presented in Figure 1 illustrates the experimental design and the microbiome model used. Saliva samples were collected from 5 caries-free individuals and 4 individuals with dental caries. DNA was extracted from an aliquot of each sample (200 μ L), and the levels of *S. mutans* were quantified. Based on this quantification, healthy samples with less than 1,000 CFU/mL were pooled and used as an inoculum for the normobiotic microbiomes. The same inoculum was spiked with *S. mutans* and used to grow another set of microbiomes (to assess if *S. mutans* induces dysbiosis). Saliva samples with more than 500,000 CFU/mL were pooled and used as an inoculum for the dysbiotic (caries-associated) microbiomes. The microbiomes were grown in two types of media: sterile saliva (SS) with 0.1% sucrose and SHI with 0.1% sucrose. The culture was grown on MBEC plates and harvested after 24 hours, 48 hours without changing the media, and 48 hours with a media change. The grown biofilm was then harvested for biomass determination and microbiome analysis. Details of the experiments and methods are presented below.

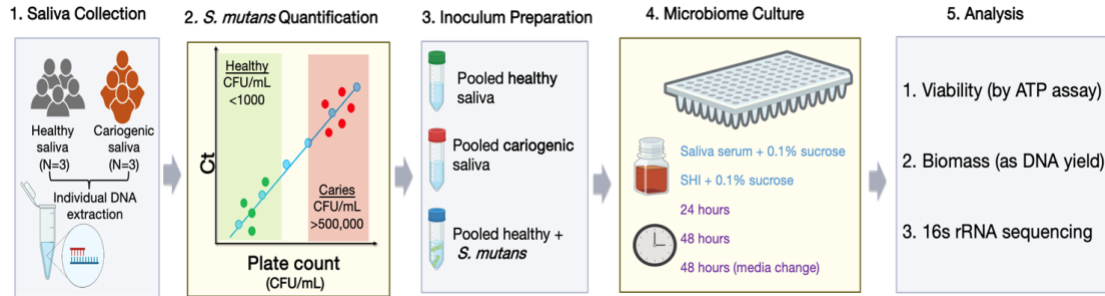


Figure 1. Flow chart of the experimental design and microbiome model. Ex vivo microbiomes were grown from three types of inocula (health-associated saliva, caries-associated saliva, health-associated saliva spiked with *S. mutans*) in sterile saliva (SS) and SHI media supplemented with 0.1% sucrose. Biofilms were harvested after 24 h, 48 h, and 48 h after a media change.

2.3. Preparation of the inocula

2.3.1. Saliva samples:

For preparation of the inocula, unstimulated saliva samples were collected from 5 patients with no dental caries (healthy) and 4 patients diagnosed with dental caries at the Kornberg School of Dentistry's clinics. Up to 5 mL of saliva was collected from each patient, immediately placed on ice, and transported to the lab.

2.3.2. Quantification of *Streptococcus mutans*:

Prior to starting the model, quantitative polymerase chain reaction (q-PCR) was employed to assess the quantity of *S. mutans* in the collected salivary samples. DNA was extracted from 200 μL of each saliva sample using the PureLink Microbiome DNA Purification kit (A29790, Invitrogen), following the manufacturer's instructions. Bead beating was done for 5×600 s cycles at 6 m/s (MP Biomedicals FastPrep). Between beating cycles, samples were placed on ice for 2 min. The q-PCR reaction was prepared by

mixing 10 μL of the PowerUp SYBR Green Master Mix, (Applied Biosystems), 2 μL of the DNA template (or negative control), and 3 μL *S. mutans*-specific primers (3 μM) (Chen et al., 2007) following the manufacturer's guidelines for PowerUp™ SYBR™ Green Master Mix. A standard curve was generated by preparing six 10-fold serial dilutions of DNA extracted from 200 μL of a *S. mutans* (UA159) solution with a known colony-count ($\sim 8 \times 10^8$ CFU/mL). Amplification was carried on a QuantStudio™ 3.0 Real-time PCR system (Applied Biosystems), including an initial uracil-DNA Glycosylase (UDG) activation at 50°C for 2 min, an enzyme activation cycle at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 1 min. The cycle threshold (Ct) values were recorded and analysed using the DA2 software (Applied Biosystems). The bacterial load in the salivary samples was calculated by correlating the cycle threshold (Ct) of each salivary sample with the corresponding CFU count on the standard curve.

2.3.3. Saliva pooling:

Saliva samples that guarantee an average *S. mutans* count $>500,000$ CFU/mL were pooled and used to grow dysbiotic (caries) microbiomes. To grow normobiotic microbiomes (health), salivary samples that guarantee an average *S. mutans* count <1000 /mL were pooled (Figure 1). A third set of microbiomes was grown using the pooled healthy saliva spiked with 1×10^6 CFU/mL of *S. mutans* cells. Spiking of healthy saliva with *S. mutans* involved the preparation of a *S. mutans* solution in Brain Heart Infusion (BHI) medium. The optical density (OD₆₀₀) of the solution was adjusted to 0.7. Then, 6.25 μL of the *S. mutans* solution was added to each mL of saliva. This rendered a saliva with a final concentration of *S. mutans* of $\sim 1\text{M}$ CFU/ml.

2.4. Growth media

The study evaluated two types of growth media: SHI media supplemented with 0.1% sucrose and SS supplemented with 0.1% sucrose.

