Oral Hygiene Regimes:

How Oil Pulling and Conventional Mouthwashes Affect Dental Biofilms,

Focus on Cariogenic Streptococci

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NOTE: Due to closures involved with the SARS-COV-2 2020 pandemic, this study was prematurely halted. All results discussed herein refer to preliminary data obtained during testing.

ABSTRACT

This research addresses whether nontraditional oral hygiene regimes provide antimicrobial efficacy comparable to commercially available synthetic mouthwashes. A common interest among holistic communities is "oil pulling"—a practice originating in ancient Ayurveda oral hygiene. It consists of rinsing the mouth with vegetable oils, of which sesame and coconut oils are preferred, to enhance oral hygiene. This study focused on whether oil pulling reduces cariogenic *Streptococcus mutans* to the same degree as does common mouthwash. Volunteers above age 18 were to participate in this UWF-IRB-approved study. Participants would provide oral rinses of 20mL with sterile saline before and after treatments of Listerine®, or pulling oil; the rinses were transported to the lab to test for S. mutans and other oral bacteria. After the first round of treatments were complete, a 1-week “reset” period allowed normal microbial flora to re-establish, after which another treatment regime began; the cycle continued until all participants underwent all treatments. Samples were plated on a highly selective medium, favoring growth of *S. mutans*. Samples were also used to inoculate two broth dilution-tube series consisting of 1) a non-selective general medium TSB, and 2) a *S. mutans*-selective medium. Preliminary trials of this protocol showed that Listerine® reduced *S. mutans* and other oral bacteria by over 1-thousand-fold. Furthermore, almost all visible background growth was eliminated from the media plates after Listerine® treatment.

Introduction and Background

Members of the bacterial genus *Streptococcus* are common inhabitants of human skin, anterior nasopharynx, and human buccal and oral cavities. *Streptococcus* species are phylogenetically grouped in Domain Bacteria, Phylum Firmicutes, Class Bacilli, Order Lactobacillales and Family Streptococcaceae. *Streptococcus mutans*, and oral streptococci in general, have been implicated in the etiology of dental caries (Vinogradov et al, 2004). They are facultatively anaerobic members of a group termed "lactic acid bacteria", which use fermentative metabolism to produce lactic acid as a waste product of their metabolism. This acid is one factor in the development of dental caries. While saliva is the primary mechanism to remove this acid from the teeth, conditions such as xerostomia prevent saliva from performing its function. The resulting buildup of acid allows cavities to form in unusual places such as the middle of the buccal surface. Without the proper saliva to keep the teeth washed, the acid byproduct that S. mutans produces continues to build-up and can lead to holes forming in the tooth's enamel, leaving the soft dentin underneath exposed. Dentin is considerably weaker than enamel and, once exposed, offers little to no protection against the formation of dental caries.

One remedy to xerostomia, and other similar issues, are mouthwashes. Mouthwashes are able to relieve the symptoms of xerostomia and halitosis in a variety of ways. Many people rely on conventional, commercially available, mouthwashes such as Scope® and Listerine®, as well as nonconventional methods such as oil pulling, to combat these issues and leave a pleasant lingering freshness. Due to such wide usage of these rinses, it is important to understand their effects. A common interest among holistic communities is "oil pulling", a practice that originated as an ancient Ayurveda therapy for maintaining oral hygiene (Tomar 2014). The practice of oil pulling consists of rinsing the mouth with various oils, although sesame and coconut oils are preferred, for anywhere between 5-10 minutes. Oil pulling claims to avoid many

of the unpleasant features of modern alcohol-containing mouthwashes, such as burning and irritation of the gum tissue, while still being able to promote oral health. Proponents of oil pulling claim that the oil generates antioxidants which damage the cell wall of microbes and kill them. They also claim that the oil will attract the lipid layer of bacterial cell membranes, causing it to stick to the oil and be removed from the teeth (Vinogradov et al, 2004)(Tomar et al, 2014). Actual mechanisms have yet to be determined and the antimicrobial efficacy of oil pulling remains the subject of much debate. Thus, this is one practice that was of particular interest during the conception of this project.

A recent study was done that determined 84.9% of post-graduate dental students prescribe some type of mouthwash to their patients (Niveda & Jaiganesh, 2019). Such widespread use of mouthwashes indicates that we must explore all potential risks and alternatives. It is crucial to understand the functions of anything that is used inside, or upon, the human body. In so doing, informed decisions can be made, and lives can be improved in the most beneficial and efficient way possible.

