Comparison of Human and Bovine Enamel in a Microbial Caries Model at Different Biofilm Maturations

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Abstract

Objectives: To compare human versus bovine enamel when used in microbial caries models; and to evaluate the use of nylon mesh to support biofilm growth over enamel. **Methods:** Twenty-four sub-subgroups were included (time factor: 4, 8, and 12 days; substrate factor: human/bovine; mesh factor: yes/no; treatment factor: 18.4 mM NaF (350 ppm F), de-ionized water [DIW]; n=9/sub-subgroup). Microcosm biofilm from human saliva (IRB approval #1406440799) was grown on enamel specimens for 24-h (Brain Heart Infusion media; 0.2% sucrose), using active attachment model. Then, pH-cycling took place. At the end of each pH-cycling period, enamel specimens were analyzed: surface microhardness (VHN_{change}); transverse microradiography (integrated mineral loss [ΔZ], lesion depth [L]). Biofilm was analyzed: lactic acid production (LDH activity); exopolysaccharide (EPS) amount; and viability (12-day sub-groups). Data were analyzed using ANOVA at a 5% level of significance. **Results:** The three-way interaction between pH-cycling duration, substrate type, and treatment type was significant for (VHN_{change} [$p<0.0005$], ΔZ [$p=0.0027$], and L [$p<0.0001$]). VHN_{change} exhibited increased lesion severity as pH-cycling time increases, in both treatments. VHN_{change} data indicated a treatment effect in all timepoints. ΔZ and L exhibited higher values with more mature biofilms. ANOVA analyses for LDH and EPS indicated a significance between variables (LDH $p=0.0100$; EPS $p<0.0001$). Mesh-covered specimens resulted in lower LDH and EPS values in all maturations. ANOVA analyses of viability (12 days) between variables was significant. **Conclusion:** within the study's limitations, human or bovine enamel can be used in microbial in vitro caries models to study biofilm's maturation and anticaries agents.

Clinical Significance: This study demonstrated how a known cariostatic effect of a fluoride concentration in toothpastes can be modulated by the maturation stage of oral biofilm. This can represent hard to reach areas in the oral cavity (e.g. in orthodontic patients or patients with intermaxillary fixation following oral and maxillofacial surgeries).

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Introduction

Dental biofilms play a major role in caries lesion development through carbohydrate fermentation and acid production. Dental biofilm maturation (i.e. the biofilm age) involves changes in biofilm composition and architecture [1]. Consequently, biofilm interactions with the surrounding environment, and with anticaries agents as well, are expected to alter. The complex oral environment, and the diversity of the dental biofilm make studying these changes during biofilm maturation challenging [2]. It has been documented that biofilm could act as a diffusion barrier preventing anticaries agents from reaching the tooth surface [3]. This happens because proteins and vitamins in the environment (i.e. the growth media if in-vitro) block the biofilm-enamel interface and restrict the diffusion of the ions into enamel surfaces [4, 5]. However, there is a need for further research in this area for better understanding.

An approach taken in utilizing caries preventive agents is targeting the biofilm virulence factors. For example, fluoride is used as an antibacterial agent; it affects bacterial acidogenicity, acidurity, as well as exopolysaccharide (EPS) formation [6]. A wide range of model systems have been introduced to test anticaries agents, with in vitro caries models being used most frequently.

Many studies have tested biofilm and its cariogenicity [7-12]. Some studies limited their methodologies to simpler approaches to achieve a reproducible, controllable model (e.g. microtiter plate, single-species biofilm); other studies aimed to maintain clinical relevance (e.g. artificial mouth models, constant-depth film fermenters) [7, 9, 10, 12, 13]. Each model has its strengths and limitations. Some of the characteristics to be tested as components of an ideal model may include: biofilm composition (i.e. single- vs multi-species biofilm); dental substrate type (human vs. bovine); growth medium conditions (i.e. mineral contents and clinical relevance-pH challenges); and hard tissue status (i.e. sound enamel or [biofilm-induced] lesion).

Both human and bovine teeth are widely used in dental studies. Although they were tested previously for their structural differences in caries studies, [14] no prior studies have explored the difference in patterns of biofilm-induced lesions between substrate types.

Studying the interaction between fluoride compounds and dental biofilm at different maturation stages is critical to fully understand the role of the biofilm in the caries process and how, as it matures, it modifies the antibacterial/anticaries effect of fluorides. Another factor that has been reported previously as a limitation to the reliability of microbial studies is achieving an evenly grown biofilm over the substrate surface, especially when growing the biofilm for relatively long periods [15-18]. This is a challenge because the variability in thickness, and therefore composition, of the biofilm over the surface may result in a large variability in the lesions formed and the characteristics of the biofilm itself from one area to another within the same surface [15-18].