2.4.1. SHI media preparation:

The SHI medium has been previously shown to grow a diverse community of species from pooled saliva that closely resemble oral microbiota (Tian et al., 2010). The composition of the medium includes proteose peptone 10 g/L; trypticase peptone 5.0 g/L; yeast extract 5.0 g/L; KCl 2.5 g/L; hemin 5 mg/L; Urea 0.06 g/L, arginine 0.174 g/L; mucin 2.5 g/L; sheep blood; 5% and *N*-acetylmuramic acid (NAM) 10 mg/L (Tian et al., 2010). For preparation, the components of the media were mixed with DI-water and autoclaved at 121°C for 15min. After sterilization, the media was cooled down to 50°C. Then, the sheep blood and NAM (N-Acetyl Muramic Acid) were added. SS was prepared as explained earlier. Supplementation was made by adding 1 g/L of sucrose. After preparation media was stored at -20°C until use.

2.4.2. Sterile saliva preparation:

Saliva for media preparation and saliva coating was collected using previous protocols (Fischer & Aparicio, 2021). Saliva was collected from 15 young, healthy donors who did not have any active caries or periodontitis, aged between 20 to 30 years, using a sterile 50 mL centrifuge tube. The donors refrained from eating food or taking liquids 2 hours prior to donation. Furthermore, the donors were free of systemic disease and not under any antibiotics. The collection time for saliva was less than 30 minutes. The collected saliva was immediately placed on ice and transported to the laboratory. Upon arrival, all

the collected saliva was pooled and centrifuged at 5,000 rpm for 15 minutes. The resulting pellet was discarded. To reduce viscosity, dithiothreitol (DTT) was added to the saliva at a final concentration of 2.5 mM before adding an equal volume of phosphate-buffered saline (PBS) (Fischer & Aparicio, 2021). The resulting mix was filter sterilized using a disposable vacuum filter with a 0.22 µm mesh filter. The saliva was stored at -80°C, in aliquots of 10 mL to prevent freezing/thawing cycles.

2.5. Biofilm Growth:

The microbiomes were grown in a Calgary device, currently called Minimum Biofilm Eradication Concentration (MBEC) plates, with hydroxyapatite-coated pegs (Innovotech, Canada). The pegs were first pre-conditioned by immersing them in SS (180 µL in each well) overnight under anaerobic conditions at 37°C (5 % H₂, 5 % CO₂, 90 % N₂). Later, in a separate plate, 170 µL of pre-reduced culture medium (SHI or SS) and 10 µL of the inoculum (Health, *S. mutans*-spiked, or caries) were added according to the layout presented in Figure 2. To avoid media evaporation, 180 µL of PBS was added to the unused wells. The lid with the pre-conditioned pegs was placed on the baseplate with the growth media. The plates were incubated under anaerobic conditions at 37°C. The microbiome was allowed to grow for durations of 24 or 48 hours with or without replenishment of the medium.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			H1	H2	H3		H1	H2	H3			
C			C1	C2	C3		C1	C2	C3			
D			M1	M2	M3		M1	M2	M3			
E												
F			NTC1	NTC2	NTC3		NTC1	NTC2	NTC3			
G												
H												

Sterile saliva + 0.1% sucrose
 SHI + 0.1% sucrose
 PBS

Figure 2. Experimental layout. Six such plates were used for growing the microbiome at 24 hours, 48 hours without changing the media, and 48 hours with changing the media in two sets (one for viability analysis and another for microbiome analysis). Three technical replicates were carried out for each condition. H= Healthy saliva inoculum, C= cariogenic inoculum, M= Healthy inoculum spiked with *S. mutans*, NTC= negative control.

2.6. Viability assessment

The microbiomes were assessed for their viability using the ATP assay (BacTiter-Glo assay, Promega, USA) based on the instructions given by the manufacturer. Briefly, after the incubation period, pegs were washed gently with PBS and transferred to a 96-well plate with 100 μ l of PBS and 100 μ l of the ATP reagent. The plate was then incubated for 5 minutes in the dark and under aerobic conditions. A multi-mode microplate reader (Synergy HTX, Biotek, USA) recorded the luminescence signal.

2.7. DNA extraction and 16S rRNA gene preamplification

The PureLink Microbiome DNA Purification kit (A29790, Invitrogen) was used to extract DNA from the biofilms by performing bead-beating on the pegs directly. Qubit Fluorometric Quantification was used to assess the DNA yield and quality. Microbiome biomass was measured as DNA yield in nanograms (ng). In preparation for sequencing, a pre-amplification step was performed using 25 cycles with the degenerate primers 27FYM (Frank et al., 2008) and 519R (Lane et al., 1985), and the Platinum™ SuperFi™ II PCR cat.no. 12368010 master mix. The PCR product checked using agarose gel electrophoresis.

2.8. Sequencing and bioinformatic analysis

For sequencing, degenerate primers 27FYM (Frank et al., 2008) and 519R (Lane et al., 1985) with index sequences were used to amplify the V1-V3 region of the 16S rRNA gene. The resultant indexed amplicon libraries were sequenced on an Illumina Miseq platform using 2*300 bp chemistry at the Integrated Microbiome Resource (IMR, Halifax, Canada). Resultant paired-end reads were merged with PEAR (Zhang et al., 2014) and then pre-processed (trimming, quality filtration, and chimera check) with mortar (Schloss et al., 2009) as previously described (Al-Hebshi et al., 2017). The high-quality reads were then classified to the species level using our previously described BLAST n-based algorithm (Al-Hebshi et al., 2015; Al-Hebshi et al., 2017). Taxonomy tables and alpha diversity analysis were generated using Quantitative Insights into Microbial Ecology (QIIME2) (Caporaso et al., 2010). The observed species, Chao index, and Shannon indexes were calculated to measure alpha diversity. For assessing beta diversity, principal component analysis (PCA) was performed on centred log-ratio (CLR)-transformed species counts

using microbiome (Xia et al., 2018) and phyloseq (McMurdie & Holmes, 2013) packages in R. MaAsLin2 (Microbiome Multivariable Associations with Linear Models) package in R (Mallick et al., 2021) were used to identify differentially abundant genera and species between testing groups. The Euclidean distances based on centred log-ratio (CLR) data were computed and used to calculate the similarity index as: $s = 1 - d_norm$, where s is in the range $[0, 1]$, with 1 denotes highest similarity (the items in comparison are identical), and 0 denotes lowest similarity (largest distance). d denotes distance, s denotes similarity. To convert distance measure to similarity measure, we need to first normalize d to $[0, 1]$, by using $d_norm = d/\max(d)$.