Methods and Materials

Preparation of Specialized Media

The *S. mutans* selective media "tryptone-yeast extract-cystine with sucrose and bacitracin" (TYCSB) was selected for use in this study. The TYCSB media has been shown to have the highest recovery rate for *S. mutans,* as well as the highest ratio of *mutans* to non-*mutans* bacterial growth (Wan et al, 2002). One liter of TYCSB media was prepared. Of the 1L, 500mL was made with agar and 500mL without agar. The 500mL without agar was used as broth a dilution series. The media was made according to the following recipe (per 1L volume): 0.2g L-cystine HCl monohydrate; 15g bacto peptone; 5g yeast extract; 0.1g Na2SO3; 0.1g NaCl; 1.0g Na2HPO4.7H2O; 2.0g NaHCO3; 20g C2H3O2Na.3H2O; 20% w/v sucrose; 15g granulated agar; 0.2U/mlbacitracin; distilled water. Once autoclaved, plates were poured with the 500mL containing agar.

Collection of Samples

Samples were collected by having each participant rinse with an allotted 20mL of sterile saline. The saline was prepared in the lab prior to use at physiological conditions and sterilized in an autoclave to avoid contamination. 20mL was then dispensed into travel tubes using a serological pipette. Two saline samples were collected, per treatment, per person. One sample was taken pre-treatment, and another post-treatment. Participants were asked to rinse with the saline for one full minute, before spitting the solution back into the tube.

Treatments

Treatments were performed in a manner similar to sample collection. 20mL of treatment solution was pipetted into a travel tube and labeled with treatment type, participant number, and date.

Participants rinsed with either the Listerine® or oil for one and two minutes respectively. This duration was selected to reflect the instructions listed on the labels of each product. Once rinsing was complete, the solutions were spit back into the original tubes. Post-treatment samples were then collected.

Quantification via Dilution to Extinction

Once all samples were collected and transported back to lab, two dilution series per sample were setup. One dilution series consisted of TYCSB media, minus agar, for the quantification of *S. mutans* in the sample. Another series was performed containing the general tryptic soy broth (TSB) media that would encourage growth any bacteria in the sample. Preliminary trials were conducted to determine the most effective dilution factors for each series. These factors were determined to be 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} for the TYCSB series, and 10^{-3}, 10^{-6}, 10^{-8}, 10^{-10} for the TSB series. Samples were inoculated into the series and results were recorded every 24hrs for three days. All series were incubated at 37*C. After the three day period, final results were notated.

Growth Confirmation and Colony Count

Samples were also plated on the selective TYCSB media plates to supplement the results of the dilution series. Sterile swabs were placed into a freshly vortexed sample and plated according to Figure 1 (Right). Plates were incubated at 37*C and all growth was recorded. Colonies were verified as viridans strep. through catalase testing and gram staining. Any abnormal colonies were identified and excluded from *S. mutans* counts.

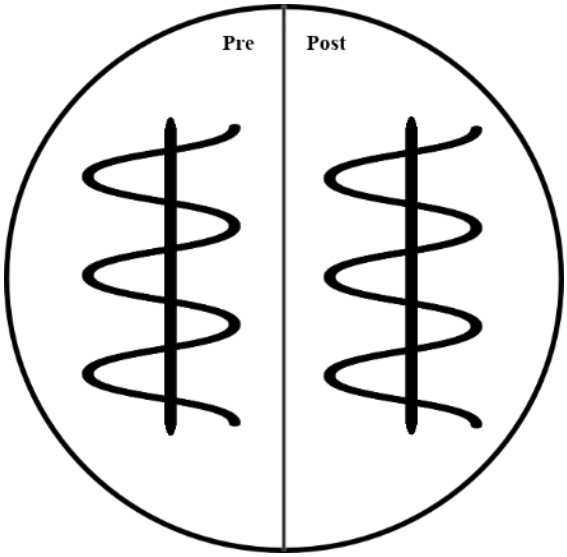

Figure 1. TYCSB Plate Inoculation

Results

Two complete Listerine® treatments were conducted. Pre-treatment samples were gram stained to verify the presence of bacteria. Figure 2 (Right) shows various gram-positive and gram-negative bacteria clinging to an epithelial cell that was suspended in the pre-treatment rinse. This confirms that 20mL saline rinses are sufficiently able to remove bacteria from the mouth. Samples were then taken and inoculated into both the TYCSB selective series and the TSB general series. The following image shows a selective series after the full incubation period (Figure 3).