Therefore, we explored in this study different variables as major components of a microbial caries model. First, we aimed to evaluate the use of human and bovine enamel specimens. Second, we tested the concept of creating niches over the substrate surface to enhance biofilm adhesion to the surface. Third, we tested these variables at different biofilm maturation stages. The null hypothesis was that the substrate type does not influence biofilm cariogenicity nor caries lesion severity.

Materials and Methods

Study Design

Biofilm, obtained from human saliva, was grown on human and bovine enamel specimens of known Vickers hardness values (VHN_{sound}) in Brain Heart Infusion (BHI) media (with 0.2% sucrose) for 24 hours. Then, the pH cycling phase began: it included two 5-minute treatment periods, three 2-hour demineralization challenges, and four 15-minute remineralization periods. Twenty-four sub-subgroups were included in the study. The variables between sub-subgroups were based on the number of pH cycling days (4 days [4D]; 8 days [8D]; 12 days [12D]), the involvement of a protective mesh over the samples (JoAnn Fabrics, item # 10173334), and treatment type (18.4 mM NaF [350 ppm F] (Acros Organics, Fisher Scientific, Fair Lawn, NJ, USA) or de-ionized water (DIW)).

At the end of each pH cycling period, enamel specimens were analyzed for caries lesion severity: surface microhardness (VHN_{change}); and transverse microradiography (integrated mineral loss [ΔZ] and lesion depth [L]). The biofilm was collected and analyzed for its cariogenicity: lactic acid production (LDH activity); exopolysaccharide (EPS) amount; and viability (12 day sub-groups only).

Specimen Preparation

Extracted human and bovine incisors were sectioned to obtain 4 × 4 mm enamel specimens using a Buehler Isomet™ low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). Approximately 108 teeth were used to obtain 216 specimens. During preparation, the teeth were stored in deionized water with thymol. Using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA), all specimens were ground and polished to ensure flat parallel dentin/enamel surfaces. For the finishing process, the dentin side was ground using 500-grit silicon carbide grinding paper. Then, the enamel side was serially ground using 1,200, 2,400 and 4,000 grit papers. After that, polishing of the specimens took place using a 1 µm diamond polishing suspension on a polishing cloth to obtain a 4 × 4 mm polished enamel surface. All specimens were checked for cracks, white spots, or any other flaws that could lead to excluding the specimen from the study, using a Nikon SMZ 1500 stereomicroscope at × 20 magnification.

Baseline Measurements and Experimental Groups

All specimens were subject to enamel surface microhardness testing (VHN_{sound}) to ensure standardization. A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s. Three indentations, approximately 100 µm apart, were created on each specimen and averaged; the inclusion range was VHN_{sound} between 300-380.

Specimens of each substrate type (i.e. human vs. bovine) were divided into three groups, based on the pH cycling duration (4, 8, and 12 days). Each group included two subgroups (n=18/subgroup), depending on whether or not the specimens were covered with a protective mesh. Each subgroup was divided into two sub subgroups, based on the treatment type used: 18.4 mM NaF (Acros Organics, Fisher Scientific, Fair Lawn, NJ, USA) vs. deionized water (DIW).

Specimens were mounted on the inside of a lid of a 24-well plate (FisherBrand, Fisher Scientific, Newark, DE, USA) using acrylic cubes to create an active attachment model, following a previously described protocol. [8, 19] For the "mesh-covered" subgroups, specimens were covered with a utility mesh-like fabric that is composed of 70% Poly Vinyl Chloride (JoAnn Fabrics, item # 10173334) (Figure 1). Prior to initiating the experiment, we tested several types of medical grade mesh materials in terms of ease of use and ability to be disinfected. The utility mesh selected for this study was easy to manipulate over the enamel specimens, easy to disinfect, and cost-effective. The model was disinfected using 70% ethanol prior to biofilm growth [20].

Salivary Bacterial Model

Saliva Collection. Ethical approval was obtained from the IUPUI institutional review board (IRB #1406440799) for saliva collection. Wax-stimulated saliva samples from three donors were collected and pooled (approximately 50 ml/donor). The inclusion criteria included: healthy participants (no systemic diseases) with normal salivary flow and no presence of active caries or periodontal disease. To ensure standardization, participants refrained from oral hygiene measures overnight.[21] Prior to bacterial inoculation or freezing, the pooled saliva was tested for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars (MSSB and Rogosa agars, respectively).[22] The results confirmed the presence of both species. Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, then mixed with 10% glycerol and frozen immediately at -80^{o}C, this microcosm bacterial mix was used as the source for bacterial inoculum.

