Effect of different atmospheres on microcosm biofilm formation and tooth demineralization

Aline Silva Braga
Bauru School of Dentistry, University of São Paulo.

Rafaela Ricci Kim
Bauru School of Dentistry, University of São Paulo.

Ana Carolina Magalhães (acm@usp.br)
Bauru School of Dentistry, University of São Paulo.

Research Article

Keywords: dental caries, dentin, enamel, microorganisms, oral biofilm.

Posted Date: September 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2081258/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

This study evaluated the effects of different atmospheres on the cariogenic potential of microcosm biofilms. Ninety bovine enamel and 90 dentin specimens were allocated into three atmospheres: 1) microaerophilia (5 days, 5% CO2); 2) anaerobiosis (5 days, jar); 3) mixed (2 days microaerophilia and 3 days anaerobiosis), which were subdivided into 0.12% chlorhexidine (positive control- CHX) and Phosphate-Buffered Saline (negative control- PBS) (n = 15). Biofilms were prepared using human saliva and McBain's saliva containing 0.2% sucrose. From the second day, the specimens were treated with CHX or PBS (1 x 1 min/day). After five days, colony-forming units (CFU) were counting and tooth demineralization was analyzed using transverse microradiography (TMR). Data were subjected to two-way ANOVA and Tukey–Sidak’s test (p < 0.05). Regarding CFU counting, most atmospheres were able to differentiate between CHX and PBS (differences of 0.3–1.48 log10 CFU/ml), except for anaerobiosis and microaerophilia for total microorganisms in enamel and dentin biofilm, respectively. In the case of dentin, no effect of CHX on Lactobacillus spp. was observed. All atmospheres were able to differentiate between CHX and PBS regarding enamel demineralization, showing lower mineral loss and lesion depth for CHX (78% and 22% reductions for enamel and dentin, respectively). The enamel mineral loss data did not differ between the models; however, the enamel lesion depth was greater under anaerobiosis. Dentin mineral loss was lower under anaerobiosis than under other atmospheres. Conclusion: The choice of atmosphere did not seem to interfere with the cariogenic potential of the microcosm biofilm.

Introduction

Dental caries is a multifactorial oral disease of great clinical relevance. It affects the tooth structure because of the presence of a biofilm in dysbiosis, which is rich in acidogenic, aciduric, and extracellular polysaccharide-producing microorganisms, which occur due to the frequent ingestion of sugar. These microorganisms can metabolize different types of sugars from the diet, mainly sucrose, forming acids that change the biofilm pH and cause tooth demineralization (Bowen 2002; Marsh et al. 2011; Walsh et al. 2015; Takahashi 2015; Pitts et al. 2017). The most common microorganisms involved in the development of dental caries are Streptococcus mutans and lactobacilli (Aas et al. 2008; Gross et al. 2010; Vieira et al. 2014; Henne et al. 2015, Mosaddad et al. 2019).

Because of the high degree of control of culture conditions and reproducibility, in vitro models have been well accepted to simulate cariogenic biofilms (Amaechi et al. 2019). In vitro models can be produced from monospecies, namely, S. mutans, or multispecies, namely, S. mutans + Lactobacillus casei biofilm models using microbial strains or from microorganisms derived from human saliva or dental biofilms, called microcosm biofilm (Filoche et al. 2007; Schwendicke et al. 2017; Amend et al. 2018).

Microcosm biofilms can accurately mimic the complexity of a dental biofilm (McBain 2009; Sim et al. 2016; Braga et al. 2021). However, within this model, different forms of cultivation have been used, comprising, 24-well microtiter plates, where specimens can be attached to the bottom of the wells (Zhang et al. 2015; Souza et al. 2018; Pires et al. 2019); meshes for the retention of microorganisms (Ayoub et al.
2020); or specimens suspended to prevent the formation of biofilm only by microorganism precipitation (Méndez et al. 2018). Some microcosm biofilm models use artificial mouths for programmed or continuous nutrient flow (Amend et al. 2018; Santos et al. 2019).

