The Effect of Mouthwash on Oral Microflora

To what extent does mouthwash containing chlorhexidine gluconate affect the growth of beneficial microflora, using lab safe *Escherichia coli* and *Streptococcus salivarius* as models?

Biology

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Introduction

In this investigation, I explored the effect mouthwash has on bacteria in the mouth. Specifically, if different mouthwash solutions are designed to differentiate between beneficial and harmful bacteria. This dilemma became apparent to me when I came across an article by "The Telegraph" on the drawbacks of using mouthwash. The article mentioned bad breath becoming a problem for frequent mouthwash users because the mouthwash dried out the mouth which allowed for certain types of bacteria to flourish (Knapton, 2017). To focus the investigation, I wanted to use *Streptococcus pyogenes*, *Streptococcus salivarius*, and *Porphyromonas gingivalis* because *S. salivarius* naturally regulates the level of *S. pyogenes and P. gingivalis* in the mouth. If mouthwash is prescribed to treat infections caused by harmful bacteria, the mouthwash may alter the levels of beneficial bacteria as well. Additionally, I decided to test both gram-positive and gram-negative bacteria to compare the impact mouthwash containing chlorhexidine gluconate (CHX) has on both. However, the ethical question of bringing pathogens into a school led me to decide to use lab safe *Escherichia coli* as a model for *P. gingivalis* because they are both gram-negative bacteria. *S. pyogenes* is a gram-positive pathogen, but I can still safely study the effect of CHX on gram-positive bacteria using *S. salivarius* as a model since *S. salivarius* is a normal part of the oral flora. By investigating both gram-negative and gram-positive bacteria, I can investigate the effect of CHX on a wider range of bacteria, while attempting to accurately represent the actual oral microflora.

Therefore, my question is: To what extent does mouthwash containing chlorhexidine gluconate affect the growth of beneficial microflora, using lab safe *Escherichia coli* and *Streptococcus salivarius* as models?

This question is worthy of investigation because if mouthwash damages the population of beneficial bacteria in the mouth, it would cause the individual to be more susceptible to other potentially harmful bacteria, possibly leading to an infection. Therefore, it is relevant to study the effect of mouthwash containing CHX on *E. coli* and *S. salivarius* in order to understand the effects of a decreased population of *S. salivarius* on the oral microflora.

Background Information

The two main categories of mouthwash are cosmetic and therapeutic. Cosmetic mouthwashes have short term benefits, such as temporarily removing unpleasant mouth odor or taste. While they do contain antiseptic ingredients, it is normally a small amount. They can be bought at any convenience store. Conversely, therapeutic mouthwashes, also referred to as oral rinses, contain active ingredients that are intended to treat symptoms by killing oral bacteria (American Dental Association, 2019). Therapeutic mouthwashes are used to treat conditions like gingivitis, tooth decay, and plaque, among others. Examples of active ingredients include fluoride, essential oils, peroxide, and chlorhexidine. Depending on the ingredient and the concentration, therapeutic mouthwashes can be prescribed or over-the-counter. Interestingly, many cosmetic mouthwash companies make claims that their product has bacteria killing properties for situations such as plaque or gingivitis. In this experiment, I will be comparing the effect of both categories of mouthwashes on *S. salivarius* and *E. Coli*.

CHX was first introduced in 1954 as an antibacterial substance. It functions by causing cell inhibition and death (Mcbain, et al., 2003) through disruption of the cell membrane, and effectively kills both gram-negative and gram-positive bacteria. There have been many studies on the benefits of CHX as an ingredient in mouthwash, as it is efficient in reducing the viability

of oral bacteria, inhibiting the regrowth of plaque, and preventing gingivitis (Mcbain, et al., 2003). However, there has not been much research on the long-term effects of mouthwash containing CHX. A study published in the journal *Frontiers in Microbiology* explains that not enough is known about the risk of oral bacteria developing a resistance toward CHX and there is little awareness on current CHX resistance in the dental community (Cieplik, et al., 2019). Even more concerning is if research on CHX continues at its current pace, potentially negative effects on one's oral health could begin to take place, likely when it is too late to reverse the effects. One effect could be the development of an infection that is hard to treat because it no longer responds to antibiotics that were once used to treat it. Additionally, there is a possibility that the bacteria could spread the resistance genes to other bacteria, leading to further complications.