Biofilm Growth. Biofilm was allowed to grow on the enamel specimens for 24 hours at 37°C in the growth media. The growth media for this model contained Brain Heart Infusion (BHI) broth, supplemented with 5 g/l yeast extract (YE), 1 mM $CaCl_2.2H_2O$ and 0.2% sucrose. After 24 hours, pH cycling protocol started, as shown in figure 2.

pH cycling Model

After the 1-day biofilm growth on enamel specimens, the biofilm model was subject to a cariogenic pH cycling model, which was modified from the model used by Zhang et al [5]. In this pH cycling model, the growth medium was used as both the remineralization (remin) and demineralization (demin) solutions (figure 2). Both the remin and demin media contained BHI broth, 5% YE, 1 mM $CaCl_2.2H_2O$.
Using 1 mM acetic acid, the pH of the culture medium was adjusted to 7 (remin), and 4.5 (demin). The sucrose concentration also differed in remineralization (no sucrose) and demineralization (1% sucrose) solutions. Between treatments, the biofilm was washed by immersing the model in 0.9% sterile saline for 2 minutes. The pH cycling model was conducted daily, the biofilm and specimens were incubated overnight in remin media. For each subgroup, two treatment types were tested: 18.4 mM NaF, which contains 350 ppm F, and de-ionized water as a negative control (DIW). The 18.4 mM NaF concentration simulates European fluoride levels (1400 ppm F), taking into consideration 1:3 saliva dilution.
At the end of each pH cycling period (4, 8, and 12 days), biofilm collection took place by carefully removing biofilm-covered enamel specimens using tweezers, then placing each specimen in an Eppendorf tube containing 1 ml of sterile saline; sonicating at 30 W for 10 seconds, and vortexing immediately for 10 seconds for complete biofilm detachment from the enamel surface. For the mesh-covered sub-subgroups, the protective mesh was removed using a tweezer and discarded before dislocating the specimen and placing it in the Eppendorf tube.

Post-treatment Analysis

Enamel Substrate

Surface Microhardness Change (VHN_{change}). Post-treatment surface microhardness was measured following the same protocol used for the VHN_{sound}. The VHN_{change} values were calculated using the formula VHN_{change} =100*(VHN_{sound} - VHN_{post})/VHN_{sound}.

Transverse Microradiography. One section, approx. 100 µm thick, was cut from the center of each specimen and across the specimen using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, Lafayette, CO, USA). All sections were placed in the TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. Digital images were analyzed using TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Biofilm Analysis

Lactic Acid Production: LDH Assay. In order to determine live biofilm metabolism, lactic acid production was determined using a LDH cytotoxicity assay, following a previously published protocol. [23] For each

sample, 45 µl of the collected, suspended biofilm was mixed with 5 µl of the LDH Assay Lysis Solution in 96-well microtiter plates, and incubated at 37°C for 45 minutes. Then, 100 µl of LDH Assay mixture was added to the cell lysate (LDH Assay Cofactor Preparation: LDH Assay Substrate: LDH Dye Solution = 1:1:1). The mixture was kept in the dark and incubated at room temperature for 30 minutes. To terminate the reaction, 50 µl of the Stop Solution was added to the mixture and absorbance readings of each well at OD_{490nm} and the background absorbance at OD_{690nm} were measured. The background absorbance values were subtracted from the primary readings.

EPS amount: Phenol-Sulfuric Acid Colometric Assay. Analyzing the EPS activity was performed using a previously described protocol [24]. This method is fast, sensitive, and allows extraction of carbohydrates from the suspended biofilm. Briefly, 50 µl of the biofilm of each sample was transferred to a 96-well microtiter plate. For each sample, 150 µl of concentrated sulfuric acid was added. Immediately after that, 30 µl of a 5% phenol solution was added to the mixture and heated to 90° C for 5 minutes. After cooling the plate at room temperature for 5 minutes the absorbance was measured at OD_{750nm} [24].
All LDH and EPS values were normalized using the protein concentration of each biofilm sample, determined using the RC DC Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) following the manufacturer's instructions.

Bacterial Viability (12-day old sub-groups). At the last time point, biofilm samples from all sub-groups were serially diluted to $1:10^{-3}$,$1:10^{-4}$, and $1:10^{-5}$ (using 0.9% sterile saline). Samples were plated on Blood Agar Plates (Thermo Scientific™, Remel, Lenexa, KS, USA) using a Spread Plate Procedure [25]. To determine bacterial counts, mean $\log_{10}$ CFU/ml values were calculated.