Studies differ concerning the sucrose concentrations and form of exposure, which can be either continuous or intermittent (Signori et al. 2018; Braga et al. 2020). Cultivation time can vary from 2–14 d depending on the selected response variables (Rudney et al. 2012; Souza et al. 2018). Incubation atmosphere is another important factor (Sim et al. 2016), which can vary between microaerophilia (5–10% CO₂) (Rudney et al. 2012; Zhang et al. 2015; Santos et al. 2019; Ayoub et al. 2020) and anaerobiosis with the use of jars, candles, or anaerobic cabins (O₂ < 0.1% and 5–10% CO₂; 10% CO₂, 10% H₂ and 80% N₂) (Maske et al. 2016; Signori et al. 2018).

Oral dental biofilms are constantly exposed to substantial fluxes of environmental conditions, namely, fast pH changes, availability of nutrients, nature of carbohydrates, and variations in redox potentials due to atmospheric conditions. These environmental parameters determine the composition of the microbial population in biofilms (Kolenbrander 2000; Dhaked et al. 2021).

Therefore, atmospheric conditions can modulate microbial changes and cariogenic potential during biofilm development (Sim et al. 2016; Maske et al. 2017). Environmental changes, physiological disturbances, or selective pressures that might occur during dental biofilm growth can stimulate the proliferation of certain species leading to the development of a potential cariogenic biofilm (Parisotto et al., 2010).

Despite this wide methodological variation between studies, the impact of the culture atmosphere on the development of microcosm biofilms and their potential to cause tooth demineralization has not yet been compared. Therefore, this study aimed to compare the three experimental cultivation models (microaerophile vs. anaerobiosis vs. experimental mixed) on the colony-forming units (CFU) of the cariogenic microorganisms and tooth demineralization. The null hypothesis is that atmospheric conditions do not influence the S. mutans and Lactobacillus spp. colonies and development of dental caries lesions (tooth demineralization).

**Materials And Methods**

**Saliva Collection**

This study was approved (CAAE: 35403320.1.0000.5417) by the local ethics committee of Bauru School of Dentistry-USP (Bauru-Brazil). The study was conducted according to the Declaration of Helsinki. After obtaining consent, saliva was collected from ten healthy participants (age: 23.8 ± 3; eight women and two men). The inclusion criteria for the saliva donors and the procedure for saliva collection (salivary flow > 1 mL/min for 10 min) followed previously reported protocols (Braga et al. 2019). Saliva samples were
collected once in the morning. In total, 132 mL (pool) of saliva was collected and diluted in glycerol (70% saliva and 30% glycerol), and 1 mL of aliquots were stored at −80°C (Pratten et al. 2003).

**Tooth Specimen Preparation**

Bovine specimens of cattle that were sacrificed in the food manufacturing industry (Frigol S. A., Lençóis Paulista, São Paulo, SP, Brazil) were obtained. The study was approved by the Ethics Committee on Animal Research (CEUA, Number 005/2020, Bauru School of Dentistry, University of São Paulo, Bauru, Brazil) following the guidelines provided by the National Council for Control of Animal Experimentation. Ninety bovine enamel and 90 bovine root dentin specimens (4 mm × 4 mm) were cut, polished, and evaluated for the average roughness (Ra; contact profilometer Mahr, Göttingen, Germany) (Braga et al. 2019) to standardize the tooth surfaces for biofilm growth. Two-thirds of the surface was covered with red nail polish (Estreia-Colorama, Rio de Janeiro, RJ, Brazil) to protect it from the biofilm and create two sound areas. This enabled appropriate analysis of tooth demineralization using transverse microradiography (TMR). Subsequently, the specimens were sterilized by exposure to ethylene oxide. Enamel and dentin specimens were randomly distributed into three groups and two subgroups (n = 15) according to their mean Ra values (Enamel Ra: 0.140 ± 0.029 µm and dentin Ra: 0.288 ± 0.056 µm), allowing the groups to have similar mean baseline Ra values.