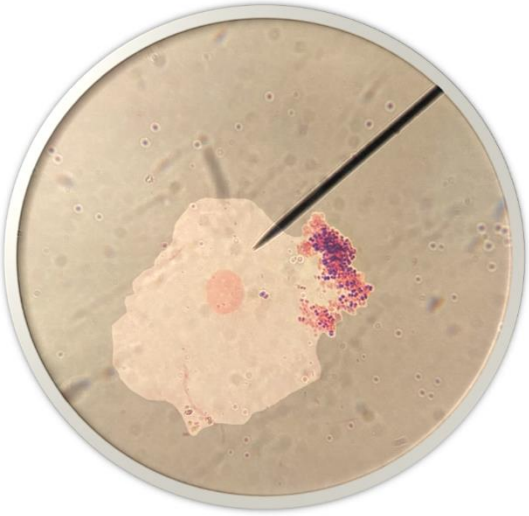

Figure 2. Rinse Gram Stain. Gram stain of pre-treatment rinse to confirm the presence of bacteria in the samples. The background has been darkened.

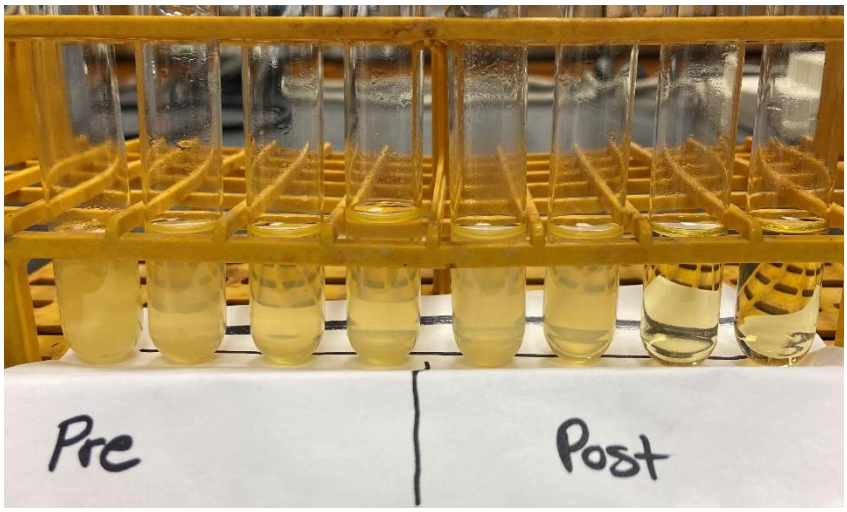

Figure 3. TYCSB Dilution Series. This dilution series shows growth in all pre-treatment tubes. Going even beyond the 1:10,000 factor of the last tube. The post-treatment tubes, however, are negative for growth after only a 1:100 dilution. This shows significant inhibition of *S. mutans* growth.

Samples were also plated onto TYCSB media plates and incubated at 37*C for 72hrs. The TYCSB media plates showed several colony types, designated as "Types #3-#5" (Figure 4):