Statistical Analysis

VHN_{change}, ΔZ and L, were analyzed using three-way ANOVA, with factors for time, substrate type and treatment type. Also, two-way, three-way interactions among the variables were analyzed.
LDH activity and EPS amount were analyzed using four-way ANOVA, with factors for time, substrate type, mesh factor, and treatment type. Also, two-way, three-way and four-way interactions among the variables were analyzed.
For the viability data (12-day subgroups), $\log_{10}$ CFU/ml were analyzed using three-way ANOVA, with factors for substrate type, mesh factor, and treatment type, as well as all the two-way and three-way interactions among the variables. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

ANOVA analyses of lesion severity indicated that the three-way interactions among pH cycling time, substrate type and treatment type were significant in VHN_{change} ($p<0.0005$), ΔZ ($p=0.0027$), and L ($p<0.0001$). The four-way ANOVA showed that applying a protective mesh over enamel samples did not produce a significant difference in lesion severity, and therefore these data are not presented (VHN_{change} [$p=0.13$], ΔZ [$p=0.78$], L [$p=0.6916$]).

The VHN_{change} data exhibited an increased severity of the lesions as pH cycling time increased, in both treatment and control groups. Moreover, the VHN_{change} data demonstrated a treatment effect in all timepoints (Table 1).

Regarding ΔZ and L data, an increased severity of the lesions was observed in longer pH cycling durations, especially in control groups. Lesions in the 12-day groups, both treatment and control, were more severe in bovine specimens; the difference was significant in controls only, for both ΔZ and L. Finally, there was an obvious treatment effect as the lesion progresses with biofilm maturation; this effect was more noticeable in bovine specimens, where there was no significant difference between 4, 8, and 12-day treatment groups (Table 1).

ANOVA analyses for both LDH activity and EPS amounts indicated that the four-way interaction among tested variables (pH cycling time, substrate type, protective mesh, and treatment type) was significant (LDH $p=0.0100$; EPS $p<0.0001$).

Within all groups, it was observed that specimens covered with protective mesh indicated lower cariogenicity (LDH and EPS data) of the biofilm, especially in 8 and 12 day groups, in both treatment and control groups, and in both substrate types (Table 2). Both substrate types allowed the same trend of cariogenic activity of the biofilm: first, biofilm's cariogenicity increased as the biofilm matures; second, an increased treatment effect was observed with time (Table 2).

For biofilm's viability, only 12 day-old biofilms were analyzed. ANOVA analyses demonstrated a significance in the three-way interactions among substrate type, protective mesh and treatment type ($p=0.0101$; table 3). Similar to biofilm cariogenicity, the protective-mesh groups exhibited lower viability values when compared to non-mesh groups in both treatment types. The difference between substrate types in non-mesh group was not significant (NaF $p=0.4953$; DIW $p=0.7082$). The difference between substrate types in protective mesh group was not significant in NaF ($p=0.1611$) but significant in DIW ($p<.0001$).

We also measured pH changes within the growth media daily. For the overnight media (remin, no sucrose, pH7), we measured the pH levels in the morning before discarding the media; the daily pH levels for all groups ranged between 5.8-6.2. The pH levels of pH cycling remin media (no sucrose, pH 7) were measured daily after the last remin period; the pH levels for all groups were always above 6.8. The same results were found for the pH cycling demin media, where we measured the pH after the last daily

demin challenge (1% sucrose, pH4.5) and found the pH levels dropped to 4.4 at the maximum (data not shown).

Discussion

The main aim of this study was to evaluate the differences between human versus bovine enamel specimens as part of a microbial cariogenic model. A secondary aim was to evaluate the effectiveness of covering enamel samples with protective mesh to grow an even biofilm layer over the surface, and monitor its uniform growth up to 12 days. To our knowledge, this is the first study evaluating the differences in biofilm-induced carious lesions between enamel substrate types. Also, limited research studies have discussed interventions used to maintain growing a uniform biofilm layer over dental substrates.

Although there are well-established, widely used chemostat caries models, [26, 27] the microbial component should be included in in-vitro caries models as a contributing factor in the initiation and progression of the disease. Creating a microbial caries model still has its challenges. A reliable, clinically relevant microbial model should allow the active attachment of biofilm to the surface. The characteristics of the model should represent daily activities such as periodic changes in pH levels during the day; periodic exposure to sucrose, and periodic exposure to caries-preventive agents. Solutions used in the model should maintain bacterial viability and growth and also mimic daily challenges. In this study, we wanted to evaluate the variables mentioned earlier (substrate type and protective mesh) as essential components of a microbial caries model. This was a follow-up study to previous pilot experiments evaluating other factors such as: surface conditioning through the creation of acquired pellicle pre-biofilm growth; mineral saturation of growth media; and sucrose concentration (data not shown).