2.9. Statistical tests

Statistical differences in the results (DNA biomass, alpha diversity, and similarity index) were evaluated using Multifactor Analysis of Variance (ANOVA). Finally, Adonis PERMANOVA (Permutational Analysis of Variance) was used to estimate the differences in beta diversity with a significance of 0.05.

CHAPTER 3

RESULTS

3.1. Microbiomes Biomass and viability

The microbial DNA biomass and viability of the microbiomes formed using different media, growth time, and inoculum were assessed (Figure 3). Statistically significant differences in the microbiome's biomass in terms of the media, growth time, and inoculum were observed ($p < 0.05$). Regardless of the inoculum type, the highest biomass was noted in microbiomes cultured in SHI media for 48 h with a media change. In addition, microbial biomass significantly increased as a function of growth time in the SS caries-derived microbiomes. The microbiome viability showed significant differences in terms of the media, growth time, and inoculum ($p < 0.05$). Overall, SHI was associated with a significant drop in cell viability after 48 h of incubation (with or without media change). Overall, the highest viability was observed for microbiomes grown in SS for 48h with medium change. As an exception, very high viability was measured after 24 h on the caries-derived microbiomes.

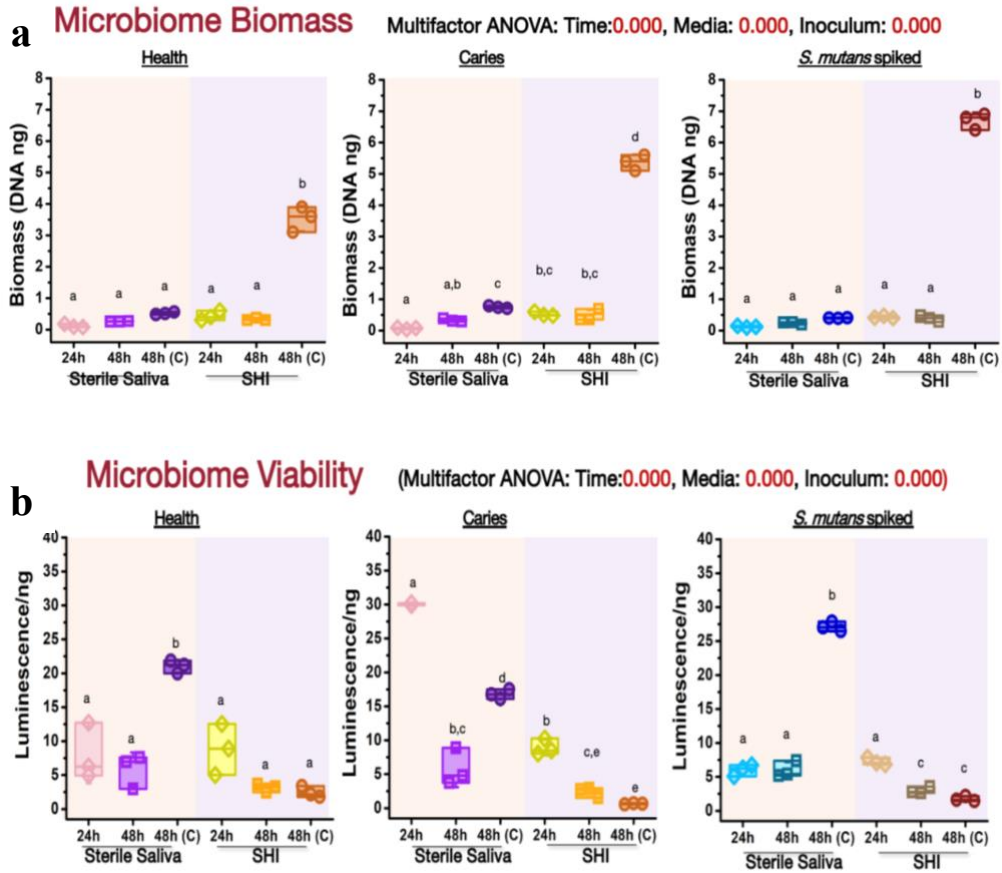


Figure 3. Microbial biomass and viability of microbiomes grown in the different media.

(a) The DNA biomass of the microbiomes was measured as the DNA yield in nanograms (ng). (b) Viability of the microbiomes. The relative luminescence was normalized with the microbiome biomass. Means with different letters are significantly different ($p \leq 0.05$). 24h: 24 hours without media change, 48h: 48 hours without media change, 48h (C): 48 hours with a media change.

3.2. *S. mutans* quantification results

The standard curve generated from serial dilution of *S. mutans* DNA is presented in Figure 4, showing excellent correlation ($R^2= 0.99$) and primer efficiency (92.3%). The bacterial load in the salivary samples was calculated by correlating each salivary sample's

cycle threshold (Ct) with the corresponding CFU count on the standard curve. 3 of the 5 samples of healthy saliva, when pooled, showed a *S. mutans* count <1000/ml. These samples were pooled together to grow the normobiotic microbiomes. Three out of the 4 caries-associated saliva samples had an *S. mutans* count >500,000 CFU/mL and were pooled together to grow dysbiotic microbiomes.