**Study conditions and sample size calculation**

The experimental models differed regarding the atmosphere, namely, microaerophilia (greenhouse with 5% CO₂), anaerobiosis (greenhouse with jar and Microbiology Anaerocult A, Merck, Darmstadt, Germany, < 0.5% O₂), and mixed (two days in the microaerophilic model and three days in the anaerobic model). Under each atmospheric condition, half of the specimens were treated with Chlorhexidine (CHX), and the other half were treated with Phosphate buffered saline (PBS). The entire experiment was performed in biological triplicates (n = 5 each), with a final n = 15 (Table 1). The sample size was calculated using the website, http://powerandsamplesize.com, based on a previous study that indicated an integrated mineral loss (ΔZ) of 3237.1 vol%. µm (SD: 781.0 µm) for CHX (Periogard) versus ΔZ 6151.3 vol%. µm (SD: 1084.5) for the PBS group (Braga et al. 2020), under a power of 80% and α error of 5% (n = 2/group).
Table 1
The tested atmospheres conditions and treatments in this experiment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Atmospheres conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microaerophilia</td>
<td>Greenhouse with 5% CO₂</td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>Greenhouse with jar and Microbiology Anaerocult A (&lt; 0.5% O₂)</td>
</tr>
<tr>
<td>Mixed</td>
<td>2 days in the microaerophilia and 3 days anaerobiosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Company/City-Country</th>
<th>Composition/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PerioGard (positive control)</td>
<td>Colgate-Palmolive/São Paulo-Brazil</td>
<td>Active component: 0.12% chlorhexidine gluconate, sorbitol and cetylpyridinium chloride.</td>
</tr>
<tr>
<td>PBS (negative control)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Preparation of Artificial Saliva

McBain artificial saliva (McBain 2009), containing 2.5 g/L mucin (type II) from porcine stomach, 2.0 g/L tryptone, 2.0 g/L bacteriological peptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L CaCl₂, 0.2 g/L KCl, 0.1 g/L cysteine hydrochloride, 0.001 g/L hemin, and 0.0002 g/L vitamin K1, was prepared. Some components were sterilized using an autoclave. Mucin was sterilized by pasteurization to protect its molecular structure and function and cysteine hydrochloride, hemin, and vitamin K1 were sterilized using a syringe filter.

Microcosm Biofilm Formation

In a 24-wells microtiter plate, each enamel or dentin specimen was exposed to 1.5 mL of inoculum (human saliva-glycerol + McBain saliva, 1:50) for 8 h. After the first 8 h, the inoculum was removed, the specimens were washed with PBS (5 s) and then received 1.5 mL fresh medium (McBain artificial saliva) with 0.2% sucrose for 16 h, for the first 24 h. From the second to the fifth day, the medium (McBain saliva) containing 0.2% sucrose was changed once a day. Before medium replacement, half of the specimens were treated daily with 0.12% CHX/PerioGard (positive control) and the other half with PBS (negative control) for 1 min (v = 1 mL/well). The experiment was performed at 37°C and each plate was stored under different atmospheric conditions.

Colony-forming units (CFU) counting

After the fifth day, the specimens were washed once with PBS solution to remove dead bacteria or those that did not adhere to the biofilm. Subsequently, the specimens were transferred to microtubes containing 1 mL of 0.89% NaCl solution and sonicated (Sonifier Cell Disruptor B-30, Branson, Danbury, USA) for 30 s at 20 W. For CFU counting, 100 µL of the microbial suspension from microcosm biofilm were diluted to 10⁻⁵ and spread on petri dishes (25 µL/dish) containing three types of agar: 1) Brain Heart Infusion agar
(BHI, Kasvi, Curitiba, Brazil) for total microorganisms; 2) De Man, Rogosa and Sharpe (MRS- Kasvi, Curitiba, Brazil) supplemented with 0.13% glacial acetic acid to assess the number of total lactobacilli (Lima et al. 2009); and 3) SB-20M containing 15 g bacto-casitone (Difco, Detroit, USA), 5 g yeast extract (Kasvi, Curitiba, Brazil), 0.2 g L-cysteine hydro-chloride (Sigma, Steinheim, Germany), 0.1 g sodium sulfite (Sigma, Steinheim, Germany), 20.0 g sodium acetate (Synth, Diadema, Brazil), 200.0 g coarse granular cane sugar, 15.0 g agar (Kasvi, Curitiba, Brazil), and 1 L distilled water (autoclaved) plus 0.2 U/mL bacitracin (Sigma, Steinheim, Germany) for determination of mutans streptococci (S. mutans and S. sobrinus) (Silva et al., 2012; Li et al., 2014).

The plates were then incubated under the same conditions as the microcosm biofilm. In the mixed atmosphere, the plates were separately incubated under both microaerophilic and anaerobic conditions. After 48 h, CFU was computed and transformed to log\(_{10}\) CFU/mL.