Streptococcus salivarius is a gram-positive bacterium found both in the gut and in the oral cavity. It has anti-inflammatory properties that counteract many pathogens' effects along the digestive tract, which suggests the bacterium plays an important role in the body's immune response to certain pathogens. A study published in the journal *Applied and Environmental Microbiology* states, "The *S. salivarius* TOVE-R strain has been reported to be a successful antagonist of virulent streptococci involved in tooth decay or pharyngitis, such as *Streptococcus mutans*, *Streptococcus sobrinus*, and *Streptococcus pyogenes*, or pathogens involved in periodontitis" (Kaci, et al., 2014). The study mentioned that the K12 strain of *S. salivarius* has been approved by the FDA and is starting to be used as a probiotic (Kaci, et al., 2014). The bacterium is a powerful weapon against pathogens becoming antibiotic resistant. An interesting similarity between *S. salivarius* and certain strains of *E. coli,* like *E. coli* UTI89 and CFT073, is that both bacteria are found in the gut and are important to the microflora of the intestines and other parts of the digestive system. Accordingly, damage to the population of either bacteria

would potentially cause drastic changes and negatively impact the entire digestive system and body overall.

Streptococcus pyogenes is a gram-positive bacterium with a known history of causing infections that can lead to further problems. If levels of the *S. pyogenes* grow out of control, the bacteria can cause a variety of infections such as strep throat, gingivitis, rheumatic fever, tonsillitis, scarlet fever, and many others (Stevens, n.d.). There have also been outbreaks of *S. pyogenes* resulting from changes in the way in which the bacterium is transmitted, as well as a change in the virulence of the organism (Stevens, n.d.) that could be attributed to the bacterium's growing resistance to antibiotics.

Porphyromonas gingivalis is a gram-negative bacterium that is known to be a main agent in causing inflammation in the oral cavity associated with periodontal disease. An article published in *Frontiers in Microbiology* claims *P. gingivalis* produces a variety of virulence factors that could penetrate the gingivae and cause tissue destruction by inducing inflammation (How, Song, & Chan, 2016). CHX is effective against gram-negative bacteria like *P. gingivalis* because it disrupts the cell membrane and prevents the growth of the population quickly, minimizing the effects of the bacteria.

In 2016, the FDA placed a ban on 19 antibacterial additives in soap, one being triclosan. Bacteria that is normally seen on the skin was found to be resistant to triclosan. This poses a problem because bacteria that is continuously exposed to triclosan develops mutations that allow the bacteria to survive the antiseptic. It is a concern of many scientists that these mutations could evolve to resist other antibacterial products (Rangel & Gerhardt, 2017). While many soap companies have taken action to remove triclosan from their products, Dial was criticized for continuing to market products with triclosan. Unfortunately, there have been many cases similar

to this where bacteria became resistant to antibiotics and caused harm to many people. *S. salivarius* may be the key to fighting against these resistant bacteria. Dr. Mignolet, interviewed by Science Today, says, "We've found that *S. salivarius* bacteriocins can kill *Staphylococcus aureus*, *Listeria* and even some enterococci – pathogenic bacteria that resist more and more antibiotic treatments" (Mignolet, 2018). If strong antibiotics continue to be unnecessarily used to treat bacterial infections, other bacteria could become resistant to antibiotics. Thus, it is even more important to investigate the effects an antiseptic like CHX can have on bacteria that function like *S. salivarius* before other bacteria become resistant to antibiotics as well.

Hypothesis

The **research hypothesis** is that mouthwash containing CHX will inhibit the growth of both *S. salivarius* and *E. coli* because CHX is not designed to differentiate between beneficial and harmful bacteria. There will be no difference in the effect of the mouthwash, the zones of inhibition, between the two bacteria.