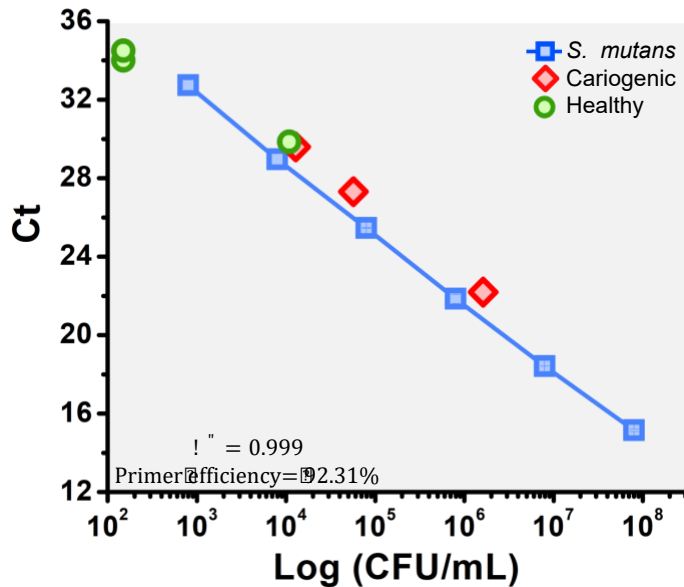


Figure 4. Standard curve. Standard curve representing the relationship between the log (CFU/mL) and threshold cycle (Ct) after running a quantitative Polymerase Chain Reaction (qPCR) using *S. mutans*-specific primers. The plot of the qPCR was generated using 10-fold serial dilutions of UA159 strain of *S. mutans*. The plot also depicts the regression equations and coefficient of determination (R^2) that was used in the experiment.

3.3. Species Richness and Alpha Diversity Analysis

The microbiome's alpha diversity was estimated using the Shannon (richness and evenness) and Chao (expected richness) indexes (Figure 5). A higher Shannon index was

measured in the healthy clinical inoculum (3.53) compared to the ex vivo healthy microbiomes (3.14). A higher Shannon index is related to a more diverse community. The opposite was observed for the caries and *S. mutans*-spiked microbiomes (3.04 for the clinical inoculum and 3.17 for the cultured microbiomes). The highest Shannon index was measured for caries-derived microbiomes grown in SS for 24 hours. The lowest Shannon indexes were measured in healthy-derived microbiomes grown in SS for 48 h with media change, caries-derived microbiomes grown in SHI for 48 h, and *S. mutans*-spiked derived microbiomes cultured in SHI for 48 h (~2.83).

Overall, a similar Chao index was observed for both clinical inoculums (135). Chao index measures the true species richness in a community, accounting for rare or undetected species. No significant differences were observed in the Chao index of the healthy microbiomes, regardless of the media and growth time (135). However, slight changes were observed in caries and *S. mutans*-spiked derived microbiomes. The highest Chao index was measured for *S. mutans*-spiked microbiomes cultured in SHI and 24 h (156). The lowest index was found in caries microbiomes grown in SS for 48 h and media change (129).

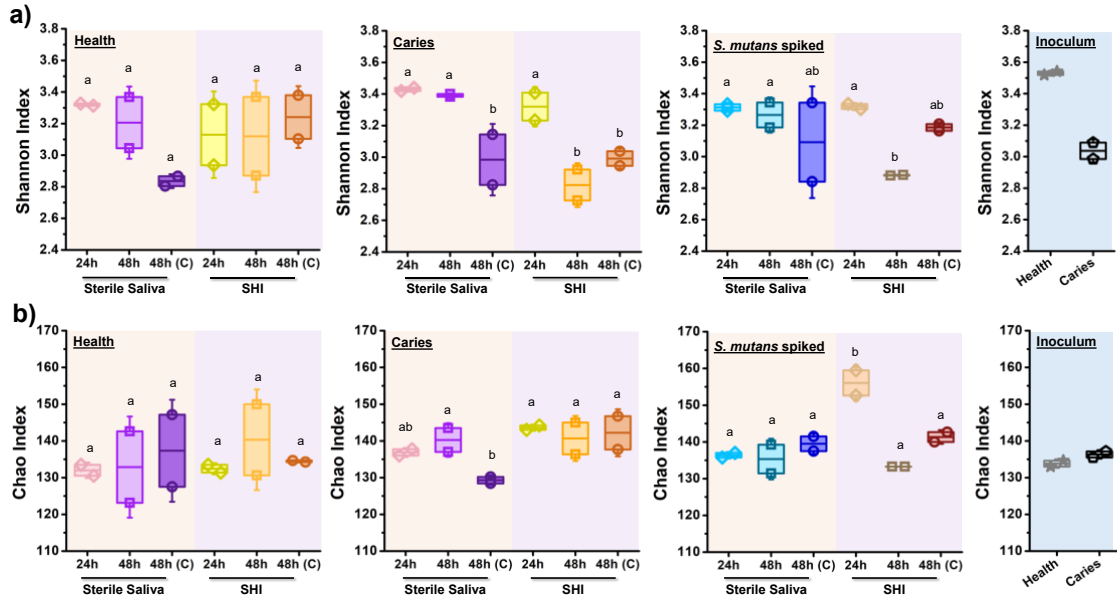


Figure 5. Species richness and alpha diversity analysis. Diversity of the healthy, cariogenic, and *Streptococcus mutans*-spiked derived microbiomes as a function of the used media and incubation time. The alpha diversity metrics included the a) Shannon and b) Chao indexes. Means with different letters are significantly different ($p \leq 0.05$).