**Transverse microradiography (TMR)**

After removing the biofilm from the specimens, all enamel and dentin specimens were transversally sectioned and polished to obtain slices of 80–100 µm (enamel) and 100–120 µm (dentin) thickness (Braga et al. 2020; Vertuan et al. 2021). The slices were fixed in a sample holder together with an aluminum calibration step wedge with 14 steps. Ethylene glycol (Sigma-Aldrich, Steinheim, Germany) was applied on the dentin specimens for 24 h to avoid shrinkage due to desiccation during X-ray exposure desiccation (Buchalla et al. 2003). A microradiograph was obtained using an X-ray generator (Softex, Tokyo, Japan) on a glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates (high-precision photo plate, Konica Minolta Inc., Tokyo, Japan) were processed and analyzed using a transmitted light microscope fitted with a 20x objective (Zeiss, Oberkochen, Germany), a CCD-Charge-Coupled Device camera (Canon, Tokyo, Japan), and a computer. Two images per specimen were acquired using data acquisition (version 2012) and interpreted using the Inspektor Research System BV calculation software (version 2006) (Amsterdam, Netherlands). The mineral content was calculated based on the assumption of 87% volume of mineral content for sound enamel and 50% mineral content for sound dentin. Lesion depth (LD, µm) integrated mineral loss (\(\Delta Z\), vol. µm), and the average mineral loss over the lesion depth (R, vol%) were obtained.

**Statistical analysis**

Data were statistically compared using the GraphPad Prism software (San Diego, CA, USA). Distribution and homogeneity were tested using the Kolmogorov–Smirnov and Bartlett’s tests, respectively. A two-way ANOVA (factors: atmospheres and treatments), followed by Tukey’s test or Sidak’s test, was applied. The level of significance was set at 5%.

**Results**

**CFU counting**
Enamel

Table 2 presents the CFU values (log$_{10}$/mL) for the total microorganisms, *Lactobacillus* spp., and *S. mutans*/ *S. sobrinus* from the microcosm biofilm cultivated under various atmospheric growth conditions on the enamel specimens.

All atmospheres, except the anaerobic condition, were able to differentiate CHX from PBS, displaying the antimicrobial effect of the CHX on total microorganisms. A minor difference in the growth of total microorganisms was seen in microaerophila compared to the other atmospheres, which did not differ from each other (anaerobic and mixed) regardless of the treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subgroups</th>
<th>Total microorganisms</th>
<th>Lactobacillus spp.</th>
<th>S. mutans/S. sobrinus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microaerophilia</strong></td>
<td>CHX</td>
<td>6.38 ± 0.52$^{Aa}$</td>
<td>5.87 ± 0.86$^{Aab}$</td>
<td>6.41 ± 0.36$^{Aa}$</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.37 ± 0.43$^{Ba}$</td>
<td>7.08 ± 0.11$^{Bab}$</td>
<td>8.05 ± 0.12$^{Ba}$</td>
</tr>
<tr>
<td><strong>Anaerobiosis</strong></td>
<td>CHX</td>
<td>7.35 ± 0.47$^{Ab}$</td>
<td>6.22 ± 0.39$^{Abc}$</td>
<td>6.53 ± 0.33$^{Aab}$</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.68 ± 0.71$^{Ab}$</td>
<td>7.46 ± 0.21$^{Bbc}$</td>
<td>7.35 ± 0.43$^{Bb}$</td>
</tr>
<tr>
<td><strong>Mixed-Anaerobic</strong></td>
<td>CHX</td>
<td>7.15 ± 0.59$^{Ab}$</td>
<td>5.69 ± 0.47$^{Aa}$</td>
<td>6.88 ± 0.54$^{Ab}$</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.91 ± 0.25$^{Bb}$</td>
<td>7.17 ± 0.10$^{Ba}$</td>
<td>7.74 ± 0.11$^{Bab}$</td>
</tr>
<tr>
<td><strong>Mixed-Microaerophilia</strong></td>
<td>CHX</td>
<td>7.20 ± 0.57$^{Ab}$</td>
<td>6.31 ± 0.32$^{Ac}$</td>
<td>6.50 ± 0.81$^{Aab}$</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.84 ± 0.18$^{Bb}$</td>
<td>7.70 ± 0.07$^{Bc}$</td>
<td>7.72 ± 0.09$^{Bab}$</td>
</tr>
</tbody>
</table>