We chose the source of the microcosm bacterial biofilm to be pooled saliva from three donors. Collecting three samples, and having sucrose in our demineralization media, allowed overcoming any expected variability between the samples, as reported in the literature previously [28-31]. We allowed the biofilm to attach actively to the samples through mounting our samples on the inside of the lid of 24-well plates [8].

The reproducibility of using microcosm biofilms where saliva from one or more donors is being used is undoubtedly challenging. Several approaches can be taken to confirm the results, such as increasing the power or repeating the experiment several times. We have utilized both approaches in our studies and found consistent results (manuscripts in preparation).

Another characteristic in the model tested was the mesh. In a previous pilot study we conducted, it was visually apparent that the biofilm grown for an extended period of time does not grow evenly over the surface. It was reported previously by Mei et al. and other studies that uneven distribution of the bacteria over the surface may result in larger variability in the results [15-18].

We used a pH cycling model in this study. The model we used was modified from a previously published model [5]. The use of a pH cycling model has its significance. It mimics daily activities, resulting in high clinical relevance. It is also applicable in-vitro, allowing the reproducibility of the model in future studies. We followed Zhang et al. (2015) protocol in the treatment types used in their study: 18.4 mM NaF (treatment) and DIW (negative control) [5]. It should be noted that 18.4 mM NaF contains 350 ppm F; this concentration is equivalent to a toothpaste at European fluoride levels after saliva dilution during brushing (1400 ppm F in a 1:3 slurry). Prior to biofilm growth, we analyzed the fluoride, calcium, and phosphate concentrations of the BHI growth media. We found that the BHI media had a high concentration of phosphate (19027.1 ppm [200.4 mM]) and low calcium concentration (0.344 mM). Therefore, we supplemented the growth media with 1 mM $CaCl_2.2H_2O$.

When looking at carious lesion severity, the actual values of the outcome analyses were different between human and bovine specimens. Based on these results, the null hypothesis was rejected. However, the pattern of lesion severity was still similar in most of the data (Table 1). A previous study by Lippert and Lynch (2014) evaluated the differences in carious lesions created on human and bovine samples [14]. The study used a chemostat caries model, whereas we incorporated the microbial component in our model. Our study was still consistent with the findings by Lippert and Lynch (2014) which concluded that lesions in bovine enamel progress at a faster rate than human enamel.

Analyzing carious lesion severity (VHN_{change}, ΔZ, L) resulted in a significant interaction between pH cycling time, substrate type, and treatment type. However, the mesh factor was not a significant factor in the three or four-way ANOVA analyses. As the pH cycling time increases, an increased treatment effect was observed in both human and bovine samples.

We wanted to test the feasibility of using bovine enamel as a substitute to human enamel in microbial models for cariology research. Bovine enamel is readily available and cost-effective [14]. Human and Bovine enamel samples are widely used in in-vitro caries models. It has been documented that Bovine enamel has a faster rate of lesion progression that human (approximately 1.4:1). [14] In addition to the differences in prism arrangement, they differ in the uniformity of enamel thickness, porosity, and surface area size. [14] Bovine enamel has higher carbonate and lower fluoride contents when compared to human enamel.[32, 33] As we found in this study, having similar patterns in biofilm cariogenicity and lesion severity with biofilm's maturation in both substrate types makes bovine enamel a suitable substitution to human enamel.

The outcome analyses we chose when testing biofilm cariogenicity included biofilm virulence factors (LDH activities and EPS amounts). In general, testing biofilm virulence was more significant than biofilm viability as an indicator of the effectiveness of anticaries/antibacterial compounds; this is because the main goal of using these compounds is interference with biofilm cariogenicity, especially that killing bacteria within the biofilm requires high concentrations of fluoride compounds [6, 7, 9, 10, 34-38].

When comparing substrate types, differences in normalized LDH and EPS values were observed between human and bovine specimens at some timepoints. However, the pattern of biofilm cariogenicity over time was similar among the two substrate types (Tables 2 and 3), which is consistent with lesion severity data.

In both human and bovine groups (non-mesh groups), a trend of an increased biofilm cariogenicity (i.e. normalized LDH and EPS values) was observed. In earlier biofilm (4 days old), there was no statistically significant difference between substrate types (Table 2). More mature biofilm behaved differently according to substrate type: the statistically significant difference started in 8-day biofilm EPS data. One could argue that since there was a significant difference between substrate types we should always use human specimens to maintain clinical relevance. Our study is the first study to explore substrate type as part of a microbial cariogenic model. Since the pattern of biofilm cariogenicity over time found in this study was the same between the two substrate types, using bovine specimens in microbial studies (especially studies exploring biofilm maturation) is still valid. Moreover, within the context of this study, if a future study does not evaluate biofilm maturation, the selection of substrate type can be based then on the study's duration.