3.4. Beta Diversity Analysis

Microbiome compositional variations (beta diversity) associated with the inoculum, time, and growth media are presented in Figure 6 and 7. The x-axis represents the first principal component (PC1) with a total variation of 24.9% in the bacterial community data. The y-axis represents the second principal component (PC2) with a total variation of 20.7% in the bacterial community data. Figure 6 represents the effect of inoculum on the experimental groups. Regardless of the growth conditions, the health-, and caries-derived microbiomes clustered separately (PERMANOVA, $p: 0.002$), along with their respective inocula. However, there wasn't a statistical difference in composition of microbiomes grown from the health-associated inoculum with or without *S. mutans*

(PERMANOVA, $p > 0.05$). Figure 7 represents the effect of medium type and time on the experimental groups.

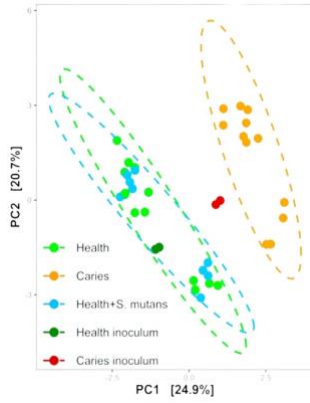


Figure 6. Principal Component Analysis (PCA) Plot for the effect of inoculum. The PCA plot is based on centred log-ratio transformed data, which is a function of the effect of the inoculum.

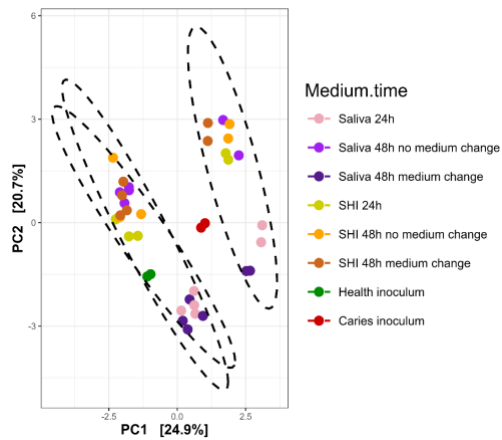


Figure 7. Principal Component Analysis (PCA) Plot for effect of medium type and time. The PCA plots are based on centred log-ratio transformed data, which is a function of the effect of the medium type and time.

3.5. Microbiome profiles of the ex vivo microbiomes compared to clinical inocula

In the health inoculum, 131 species, distributed in 7 phyla and 36 genera, were observed. A total of 130 species distributed in 6 phyla and 33 genera were identified in the caries inoculum. In the *ex vivo* grown microbiomes, the number of observed species differed by media, growth time, and inoculum. The lowest number of species were observed in healthy microbiomes grown in SS for 48 h with a media change (126 species) and the highest on SHI caries microbiomes grown for 24 h (139 species). The most dominant phyla in the healthy inoculum included Firmicutes (70%), Bacteroidetes (11%), Proteobacteria and Actinobacteria in the same proportion (9%), and Fusobacteria (1%). The caries clinical inoculum included Firmicutes (57%), followed by Bacteroidetes (27%), Actinobacteria (9%), Proteobacteria (6%), and Fusobacteria (1%) (Figure 8). At the phylum level, the health-derived microbiomes were dominated by Firmicutes (86%), Bacteroidetes (10%), and Proteobacteria (3%). Compared to the healthy clinical inoculum, all healthy *ex vivo* microbiomes showed increased levels of Firmicutes at the expense of Actinobacteria and Proteobacteria, except for *S. mutans*-spiked microbiomes grown in SHI for 48 h without media change. Compared to the caries inoculum, these microbiomes showed a decrease in Firmicutes (54%) and Bacteroidetes (5%) but increased levels of Proteobacteria (29%). Overall, all *ex vivo* microbiomes showed reduced levels of Actinobacteria bacteria.

At the genus level, the dominant genera in the healthy clinical inoculum were *Streptococcus* (60%), followed by similar quantities of *Prevotella*, *Neisseria*, *Veillonella*, and *Actinomyces* (8%). In the caries inoculum, *Streptococcus* (49%), *Prevotella* (24%), *Actinomyces* (5%), *Veillonella* (5%), and *Neisseria* (4%) accounted for 83% of the genus

population (Figure 8). Both healthy and *S. mutans*-spiked microbiomes showed similar genus distribution, comparable to the respective clinical inoculum in terms of *Streptococcus* (56%), and *Prevotella* (9%), but with expansion of *Veillonella* (21%) and depletion of *Actinomyces* and *Neisseria*. Caries-derived microbiomes comparable levels of *Streptococcus* (44%) and *Prevotella* (20%) to the caries inoculum, but increased levels of *Veillonella* (6%) and failure to capture *Actinomyces* and *Neisseria*. The highest deviation from the clinical inoculum was observed in the *S. mutans*-spiked microbiomes grown in SHI for 48 h without media change (Figure 8).

At the species level, the average health inoculum was dominated by *Streptococcus salivarius/vestibularis* (33%), *Neisseria flavescens/subflava* (8%), *Prevotella melaninogenica* (6%), and *Veillonella parvula* group (6%), together making ~52 % of the inoculum (Figure 8). The caries inoculum was composed mainly of *S. salivarius/vestibularis* (18%), *Prevotella sp. oral taxon 313* (11%), *S. parasanguinis II* (6%), and *Streptococcus sp. oral taxon 431* (5%). Again, healthy and *S. mutans*-spiked microbiomes showed similar bacterial distribution, mainly composed of *S. salivarius/vestibularis* (9%), *Prevotella melaninogenica* (5%), and *V. parvula* group (15%). Caries-derived microbiomes showed to be more similar to their clinical inoculum and composed of *S. salivarius/vestibularis* (19%), *Prevotella sp. oral taxon 313* (7%), *S. parasanguinis II* (4%), *P. melaninogenica* (7%). Both health- and caries-derived microbiomes showed depletion of *Neisseria flavescens/subflava* and *Actinomyces odontolyticus*.