Capital letters show significant difference between treatments, for each type of atmosphere. Lowercase letters show significant difference between atmospheres, for each type of treatment. For total microorganisms, two-way ANOVA was applied followed by Tukey’s test (treatment $p < 0.0001$, atmosphere $p < 0.0001$ and interaction $p = 0.0481$). For *Lactobacillus* spp., two-way ANOVA was applied, followed by the Sidak’s test (treatment $p < 0.0001$, atmosphere $p < 0.0001$, no interaction, $p = 0.7677$). For *S. mutans*/ *S. sobrinus*, two-way ANOVA was applied, followed by the Tukey’s test (treatment $p < 0.0001$, atmosphere $p = 0.0041$ and interaction $p = 0.0039$).
All atmospheres were able to differentiate CHX from PBS, indicating the antimicrobial effect of CHX on *Lactobacillus* spp. and *S. mutans/ S. sobrinus*.

Lower growth of *Lactobacillus* spp. was observed under a mixed atmosphere (anaerobic CFU) than in the other conditions, except for the microaerophilic condition. The microaerophilic atmosphere was similar to the anaerobic atmosphere but displayed lower *Lactobacillus* spp. growth compared with the mixed atmosphere (microaerophilic CFU), which did not differ from the anaerobic and mixed-anaerobic CFU.

Regarding *S. mutans/ S. sobrinus*, lower microbial growth was observed in the microaerophilic atmosphere, which was similar to the mixed atmosphere (microaerophilic CFU). However, the microaerophilic atmosphere significantly differed from the anaerobic atmosphere when considering PBS and from the mixed atmosphere (anaerobic CFU) when considering CHX.

**Dentin**

Table 3 displays the CFU values (log₁₀/mL) for the total microorganisms, *Lactobacillus* spp., and *S. mutans/ S. sobrinus* from a microcosm biofilm cultivated under different atmospheric growth conditions on dentin specimens.
Table 3
CFU counting (log_{10} CFU mL) of total microorganisms, *Lactobacillus* spp. and *Streptococcus mutans/ Streptococcus sobrinus* from microcosm biofilm produced on dentin specimens.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subgroups</th>
<th>Total microorganisms</th>
<th>Lactobacillus spp.</th>
<th>S. mutans/S. sobrinus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microaerophilia</td>
<td>CHX</td>
<td>7.62 ± 0.22^{Aa}</td>
<td>6.95 ± 0.46^{Aab}</td>
<td>7.12 ± 0.39^{Aa}</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.74 ± 0.11^{Aa}</td>
<td>7.06 ± 0.38^{Aab}</td>
<td>7.63 ± 0.19^{Ba}</td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>CHX</td>
<td>7.45 ± 0.38^{Aa}</td>
<td>7.13 ± 0.46^{Ab}</td>
<td>7.24 ± 0.35^{Aa}</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.94 ± 0.22^{Ba}</td>
<td>7.51 ± 0.20^{Ab}</td>
<td>7.60 ± 0.19^{Ba}</td>
</tr>
<tr>
<td>Mixed-Anaerobic</td>
<td>CHX</td>
<td>7.21 ± 0.53^{Aa}</td>
<td>7.25 ± 0.59^{Ab}</td>
<td>7.05 ± 0.35^{Aa}</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.84 ± 0.16^{Ba}</td>
<td>7.33 ± 0.51^{Ab}</td>
<td>7.69 ± 0.21^{Ba}</td>
</tr>
<tr>
<td>Mixed-Microaerophila</td>
<td>CHX</td>
<td>7.49 ± 0.28^{Aa}</td>
<td>6.76 ± 0.36^{Aa}</td>
<td>7.21 ± 0.41^{Aa}</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7.80 ± 0.16^{Ba}</td>
<td>6.87 ± 0.56^{Aa}</td>
<td>7.77 ± 0.22^{Ba}</td>
</tr>
</tbody>
</table>

Capital letters show significant difference between treatments, for each type of atmosphere. Lowercase letters show significant difference between atmospheres, for each type of treatment. For total microorganisms, two-way ANOVA was applied, followed by Tukey’s test (treatment p < 0.0001, atmosphere p < 0.1222 and interaction p = 0.0099). For *Lactobacillus* spp., two-way ANOVA was applied, followed by the Tukey’s test (treatment p = 0.0602, atmosphere p = 0.0002, no interaction, p = 0.5955). For *S. mutans/ S. sobrinus*, two-way ANOVA was applied, followed by the Tukey’s test (treatment p < 0.0001, atmosphere p = 0.4706 and interaction p = 0.4637).