The **alternative hypothesis** is that mouthwash containing CHX will create a greater zone of inhibition with *E. coli* than *S. salivarius*.

Method

Ethical Considerations

Along with collecting all materials, I signed a permission slip with my dentist to ensure I used the oral rinse properly (**Appendix A**). *S. salivarius* and K12 *E. coli* were verified as safe bacteria to use for this experiment (**Appendix B**). Sterile procedures include wearing gloves and goggles, using sterile equipment for each trial, using a Bunsen burner to sterilize the inoculation

loop after every smear, and at the end all agar plates were treated with a 10% bleach solution and all materials were sterilized in a hydroclave.

Materials List

- Culture of *Escherichia coli* K12 (BioRad)
- Culture of *Streptococcus salivarius* (Carolina Biological)
- Cosmetic mouthwash: Listerine Fresh Burst (Eucalyptol 0.092%, Menthol 0.042%, Methyl salicylate 0.060%, Thymol 0.064%)
- Therapeutic mouthwash: Xttrium Laboratories Oral Rinse (0.12% CHX)
- Agar plates (150x15 mm)
- Sterile cotton swabs (Fisherbrand)
- Sterile disks 6 mm (BBL brand)
- Sterile forceps
- Ruler (± .5 mm)
- Sharpie
- Sterile plastic 1mL (± 0.1 mL) pipette (BioRad)
- Harvey-Barnstead MC10 Hydroclave
- Sterile 50 mL (± 5 mL) beaker (Pyrex)
- Sterile water
- Vortex mixer (Fisherbrand)
- Inoculation loops
- Parafilm “M”
- Bunsen burner
- Additional Agar Materials

- Nutrient agar (see **Appendix C**)
- Tryptic soy agar (see **Appendix C**)
- Thermometer (±1 ºC) (Flinn)
- Sterile 1 L (± 0.0005 L) borosilicate glass flask (Kimax)
- Hot plate (Corning)

Variables

Independent variables:

- Type of bacteria: *E. coli* or *S. salivarius*
- Type of mouthwash: cosmetic or therapeutic (containing CHX), and the concentration in the therapeutic mouthwash (0.06 %, 0.12%)

Dependent variables:

- Diameter of the zones of inhibition in millimeters measured with a ruler (± 0.5 mm)

Control variables

- Type of agar for each bacterium
- Temperature at which the inoculated plates were stored (22°C)
- Location (school laboratory benchtop) and time (3 days) at which the inoculated plates were stored
- Size of the disks, which also ensures the same amount of mouthwash solution is used in each individual trial
- Sterile techniques to ensure no contamination takes place

Control

- The control for the *S. salivarius* is trial 1, the *S. salivarius* and cosmetic mouthwash disks
- The control for the *E. coli* is trial 2, the *E. coli* and cosmetic mouthwash disks

Procedures

Tryptic Soy Agar (TSA) was utilized for *S. salivarius,* and Nutrient Agar for *E. coli* as those were the non-selective agar types recommended by the bacteria companies for ideal colony growth. The agar procedures can be found in **Appendix C**.

Preparation

1. Set aside 4 mL of sterile nutrient broth.
2. Pour 20 mL of 0.12 CHX into a 50 mL beaker. Soak 27 disks.
3. Pour 20 mL of the Listerine mouthwash into a 50 mL beaker. Soak 32 disks.
4. Make a 0.06% CHX dilution by adding 10 mL of 0.12% CHX to a 50 mL beaker and 10 mL of sterile water. Soak 32 disks.

Trial 1

1. Label nutrient agar plate "K: cosmetic - *E. coli*" with the Sharpie
2. Measure 2 mL of nutrient broth using a pipette and dispense into a sterile test tube. Use the same tube for all trials with the same bacteria but use a new sterile swab each time.
3. Scrape through colonies using an inoculation loop, suspend colonies into the test tube of broth, cover the top of the tube with parafilm, then agitate for 30 seconds using a vortex mixer. Let tube rest for 5 minutes.
4. Dip into the mixture of the bacteria and broth using a sterile swab, pressing out excess on the side of the test tube.
5. Spread the inoculum over the agar plate using the swab, ensuring the entire surface is completely covered.
6. Using sterilized forceps, place 5 disks that have been soaked in the appropriate mouthwash solution onto the plate equidistant apart.