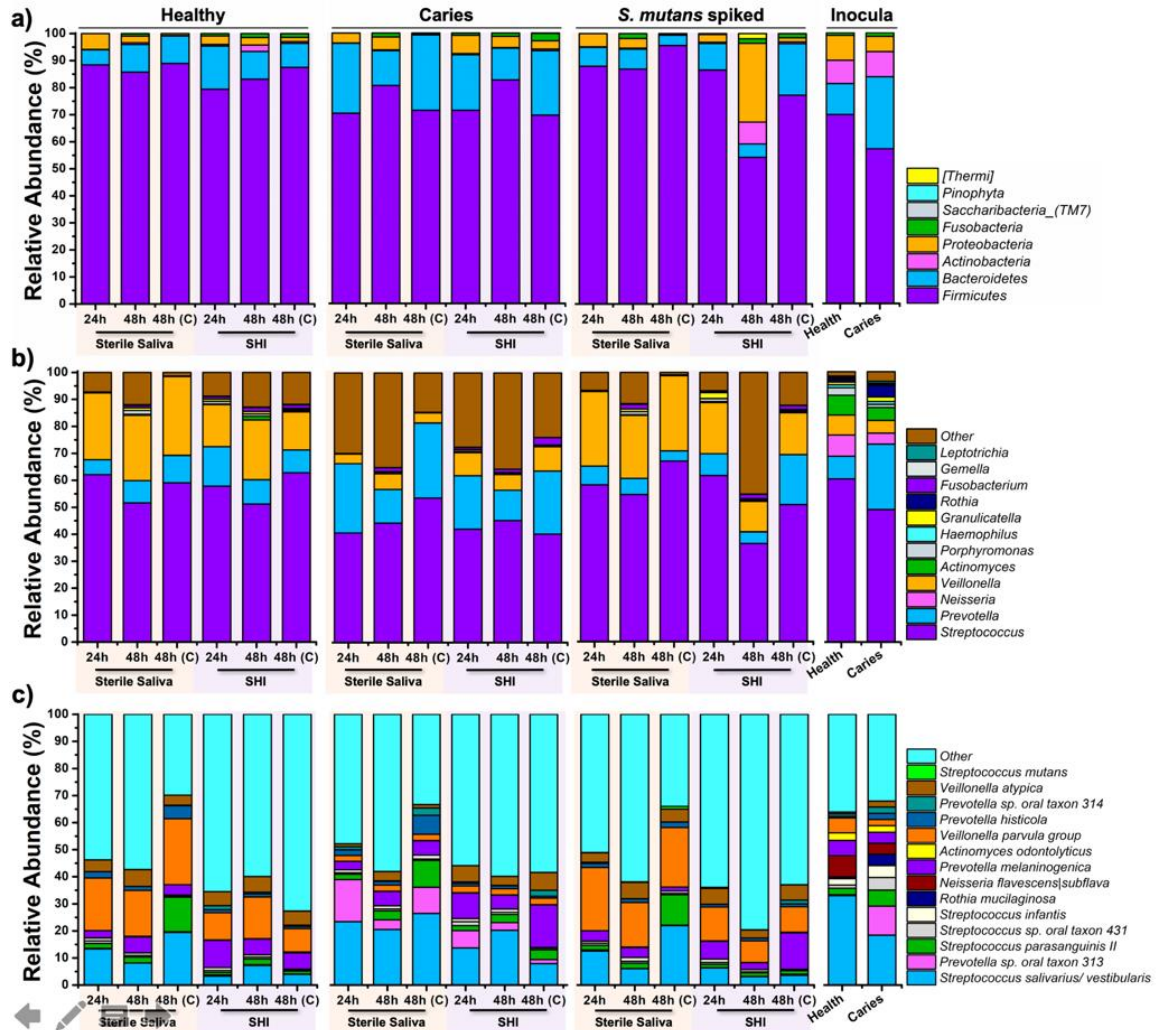


Figure 8: Microbial taxonomy profiles. Microbial taxonomy profiles of healthy, caries-derived, and *Streptococcus mutans*-spiked derived microbiomes grown in different media and compared to the clinical inoculum. Data is presented at the a) phylum, b) genus, and c) species levels. Taxa are ordered by abundance with respect to the clinical inoculum.

3.6. Effect of media, growth time, and inoculum on specific bacterial species

Differential abundance analysis was conducted to identify statistically significant differences (FDR ≤ 0.05) in the abundances of the genus and species (Figure 9) between the health and caries-derived microbiomes (groups in which we found statistical differences in the PERMANOVA analysis). At the genus level, caries-derived microbiomes significantly enriched *Selenomonas*, *Prevotella*, and the *Catonella* genera. Health-derived microbiomes mainly enriched the *Veillonella* and *Streptococcus* genera. At the species level, caries-derived microbiomes enriched newly identified caries-related species including several *Selenomonas spp.* and *Prevotella spp.*, besides *Streptococcus anginosus*, and *Alloprevotella rava*. Healthy-derived microbiomes enriched mainly commensal species including *S. vestibularis*, *S. oralis*, and *Granulicatella elegans*. These results suggest that the model was able to identify microbial differences between the health and caries -derived microbiomes that are consistent with known differences in the cariogenic and healthy saliva.