All atmospheres, except microaerophilia, were able to differentiate between CHX and PBS, showing the antimicrobial effect of CHX on all microorganisms. When the atmospheres were compared, no differences were observed regardless of the treatment.

Regarding *Lactobacillus* spp. CFU, none of the atmospheres was able to differentiate between CHX and PBS. When the atmospheres were compared, a mixed atmosphere (microaerophilic CFU) presented lower growth of *Lactobacillus* spp. than the other atmospheres, except for microaerophilia.

In contrast, all atmospheres significantly differed in CHX and PBS regarding *S. mutans/ S. sobrinus* growth, whereas no differences were observed when the atmospheres were compared regardless of the treatment.

**TMR analysis**

Tables 4 and 5 present the TMR data for the enamel and dentin specimens, respectively. Figures 1 and 2 display representative TMR images of the enamel and dentin from each group, respectively.
Table 4
Mean ± SD of the integrated mineral loss (ΔZ, vol%. µm), the lesion depth (LD, µm) and the average mineral loss (R, vol%) of enamel specimens.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subgroups</th>
<th>ΔZ (vol%.µm)</th>
<th>LD (µm)</th>
<th>R (vol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microaerophilia</td>
<td>CHX</td>
<td>1035.62 ± 561.38&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>37.89 ± 15.46&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>27.92 ± 8.16&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7223.33 ± 1206.52&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>119.84 ± 20.27&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>59.14 ± 3.11&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>CHX</td>
<td>1702.22 ± 1056.60&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>61.19 ± 24.22&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>30.57 ± 10.52&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>6555.00 ± 1681.07&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>134.22 ± 28.45&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>54.71 ± 4.33&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed</td>
<td>CHX</td>
<td>1436.11 ± 495.77&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>49.46 ± 13.01&lt;sup&gt;Aab&lt;/sup&gt;</td>
<td>23.94 ± 2.05&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>7246.11 ± 350.14&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>119.54 ± 8.47&lt;sup&gt;Bab&lt;/sup&gt;</td>
<td>59.66 ± 2.06&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Capital letters show significant difference between treatments, for each type of atmosphere. Lowercase letters show significant difference between atmospheres, for each type of treatment. For ΔZ, two-way ANOVA was applied, followed by Tukey’s test (treatment p < 0.0001; atmosphere p = 0.7627 and interaction p = 0.0168). For lesion depth, two-way ANOVA was applied, followed by Tukey’s test (treatment p < 0.0001, atmosphere p = 0.0148 and interaction p = 0.6412). For the average mineral loss, two-way ANOVA was applied, followed by Sidak’s test (treatment p < 0.0001, atmosphere p = 0.7709, interaction, p = 0.0284).
Table 5
Mean ± SD of the integrated mineral loss (ΔZ, vol%·µm), the lesion depth (LD, µm) and the average mineral loss (R, vol%) of dentin specimens.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subgroups</th>
<th>ΔZ</th>
<th>LD</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(vol%·µm)</td>
<td>(µm)</td>
<td>(vol%)</td>
</tr>
<tr>
<td>Microaerophilia</td>
<td>CHX</td>
<td>4389.2 ± 603.51&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>159.2 ± 13.7&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>29.2 ± 3.4&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>5768.5 ± 523.95&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>184.2 ± 24.5&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>31.5 ± 2.9&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>CHX</td>
<td>4242.3 ± 581.68&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>144.0 ± 14.0&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>27.8 ± 2.2&lt;sup&gt;Ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>5149.6 ± 551.40&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>194.3 ± 28.7&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>28.7 ± 3.1&lt;sup&gt;Ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed</td>
<td>CHX</td>
<td>4744.3 ± 472.60&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>160.3 ± 18.4&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>29.0 ± 2.3&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>5692.5 ± 243.04&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>183.1 ± 13.2&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>31.6 ± 2.5&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Capital letters show significant difference between treatments, for each type of atmosphere. Lowercase letters show significant difference between atmospheres, for each type of treatment. For ΔZ, two-way ANOVA was applied, followed by Tukey’s test (treatment p < 0.0001; atmosphere p = 0.0034 and interaction p = 0.2346). For lesion depth, two-way ANOVA was applied, followed by Tukey’s test (treatment p < 0.0001, atmosphere p = 0.809 and interaction p = 0.0614). For average mineral loss, two-way ANOVA was applied followed by Tukey’s test (treatment p = 0.0039, atmosphere p = 0.0093, interaction, p = 0.4243).