7. With sterile forceps, gently press down on disks to maximize contact with surface of agar, taking care not to damage the agar.
8. Place agar plates on benchtop for 3 days at room temperature (22°C).
9. After 3 days, use a ruler to measure the diameter of the zones of inhibition.
10. Dispose of all materials using sterile technique to ensure there is no contamination of lab equipment or other trials.

Trial 2

1. Label the TSA agar plate "K: cosmetic - *S. salivarius*"
2. Repeat steps 2-10 from trial 1 using cosmetic mouthwash and *S. salivarius*.

Trial 3

1. Label the nutrient agar plate "K: therapeutic 0.06% CHX - *E. coli*"
2. Repeat steps 2-10 from trial 1 using 0.06% CHX and *E. coli*.

Trial 4

1. Label the TSA agar plate "K: therapeutic 0.06% CHX- *S. salivarius*"
2. Repeat steps 2-10 from trial 1 using 0.06% CHX and *S. salivarius*.

Trial 5

1. Label the nutrient agar plate "K: therapeutic 0.12% CHX- *E. coli*"
2. Repeat steps 2-10 from trial 1 using 0.12% CHX and *E. coli*.

Trial 6

1. Label the TSA agar plate "K: therapeutic 0.12% CHX - *S. salivarius*"
2. Repeat steps 2-10 from trial 1 using 0.12% CHX and *S. salivarius*.

Results

Qualitative Data

Initially, I predicted that the cosmetic mouthwash would have no effect on the bacteria, as it does not have a strong antibacterial agent. However, even though the zone of inhibition was not as large as the trials with CHX, there was a ring around the antibacterial disk (**Appendices D and E**). Thus, it is reasonable to conclude that the cosmetic mouthwash did inhibit the growth of both bacteria.

Furthermore, the results of the trials of bacteria in both the 0.06% and 0.12% CHX show a clear inhibition of growth in generally uniform circles (**Appendices F and G**). Data points that were not uniform circles were discarded from the analysis and were attributed to researcher error when placing the soaked disks. Also, the zones of inhibition on the 0.06% and 0.12% CHX didn't appear different in size through visual observation. I concluded that more testing is needed to determine at which dilution the CHX would show a decrease in size of the zone of inhibition.

Quantitative Data

In general, there was no significant difference in the effect of any mouthwash trial between the *E. coli* and *S. salivarius*. The raw data is in **Appendix H**, but the means of the measurements of the zones of inhibition in millimeters are displayed in **Table 1**.

Table 1:

Means of the measurements of the zones of inhibition in millimeters rounded to the hundredth place

Trial	**Zone of Inhibition (± 0.5 mm)**
Trial 1: cosmetic - *E. coli*	6.63
Trial 2: cosmetic - *S. salivarius*	6.50
Trial 3: therapeutic 0.06% CHX - *E. coli*	20.88
Trial 4: therapeutic 0.06% CHX- *S. salivarius*	21.17
Trial 5: therapeutic 0.12% CHX- *E. coli*	22.48
Trial 6: therapeutic 0.12% CHX - *S. salivarius*	22.58

Calculations:

I decided to conduct a 2-sample t-tests of independent means to determine if there is a significant difference in the means of the trials. This test was a good fit to see if any of the mouthwashes created greater zones of inhibition in one bacterium over another. Using a TI-84 calculator, I conducted 3 2-sample t-tests of independent means, one for each pairing of the two types of bacteria with the same mouthwash. The pairings are as follows: trials 1 and 2, trials 3 and 4, and trials 5 and 6. Table 2 displays the t-values and p-values of each test, along with whether the p-value leads to a rejection of the alternative hypothesis. The assumption is that the measurements of the zones of inhibition for each separate trial are normally distributed. Another

assumption is that trials 1 and 2 are independent of one another, trials 3 and 4 are independent of one another, and trials 5 and 6 are independent of one another. The t-test will test under the condition $\mu1>\mu2$. The $\mu1$ is the mean diameter of the zone of inhibition of the *E. coli*, and the $\mu2$ is the mean diameter of the zone of inhibition of the *S. salivarius*.