When evaluating the application of a protective mesh, the results of biofilm analyses exhibited a significant effect of the protective mesh. All groups that included a protective mesh demonstrated a lower biofilm cariogenicity (i.e. LDH activity, EPS amounts, and viability).

The main purpose of covering the specimens with mesh was to create niches for the biofilm to attach to, and allow biofilm to be adherent to the surface without dispersal/detachment, especially considering that biofilm maturation was a variable evaluated in this study (up to 12 days). In the "mesh-groups", the values of LDH activity and EPS amounts were not only low when compared to non-mesh groups, but also continued to be low among all timepoints (up to 12 days). When we collected the biofilm at each time-point, we used clean tweezers to remove the mesh first, as we were interested in analyzing the biofilm formed directly over the enamel surfaces. Then we loosened the specimen (using tweezers) and placed it in sterile saline for further analyses. Although we made sure not to disturb the biofilm layer formed over the sample itself, we believe that the physical removal of the mesh resulted in removing a large portion of the biofilm grown over the enamel surface. This may explain the low cariogenicity values when compared to non-mesh groups.

Based on the results of our study, we believe that applying a protective mesh was not a practical solution, even though it ensures the growth of a more evenly distributed biofilm layer (evaluated visually) over the mesh-covered samples. Zero (1995) emphasized the importance of biofilm's thickness and the influence of this factor on lesion severity as well as the pattern of lesion's remineralization (when the biofilm serves as a diffuser to different ions). Zero (1995) compared gauze-free and gauze-covered enamel samples in in-situ experimental models. He concluded that applying the gauze, even if it resulted in less clinical relevance, is critical; it allows the creation of a thicker biofilm and higher demineralization/ remineralization to the enamel surface, and therefore should be explored more [39]. However, Zero did not evaluate the biofilm's characteristics; he only focused on the lesion's characteristics. Based on the findings from our study and based on previous literature, this is found to be a great area of research and exploration in the future as a main characteristic of a standard microbial cariogenic model (i.e. finding interventions to maintain an even, thicker, actively attached biofilm in studies that extend to longer periods). Fluoride in the environment (i.e. growth media) can be a contributing factor influencing the pattern of enamel demineralization/remineralization. We did not measure fluoride concentrations in our culture media at different challenges. However, as mentioned earlier that biofilm can play a role as a diffusion barrier [3], we assumed that fluoride within the environment only affects biofilm-free specimens. Moreover, at each challenge, we used fresh media to minimize cross-contamination. We also washed the enamel with sterile saline between challenges to prevent contamination. However, this is still a critical area of research that needs to be evaluated in future studies.

Limitations and Future Studies

This study allowed a better understanding of the components of a controlled, more clinically-relevant, in-vitro microbial model. It established a further step to achieve an optimum microbial model. However, there are some limitations to this study, First, the large variability in the results has been a challenge in previous studies and was still a limitation to our study and needs to be addressed. Future studies may include the modification of the substrate's surface, such as omitting the polishing step or creating plaque stagnation areas, such as those on occlusal surfaces on the surface. Other studies may also include a more detailed analysis of the substrate surface to overcome the large variability. This can be achieved by obtaining multiple VHN_{change} readings from different areas on the enamel surface, and analyzing two or more enamel sections for ΔZ and L. Another limitation in our study is related to how pH cycling models may limit the inclusion of continuous pulsation of sucrose that establishes clinical relevance. Future studies may take this factor in consideration and its effects on the study's outcomes. Finally, this study focused mainly on alterations on biofilm's function (i.e. cariogenicity) and not composition (i.e. dominant bacteria within each maturation). This can be a critical future research area

for a full understanding of the fluoride-related alterations in the biofilm's function as well as the composition. Future studies may also include testing different fluoride concentrations to confirm the sensitivity of such models in testing anticaries agents.

Conclusion

In conclusion, within the study's limitations, human or bovine enamel specimens can be used in a microbial in vitro caries model to study biofilm maturation and the effect of anticaries agents. The use of utility protective mesh over the samples prevents accurate evaluation of the biofilm that is formed over the enamel surface, and therefore should be avoided.