Chlorhexidine reduced enamel demineralization in all the tested atmospheres. Similar carious lesions were produced in enamel in all atmospheres, except for microaerophilia, which induced a shallow lesion compared with the anaerobic atmosphere, both being similar to the mixed atmosphere (Table 4 and Fig. 1).

Chlorhexidine was also able to reduce dentin demineralization in all tested atmospheres, except for the average mineral loss in the case of microaerophilia and anaerobiosis. Microaerophilic and mixed atmospheres produced similar carious lesions in dentin with greater mineral loss (integrated and mean) than those produced under anaerobiosis (Table 5 and Fig. 2).

Discussion

Although the microcosm biofilm model is a well-established practice, there are several differences between the protocols, as for example the growth atmosphere. Accordingly, the atmosphere that better represents oral conditions needs to be investigated. This study did not aim to validate a type of
atmosphere based on the oral environment since no comparison was made between the tested protocol and \textit{in vivo} conditions. This study intended to check the differences between the atmospheric models (with or without O$_2$), \textit{in vitro}, and their relevance in the development of artificial dental caries.

Some minor differences were observed among the atmospheric conditions, but they were able to produce very similar carious lesions in both enamel and dentin. Furthermore, most atmospheres were able to differentiate between CHX and PBS, which is essential outcome, since the antimicrobial effect of CHX has been well established (Shapiro et al. 2002).

From a microbiological perspective, to analyze the effect of the atmosphere on the microorganisms from the microcosm biofilm, we selected CFU counting, which is a well-established, reproducible, and simple quantitative method (van Houte et al. 1993). However, it is not as precise and sensitive as PCR-Polymerase chain reaction method and does not provide an overview of the entire microbiota (Tanner et al. 2018). Based on the findings of the CFU count, there was a lesser number of \textit{Lactobacillus} spp. and \textit{S. mutans} observed under the microaerophilic than in the anaerobic environment, especially for enamel. The differences less than 0.2 log$_{10}$ CFU/mL might be clinically irrelevant and, in fact, they were important when TMR data were taken into account.

Both bacteria were chosen because they are highly established cariogenic species (Gao et al. 2016). A limitation of the study is that other species, which might be present in the microcosm biofilm, were not examined. Therefore, future studies using “omics” are desirable, especially to understand the reason that CHX did not reduce \textit{Lactobacillus} spp. in microcosm biofilms produced on dentin. Interestingly, the number of total microorganisms was reduced under anaerobic conditions for CHX. This led to speculations regarding the contribution of other species. Herein, it is important to consider that the term total microorganism does not include species that require supplementation for their growth on BHI agar.

The association between lactobacilli and dental caries dates back to a century (Kligler 1915; Caufield et al. 2015). In another study, 0.2% chlorhexidine, utilized in an \textit{in situ} model, was not able to reduce the CFU count for lactobacilli on dentin specimens when compared with the control (van Strijp et al., 1997). The author suggested that dentin can act as a “shelter” for this bacterium against the action of chlorhexidine (van Strijp et al., 1997).