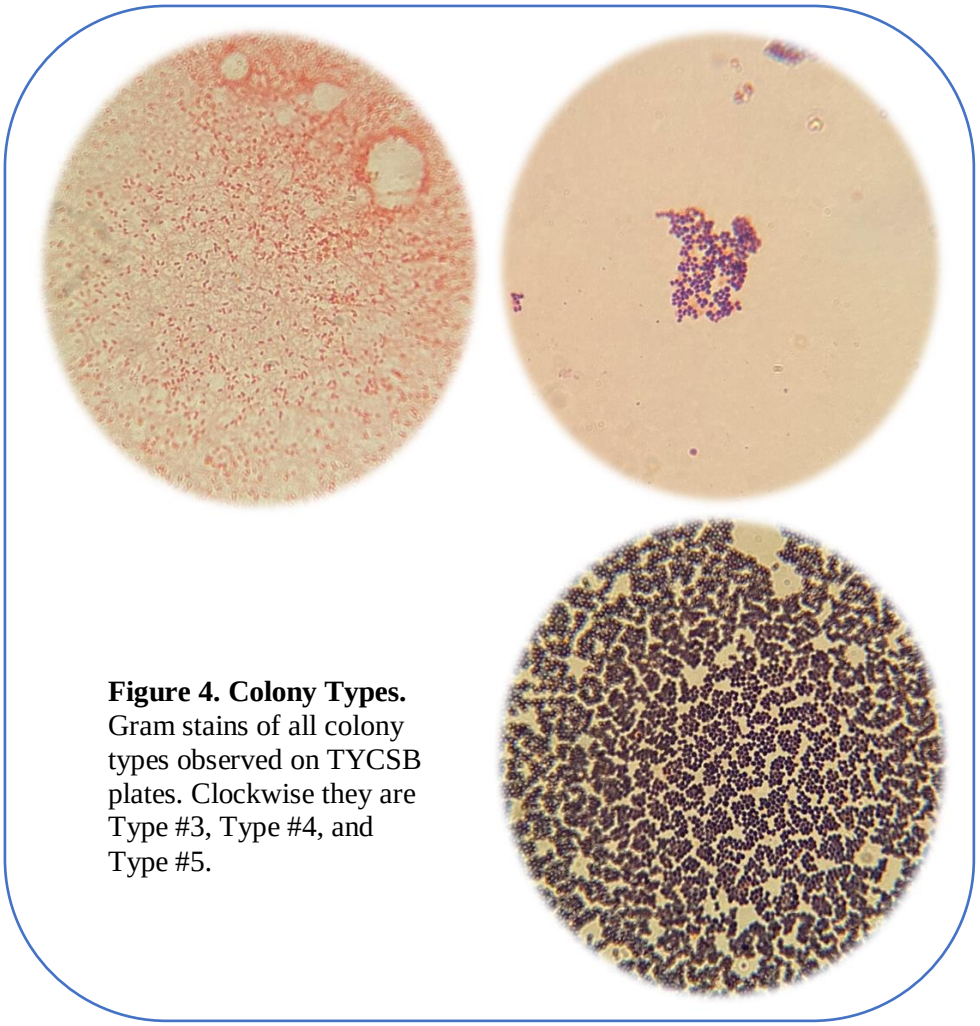

Figure 4. Colony Types. Gram stains of all colony types observed on TYCSB plates. Clockwise they are Type #3, Type #4, and Type #5.

Type #3 was a single colony outlier that was observed only on a single plate. It was a catalase positive, bacitracin-resistant, gram-negative rod. This is not unusual, as there are many such bacteria that can be found as normal oral flora. Notable colony morphology included a distinct beige color.

Type #4 appeared only as small, hard colonies. Its catalase result was equivocal due to the hard nature of the colonies. It is, however, a gram-positive coccus. There was a visible substance around the cells (Figure 4) that may be contributing to the hard nature of the colony. This is likely some way of coping with the small amount of bacitracin present in the media. This colony type was entirely eliminated in the post-treatment Listerine® samples.

Type #5 is suspected to be *S. mutans.* It showed uninhibited growth on the selective media, was catalase negative, and gram stained as gram-positive cocci in chains (Figure 5).

Broth cultures were also gram stained to confirm the identity of the dominant microbe.