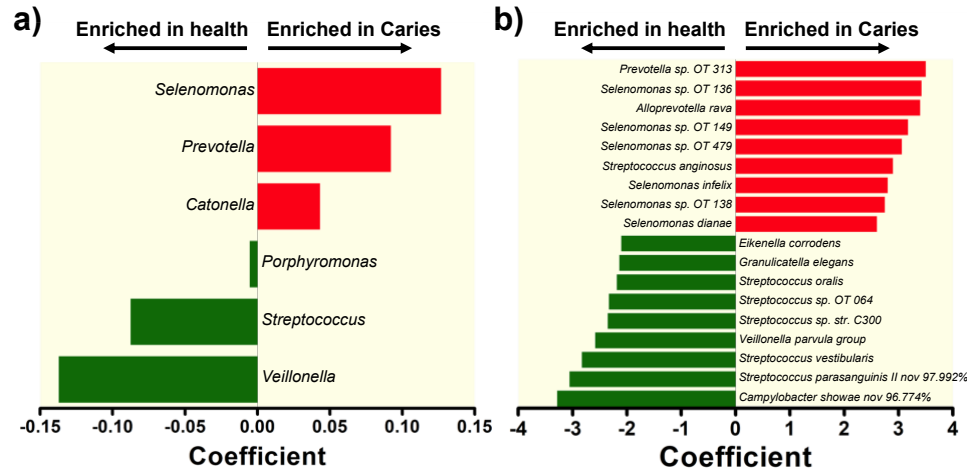


Figure 9. Effect of media, growth time, and inoculum on specific bacterial species. Centred log ratio (CLR) transformed data were analysed with Multivariate Association with Linear Models (MaAsLin2) to identify differentially abundant a) genus and b) species in the healthy and caries-derived microbiomes. Differences with $FDR \leq 0.05$ were considered significant.

3.7. Similarity of the microbiomes with the clinical inoculum

The similarity of the ex vivo microbiomes with their respective clinical inoculum (healthy or caries) was measured using a similarity index (Figure 10). A higher index indicates higher similarity with the clinical inoculum. Overall, higher similarity with the clinical inoculum was measured in the healthy-derived microbiomes. For both healthy and caries-derived microbiomes, the highest index was observed when microbiomes grew in SHI media for 24 h. However, statistical differences among groups were not observed. Increasing the incubation time in SHI to 48 h resulted in a slightly decreased similarity (regardless of whether the media changes). The lowest similarity was observed in caries-derived microbiomes grown in SS for 48 h without media change.

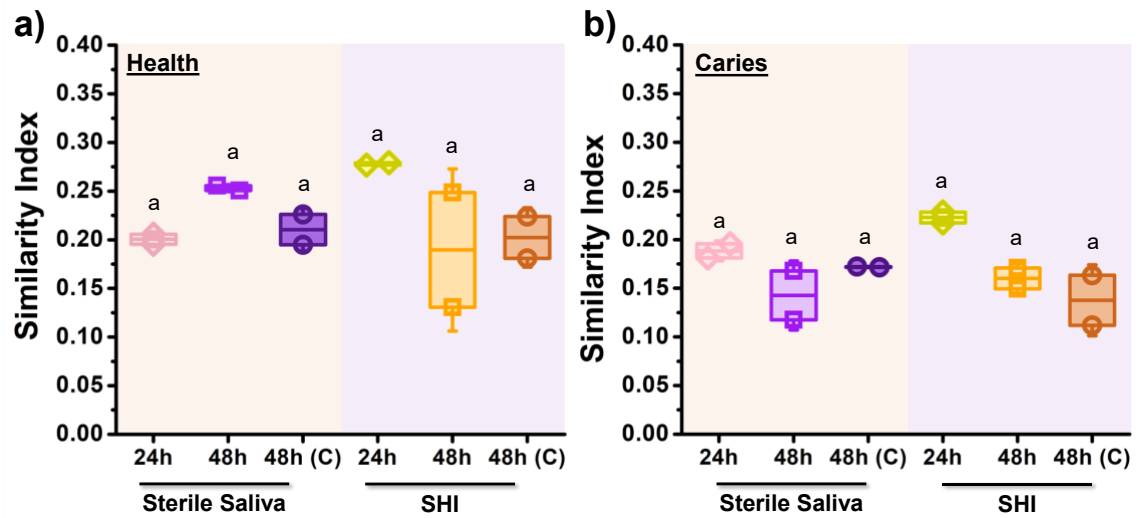


Figure 10. Similarity index. Similarity index of the health and caries-derived microbiomes to their respective clinical inoculum. Higher index indicates a higher similarity to the clinical inoculum. Means with different letters are significantly different ($p \leq 0.05$).

CHAPTER 4

DISCUSSION

The oral microbiome plays an important role in maintaining the oral health and disease progression, with microbial diversity and composition serving as key indicators of dysbiosis (Maier, 2023). In vitro/ex vivo modelling of the oral microbiome associated with dental caries provides a valuable tool for studying microbial interactions with the environment and assessing factors such as nutrient availability, drivers of dysbiosis, and biofilm-formation dynamics. In this study, we developed an ex vivo model by growing health- and caries- derived microbiomes under different conditions.

Based on the results, microbiomes grow more robustly (higher DNA yield) under nutrient-rich conditions (SHI) specially when incubated for 48 h with media replenishment (Figure 2). However, it's important to note that those microbiomes were less viable than the ones cultured in SS especially when grown for 48 h even with media replenishment (Figure 2). SHI is a nutrient-rich media that promotes rapid microbial growth, which explains the higher biomass (Lamont et al., 2021). The SHI medium closely mimics the carbohydrate rich environment of the oral cavity (Tian et al., 2019), thus promoting a rapid initial growth of the microbiome; however, it fails to sustain viability over an extended time period, possibly due to the accumulation of metabolic byproducts (e.g., acids, ROS), toxic microenvironments, osmotic or oxidative stress (Görlach et al., 2015), and rapid nutrient depletion. The results highlight the importance of balancing between biofilm biomass and viability when designing experiments. For example, SS may be more suitable for studies requiring the generated biofilms to be viable, for example antimicrobial assays.