The outside atmosphere had little impact on the microbiological analysis of the microcosm biofilms because the biofilm itself can create its atmosphere, with the deeper layers rich in strict anaerobic microorganisms and the superficial layers rich in facultative microorganisms (Diaz et al. 2006; Schoilew et al. 2019). Although dental biofilms are composed primarily of obligate anaerobic species with preferential growth in the presence of carbon dioxide (CO$_2$), these microorganisms may be protected from the toxic effects of oxygen, enabling their growth under microaerophilic conditions (Bradshaw et al. 1997; Sim et al. 2016). It should be noted that the findings of this study cannot be extrapolated to monospecies or other multispecies models.
Despite knowing the microbiological composition of the biofilm, it was clear by the tooth lesion analysis that even in the presence of different species, the set of metabolic products released into the biofilms under different atmospheres was able to induce similar artificial carious lesions. Metabolome analysis could be better used to discuss the results; however, the most important response variable is related to the tooth (TMR data).

Regarding enamel demineralization, the mineral loss (integrated and mean) was similar among different atmospheres; however, the anaerobic condition induced a deeper lesion, showing that in this case, the acids could have penetrated deeper into the environment, which may be related to the metabolic profile of the biofilm. While dentin lesion depth was similar among the atmospheres, dentin mineral loss (integrated and mean) was lower for anaerobic than for microaerophilic and mixed atmospheres, which behaved similarly. Therefore, as previously discussed, the metabolic products (primarily acids) induced by the anaerobic biofilm may have been neutralized by the organic content of dentin (Takahashi 2015; Wicaksono et al. 2020).

The mixed biofilm model was first performed by Braga et al. (2021), but with a different sequence: the first three days in an anaerobic and the last two days in a microaerophilic atmosphere. In their study, the biofilm model was able to differentiate CHX from PBS. The results of this study warrant further metabolome analysis to better explain the slight differences found in CFU counting and TMR analysis.

For both tissues, especially enamel, lesions induced in the absence of treatment (PBS group) were highly demineralized, and most of them were cavitated (about 85–100% for enamel and 31–55% for dentin), with no differences among the atmospheres. It is suggested that the higher cavitation of enamel is due to its lower organic content compared to dentin, which plays an important role in the modulation of de-mineralization progression. Also, the percentage of prevention fraction of CHX was much higher for enamel (~ 78%) than for dentin (~ 22%), which may be related to the high degree of de-mineralization of the enamel and the type of interaction of CHX with the biofilm, in agreement with previous reports (Santos et al. 2019; Braga et al. 2020; Pelá et al. 2021).

Our results provide new information about the effect of the atmosphere on microcosm biofilm growth and its potential to induce tooth demineralization. These different protocols should be analyzed in the future to identify their impact on the results and interpretation of metabolome. In addition, the biofilm should be analyzed by laser scanning microscopy or scanning electron microscopy using the 3D architecture of the biofilm to identify if the specific organization of microbial communities could be created by different atmospheric conditions.

Conclusion

In conclusion, this study identified some minor differences among the atmospheric conditions; however, in general, microcosm biofilms produced under microaerophilia, anaerobiosis, or mixed models produced very similar artificial carious lesions in both enamel and dentin.
Declarations

Ethics approval and consent to participate: We collected human saliva from ten participants, who approved and signed the consent to participate of the present study. The study was first approved by the local ethics committee of Bauru School of Dentistry-USP (Bauru-Brazil), number CAAE: 35403320.1.0000.5417.

Consent for publication: Not applicable.

Availability of data and material: Please contact the corresponding author for data requests.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP Proc. 2019/21797-0; 2019/01730-9; 2017/17249-2). This funding source had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the article for publication.

Authors' contributions

A.S.B.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - Review & Editing, Visualization; R.R.K.: Methodology, Validation, Investigation, Resources, Funding acquisition; and A.C.M.: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Authors' information: Not applicable.

Acknowledgments

We would like to thank the anonymous saliva donors and the São Paulo Research Foundation.

Disclosure Statement

The authors declare no potential conflict of interest.

References


Figures
Figure 1

TMR representative pictures (20×) of an enamel specimen from each group: A. Microaerophilia; B. Anaerobiosis; C. Mixed, after treatment with Chlorhexidine (CHX) or PBS. The arrows show the lesion area. Specimens belonging to PBS showed cavitation, regardless of the atmosphere. Specimens treated with CHX showed shallow lesion.

Figure 2
TMR representative pictures (20×) of a dentin specimen from each group: A. Microaerophilia; B. Anaerobiosis; C. Mixed, after treatment with Chlorhexidine (CHX) or PBS. The arrows show the lesion area. Mixed-PBS was the only one not showing some cavitation of the dentin lesion.