Research Hypothesis: There will be no significant difference in the effect of the mouthwash, the zones of inhibition, between the two bacteria.

Alternative Hypothesis: There will be a significant difference in the effect of the mouthwash, the zones of inhibition, between the two bacteria. Mouthwash containing CHX will create a greater zone of inhibition with *E. coli* than *S. salivarius*.

Table 2:

A table displaying the t-values and p-values of each test, and whether the p-value is significant for a rejection of the alternative hypothesis or not

	T-Value	**P-Value**	**Reject Alternative?**
Trials 1 and 2	0.74739	0.230322	No
Trials 3 and 4	-1.06565	0.147541	No
Trials 5 and 6	-0.18639	0.426823	No

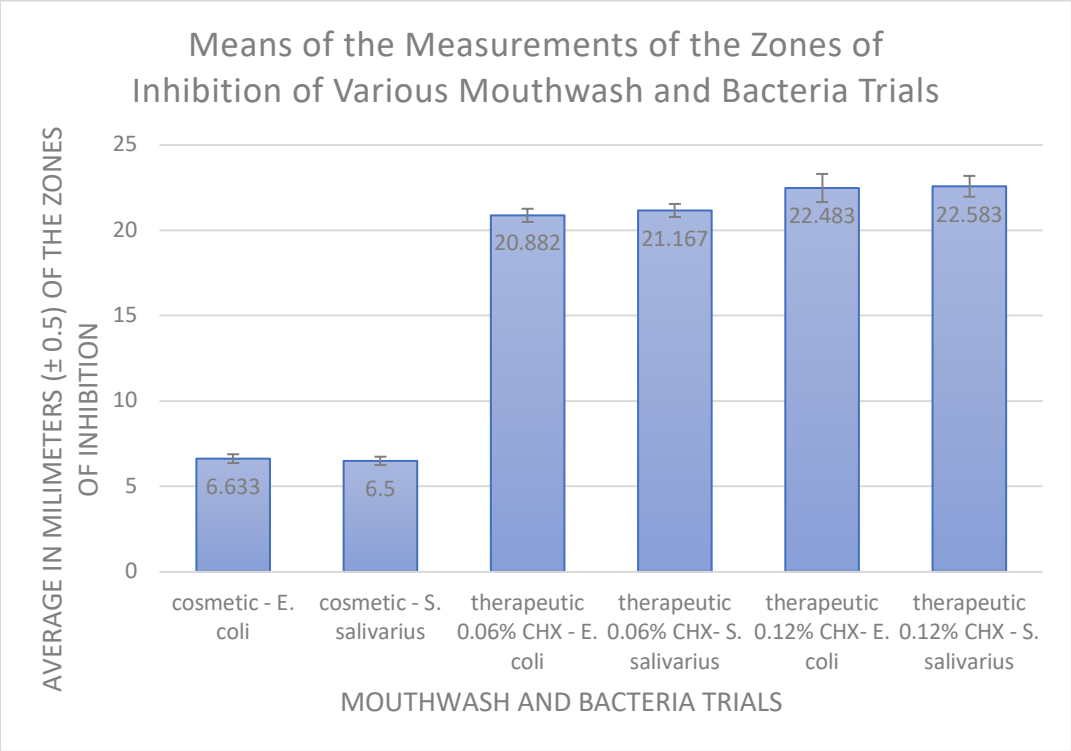
Analysis and Discussion

Figure 1 displays the zones of inhibition for the Various Mouthwash and Bacteria Trials in a bar chart with error bars. The error bars calculated through excel display the standard deviation of the data set. The standard deviation represents the average value that a data set differs from the mean value, which can be used to determine the reliability and spread of data

relative to the mean. The error bars show overlap between the pairs of the 3 groups, which suggests there is no statistically significant difference in the means, but this must be verified using a t-test.