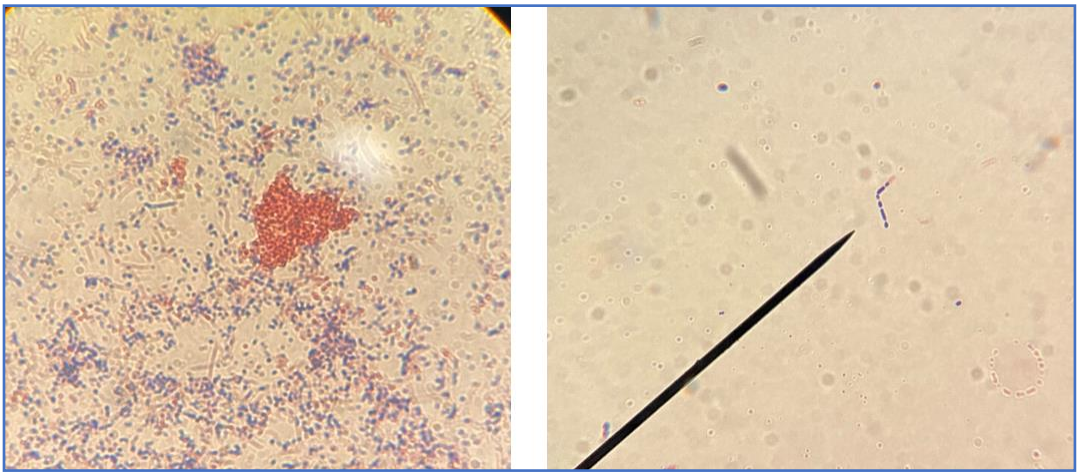

Figure 5. Gram Stains of Broth Cultures. Pre-Treatment rinses after 48hr incubation in general TSB (left) and specialized TYCSB (right).

Broth cultures allow the microbes to easily form their classic shape, such as the chains that can be seen in Figure 5. This can be lacking in the direct colony gram staining that is seen in Figure 4. The TSB media shows the expected growth of all organism present in the rinse. The TYCSB media shows significantly less growth. This is also as expected. The only growth seen was that of gram-positive cocci in chains.

More detailed supporting results and analyses are available in **Appendix 1: Office of Undergraduate Research poster presentation, and imbedded PDF.**

Conclusions

This study, while not able to be completed to its original extent, serves as a valuable proof-of-concept. Overall, the Listerine® treatments were able to consistently eliminate the background colony, Type #4. This is an extremely valuable metric that oil pulling treatments can be compared directly against. *S. mutans* elimination was variable and further testing should be done to confirm the efficacy of the Listerine® against *S. mutans.* Oil treatments were not conducted due to unexpectedly restricted laboratory access. However, these results show that the methodology and techniques employed in this study are effective at determining the antimicrobial properties of oral rinses. This can be expanded in future research to encompass more organisms, as well as a variety of treatments.

Acknowledgements

This honors thesis was completed under the mentorship of Joe Lepo, PhD, of the Department of Biology. This project was funded by an award from the University of West Florida Office of Undergraduate Research; award number S20-083P. This project also received and fulfilled all requirements for approval by the University of West Florida Institutional Review Board; proposal number IRB 2020-132.

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Appendix 1: Office of Undergraduate Research poster presentation, and imbedded PDF.

Oral Hygiene Regimes: How Oil Pulling and Conventional Mouthwashes Affect Dental Biofilms, Focus on Cariogenic Streptococci

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ABSTRACT

This research addresses whether nontraditional oral hygiene regimes provide antimicrobial efficacy comparable to commercially available synthetic mouthwashes. A common interest among holistic communities is "oil pulling"—a practice originating in ancient Ayurveda oral hygiene. It consists of rinsing the mouth with vegetable oils, of which sesame and coconut oils are preferred, to promote oral and overall heath. This study focused on whether oil pulling reduces cariogenic *Streptococcus mutans* to the same degree as does common mouthwash. Volunteers above age 18 participated in this UWF-IRB-approved study. Five participants were to provide oral rinses of 20mL with sterile saline before and after treatments of Listerine®, or pulling oil; the rinses were then to be transported to the lab to test for *S. mutans* and other oral bacteria. After the first round of treatments were complete, a 1-week "reset" period allowed normal microbial flora to re-establish, after which another treatment regime began; this cycle would continue until all participants underwent all treatments. Samples were plated on a highly selective medium (TYCSB), favoring growth of *S. mutans*. Samples were also used to inoculate two broth dilution-tube series consisting of 1) a non-selective general medium (TSB), and 2) a *S. mutans*-selective medium. Preliminary trials of this protocol showed that Listerine® reduced *S. mutans* and other oral bacteria by over 1-thousand fold. Furthermore, almost all visible background growth was eliminated from the media plates after Listerine® treatment.

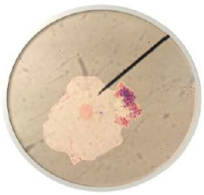

Figure 1 (Above).
A gram stain of a saline rinse that shows bacteria clinging to a cell. The background has been darkened.