The stability of alpha diversity across the different experimental conditions contrasts (Figure 5) with the changes in the bacterial composition (Figure 8), indicating that while taxonomic composition changes, overall diversity is maintained. This aligns with the findings in chronic disease microbiomes, where dysbiosis involves taxon replacement rather than diversity loss (Peterson et al., 2009). Principal component analysis (PCA); however, (Figure 6,7) revealed distinct clustering of health- and caries-derived microbiomes, along with their respective inocula, demonstrating that the model successfully recapitulated the differences between health and disease associated microbiomes. The clustering was observed regardless of medium type, which suggests that these communities maintain uniqueness despite different environmental conditions. Our findings align with a study done by Gross et al., where the principal component analysis in their study revealed a distinct separation between the microbiomes of caries affected and caries-free individuals, emphasizing that these communities can be distinguished regardless of the environmental variation (Gross et al., 2012).

Interestingly, no significant microbial shift was observed when spiking the healthy inoculum with *S. mutans* (Figure 6,7)., which is not consistent with its claimed role as a keystone cariogenic bacterium and driver of dysbiosis (E. Hurley et al., 2019). It is generally believed that health-associated oral microbiomes exhibit resilience, allowing them to maintain stability and absorb ecological pressures, thus preventing dysbiosis (Rosier et al., 2018). *S. mutans*, which is usually a commensal microorganism, requires interactions with other microbial species, such as *Selenomonas*, to effectively drive dysbiosis (Hunyoung Cho et al., 2023). Existing literature states that the presence of *S. mutans* does not always correlate directly with caries progression, as other microbial

species significantly contribute to the disease process (Becker et al., 2002; Corby et al., 2005).

Another interesting finding was the enrichment of *Prevotella* and *Selenomonas* spp. in caries-derived microbiomes (Figure 9). This aligns with very recent literature that demonstrated their involvement in caries-associated dysbiosis. Although these species have traditionally not been considered as cariogenic pathogens, high-throughput sequencing has identified their presence in caries-active populations (Chen et al., 2021; Yufen Niu et al., 2023). Furthermore, in vitro and ex vivo experiments showed that they have synergistic interactions with *S. mutans* in dental caries (Hunyong Cho et al., 2023; Y. Niu et al., 2023). Several studies have shown the role of *P. denticola* in both saliva and plaque of children with early childhood caries (Eimear Hurley et al., 2019; Kressirer et al., 2018; Zhang et al., 2020). *P. denticola* influences the virulence properties of biofilms by increasing the carbohydrate metabolism and acid productivity (Yufen Niu et al., 2023). Although *Prevotella ssp.* alone exhibits lower acidogenicity compared to *S. mutans*, co-culture studies have shown that the presence of *Prevotella* increases lactate secretion, which is a key metabolite in enamel demineralization (Kianoush et al., 2014; Yufen Niu et al., 2023). Similarly *S. sputigena*, as found to increase *S. mutans* biofilm virulence (Hunyong Cho et al., 2023). In the presence of *S. mutans*, *Selenomonas* species become entrapped within the exopolysaccharide (EPS) matrix produced by glucosyltransferases of *S. mutans*, losing motility and forming a honeycomb-like biofilm structure (Hunyong Cho et al., 2023). This forms densely packed biofilm superstructures that promote acidogenesis and aciduricity, which are key factors in enamel demineralization (Cai & Kim, 2023; Cho et al., 2022).

Overall, the proposed model captured both health-associated and caries-associated microbiomes. The results showed the highest similarity with the clinical inocula (healthy or caries) is found for microbiomes cultured for 24 h in SHI media (Figure 10). Nevertheless, the study has some limitations. Firstly, the study used saliva samples from only 5 healthy individuals and 4 individuals with dental caries for the inoculum preparation. A larger sample size could enhance the generalizability of the findings. Secondly, while the study attempted to mimic the oral environment, the in vitro model does not fully replicate the complexity of the oral cavity, including host immune responses, salivary flow, and mechanical forces like mastication. Lastly, the lack of microbial shift despite spiking with *S. mutans* suggests that additional factors, such as prolonged sugar exposure or increased *S. mutans* quantity may be required to induce dysbiosis. Future research should address the limitations of this study to increase applicability of the findings. Increasing the sample size for inoculum collection would improve the generalizability of the results. Additionally, incorporating a flow cell system could facilitate a continuous nutrient supply, better simulating the dynamic oral environment. Since sucrose concentration plays a key role in biofilm nutrition, varying sucrose levels should be studied to assess their impact on microbial growth under different conditions.

CHAPTER 5

CONCLUSION

In conclusion, this study successfully established an *in vitro* model to replicate both health- and dental caries-associated microbiomes. Including health-derived microbiomes are important as a reference, especially when assessing microbiome modulators. The findings highlight that no single culture condition is universally ideal for microbiota growth; instead, the selection of media, incubation period, and inoculum source should be tailored to the specific research objectives. Notably, SHI conditions demonstrated the highest similarity to the original clinical inoculum, making them a preferred choice when the goal is to maintain microbial diversity and closely mimic the natural oral microbiome. However, for studies prioritizing biofilm viability, SS proved to be the more suitable medium, as it provides essential nutrients and environmental conditions that support long-term microbial survival. Furthermore, for studies that need to rapidly generate a high microbial biomass within a limited time frame, SHI media is recommended due to its efficiency in promoting bacterial proliferation. Thus, these findings provide valuable insights for optimizing microbial culture conditions to better mimic *in vivo* microbiomes.

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APPENDIX A
AGAROSE GEL ELECTROPHORESIS