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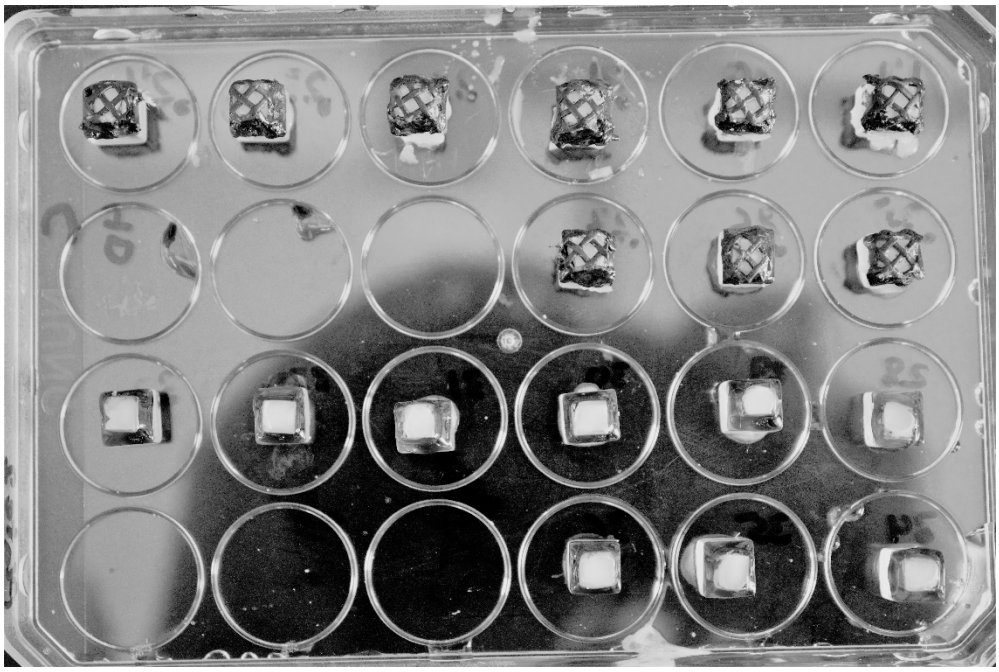

Figure 1- Protective mesh over enamel samples

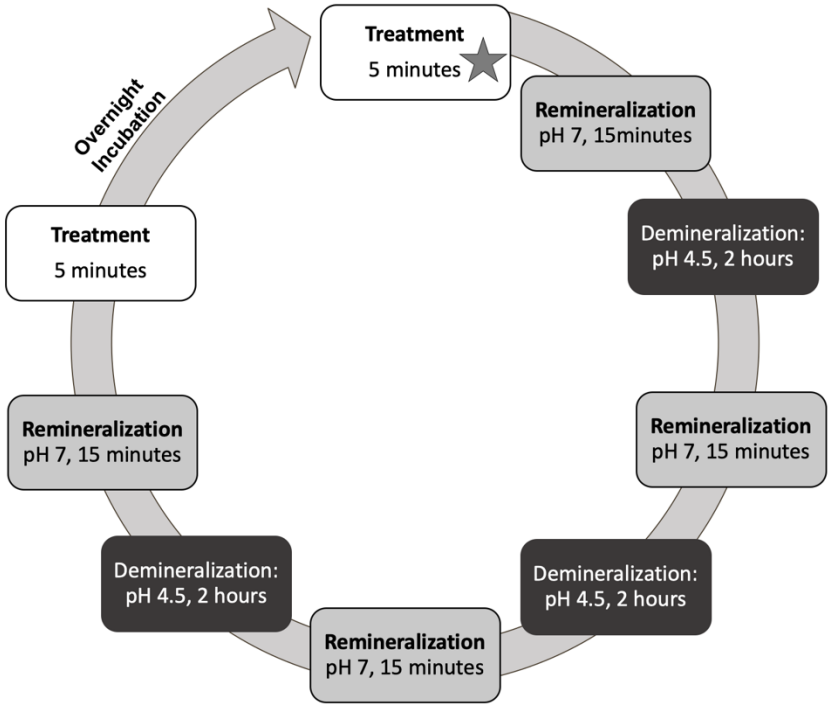

Figure 2- Daily pH cycling protocol

Table 1. Caries lesion severity: surface microhardness change (VHN_{change}); integrated mineral loss (ΔZ; %volmin.µm); and lesion depth (L; µm). All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

		VHN_{change}						ΔZ						L					
		4 Days		8 Days		12 Days		4 Days		8 Days		12 Days		4 Days		8 Days		12 Days	
Human	NaF	6.9 (5.8)	a,A,1	31.0 (16.6)	b,A,1	40.8 (16.1)	c,A,1	658 (601)	a,A	1156 (551)	b,1	828 (619)	ab,A	44.3 (50.0)	a,A	85.6 (52.0)	b,A,1	46.1 (48.2)	a
	DIW	15.8 (6.8)	a,B,1	67.2 (16.6)	b,B,1	74.2 (8.9)	b,B	1216 (676)	a,B	1092 (584)	a,1	1686 (771)	b,B,1	78.2 (40.3)	a,B,1	49.6 (29.5)	b,B,1	67.2 (25.7)	ab,1
Bovine	NaF	15.7 (6.1)	a,A,2	39.6 (16.5)	b,A,2	31.6 (12.9)	b,A,2	792 (608)	ab	509 (454)	a,A,2	963 (472)	b,A	40.9 (50.0)		24.3 (26.4)	A,2	51.3 (36.9)	A
	DIW	35.6 (13.3)	a,B,2	57.0 (18.5)	b,B,2	76.0 (7.7)	c,B	928 (721)	a	1513 (578)	b,B,2	2162 (883)	c,B,2	42.5 (46.4)	a,2	82.2 (47.0)	b,B,2	100.3 (44.1)	a,B,2