PURPOSE

This study's aim was to reveal the true connections between oral rinses and their ability to eliminate the cariogenic bacteria that reside in the mouth. Information on this topic is limited and neglects to explore the microbiological side of how rinses can affect oral health. A recently published study determined that 84.9% of post-graduate dental students prescribe some type of mouthwash to their patients. Oil pulling originated as an ancient ayurvedic therapy for maintaining oral hygiene and has increased in popularity among people looking to avoid many of the possible side effects that often accompany modern medicines. It is crucial to understand the functions of anything that is used inside, or upon, the human body. In so doing, informed decisions can be made, and lives can be improved in the most beneficial and efficient way possible.

METHOD

- Pre-Treatment samples were taken by rinsing with 20mL of sterile saline.
- Subjects then rinsed with 20mL of an assigned treatment (Listerine® or pulling oil) for one or two minutes, respectively.
- Post-Treatment samples were taken within 5min of treatment with another 20mL of sterile saline.
- Samples were transported to the lab and plated on TYCSB media plate and inoculated into dilution series.
- Results were recorded every 24hrs for three days.

Figure 2 (Below).
Example of a dilution series set.

The above photos (Figure 2) show five TYCSB dilution series before inoculation. In order from High to Low concentration they are, 1/10, 1/100, 1/1,000, and 1/10,000.

Samples were also plated onto the TYCSB selective media plates. Plates were divided in half with the Pre-Treatment sample on one side and the Post-Treatment sample on the other. Notable colonies were marked, gram stained, and biochemically tested. One outlier was discovered and labeled as colony type #3. The outlier was a gram-negative rod that tested catalase positive. It must also exhibit some level of bacitracin resistance to grow on the TYCSB plate without inhibition. Most colonies were of type #5 and were gram-positive cocci that tested as catalase negative. This is what was interpreted as *S. mutans*.

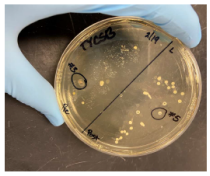

Figure 3 (Above).
TYCSB Media Plate with pre and post treatment rinses plated and colony types marked.

RESULTS

Preliminary results showed significant inhibition of *S. mutans* growth with Listerine® treatments. Figure 7 shows a minimum of a 100-fold decrease in bacterial growth.

The TYCSB media plates showed several colony types, labeled #3-#5.

Type #3 was a single colony outlier that was observed only on a single plate. It was a catalase positive, bacitracin resistant, gram-negative rod. This is not unusual, as there are many such bacteria that can be found as normal oral flora.

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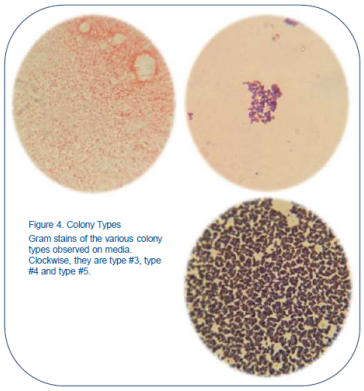

Figure 4. Colony Types
Gram stains of the various colony types observed on media. Clockwise, they are type #3, type #4 and type #5.

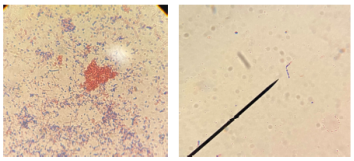

Figure 6 (Above). Broth Gram Stain.
Pre-Treatment rinses after 48hr incubation in general TSA broth (left) and specialized TYCSB broth (right).

CONCLUSIONS

Overall, the Listerine® treatments were able to consistently eliminate the background colony type #4. *S. mutans* elimination was variable and further testing should be done to confirm the efficacy of the Listerine® against *S. mutans*. Oil treatments were not able to be conducted due to unexpectedly restricted laboratory access. However, these results show that the methodology and techniques employed in this study are effective at determining the antimicrobial properties of oral rinses. This can be expanded on in future research to encompass more organisms, as well as a variety of treatments.

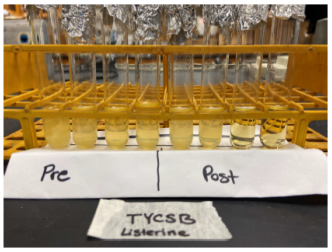

Figure 7 (Above). A dilution series from Figure 2 after being incubated for 72hrs. It consists of a Listerine® treatment in selective *S. mutans* media.

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