Lower Case: significance among pH cycling duration (4 Days; 8 Days; 12 Days). Different lower case indicate significance between groups
Upper Case: significance among treatment type (NaF; DIW). Different upper case indicate significance between groups
Numbers: significance among substrate type (Human; Bovine). Different numbers indicate significance between groups

Table 2: Biofilms cariogenicity: LDH activity (μg/ml), EPS amount (μg/ml), and viability. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment. All LDH activity and EPS amount values were normalized using protein concentration data

			LDH Activity Mean (SD)						**EPS Amount** Mean (SD)					
			4 Days		**8 Days**		**12 Days**		**4 Days**		**8 Days**		**12 Days**	
Human	**Mesh**	**NaF**	1.5 (0.7)		1.9 (0.7)		3.3 (1.9)	A,◊	2.0 (0.6)		4.0 (1.3)		3.5 (2.1)	◊
		DIW	1.7 (0.5)	a	1.5 (1.1)	a,◊	9.1 (3.7)	b,B,1,◊	2.5 (0.3)	a	2.2 (1.0)	a,◊	5.9 (1.9)	b,1,◊
	No Mesh	**NaF**	1.4 (1.2)	a	2.9 (1.2)	a,A	14.5 (7.2)	b,A,1,◊◊	2.7 (0.7)	a	4.7 (2.5)	a,A,1	10.7 (3.7)	b,A,1,◊◊
		DIW	0.6 (0.1)	a	7.6 (2.3)	b,B,◊◊	18.8 (6.0)	c,B,1,◊◊	2.7 (1.2)	a	7.9 (3.8)	b,B,◊◊	13.3 (6.8)	c.B,◊◊
Bovine	**Mesh**	**NaF**	1.0 (0.3)		0.9 (0.7)	◊	1.3 (0.3)	◊	3.7 (2.2)		2.1 (0.6)	◊	2.9 (0.7)	
		DIW	0.9 (0.6)		1.7 (0.6)	◊	0.9 (0.2)	◊,2	2.3 (0.7)		4.0 (1.2)	◊	1.7 (0.4)	2,◊
	No Mesh	**NaF**	0.9 (0.3)	a	4.8 (2.6)	b,◊◊	6.5 (2.1)	b,A,2,◊◊	2.7 (0.4)	a	10.2 (7.1)	b,A,2,◊◊	5.4 (1.2)	c,A,2
		DIW	0.6 (0.2)	a	6.3 (2.3)	b,◊◊	10.1 (1.8)	c,B,2,◊◊	1.6 (0.5)	a	6.8 (3.1)	b,B,◊◊	11.1 (3.9)	c,B,◊◊

Lower Case: significance among pH cycling duration (4 Days; 8 Days; 12 Days). Different lower case indicates significance between groups
Upper Case: significance among treatment type (NaF; DIW). Different upper case indicates significance between groups
Numbers: significance among substrate type (Human; Bovine). Different numbers indicate significance between groups
Symbols: significance among the use of protective mesh (yes/no). The presence of ◊ and ◊◊ indicates significance between the two groups

Table 3: Biofilm viability (12 day-old biofilms)

				log_{10} CFU/ml Mean (SD)
Human	**Mesh**	**NaF**	9.4 (0.7)	A,◊
		DIW	9.7 (0.2)	B,1, ◊
	No Mesh	**NaF**	10.1 (0.2)	◊◊
		DIW	10.0 (0.1)	◊◊
Bovine	**Mesh**	**NaF**	9.2 (0.2)	A, ◊
		DIW	8.8 (0.3)	B,2, ◊
	No Mesh	**NaF**	10.0 (0.1)	◊◊
		DIW	9.9 (0.1)	◊◊

Upper Case: significance among treatment type (NaF; DIW). Different upper case indicate significance between groups

Numbers: significance among substrate type (Human; Bovine). Different numbers indicate significance between groups

Single and Double Diamond Symbols: significance among the use of protective mesh (yes/no). The presence of ◊ and ◊◊ indicates significance between the two groups