Figure 1:

Vertical bar chart displaying the means of the measurements of the zones of inhibition of various mouthwash and bacteria trials



Analysis of T-Tests

At the 5% significance level, the t-test with the cosmetic mouthwash and both bacteria produced a p-value of 0.230322 under the condition $\mu 1 > \mu 2$, which is not statistically significant. Therefore, the results show insufficient evidence that the cosmetic mouthwash created a greater zone of inhibition in *E. coli* than *S. salivarius.* Thus, it is reasonable to reject the alternative hypothesis in favor of the research hypothesis at the at the 5 % significance level. There was no

statistically significant difference in effect of cosmetic mouthwash on the inhibition of the growth of *S. salivarius* and *E. coli.*

At the 5 % significance level, the t-test with the 0.06% CHX mouthwash and both bacteria produced a p-value of 0.147541 under the condition $\mu 1 > \mu 2$. Therefore, the results show insufficient evidence that the CHX 0.06% mouthwash created a greater zone of inhibition in *E. coli* than *S. salivarius.* Thus, it is reasonable to reject the alternative hypothesis in favor of the research hypothesis at the at the 5% significance level. There was no statistically significant difference in effect of 0.06% CHX mouthwash on the inhibition of the growth of *S. salivarius* and *E. coli.*

At the 5% significance level, the t-test with the 0.12% CHX mouthwash and both bacteria produced a p-value of 0.426823 under the condition $\mu 1 > \mu 2$. Therefore, the results show insufficient evidence that the CHX 0.12% mouthwash created a greater zone of inhibition in *E. coli* than *S. salivarius.* Thus, it is reasonable to reject the alternative hypothesis in favor of the research hypothesis at the at the 5 % significance level. There was no statistically significant difference in effect of 0.12% CHX mouthwash on the inhibition of the growth of *S. salivarius* and *E. coli.*

Evaluation

Evaluation of Method

The method of placing multiple disks on one large agar plate proved to be useful in order to have a large number of samples without risking overlap of the zones of inhibition, which strengthened the reliability of the results. However, zone of inhibition testing doesn't necessarily mean the bacteria was killed by the antimicrobial, just that it was prevented from growing. So,

there are limitations to the application of this experiment when discussing if using mouthwash containing CHX kills bacteria or only inhibits their growth. Also, the zones have a natural variability because the boundaries of the zones aren't always clear. In future experiments, using tests (Emerson et al.), such as those shown in **Appendix I,** to determine whether the bacteria were killed by the CHX will increase the validity of the results.

Evaluation of Online Sources

I used a variety of online scientific sources used in the process of the experiment; each source was well-researched and credible. I focused on using information from trusted databases, mostly using website libraries of research studies, such as the American Dental Association, National Center for Biotechnology Information, and the American Society for Microbiology; All of these are peer-reviewed and highly respected research institutions. However, I was limited in gathering print sources to use as most libraries shut down for the pandemic. In future experiments, I would like to gather more of a variety of sources.

Evaluation of Sample Size

I initially intended to collect 10 data points from each condition, but I had extra agar plates, so I was able to have more data points for some conditions. The sample size for each trial was sufficient enough to gain a representative average and to conduct a t-test, but due to financial restraints and the pandemic, I was limited in how many data points I could collect. Of course, having a larger sample size in future experiments would serve to strengthen the validity of the test results.

Concerns with the Repeatability and Control with using Living Organisms

Lastly, there are concerns with repeatability and control while using living organisms. I used the same population of both bacteria to control for possible differences between bacteria

populations. Although this also means my data collection and the repeatability of this experiment is limited to the population that I used. Even with strict measures in place throughout the entire duration of the experiment, there remains the possibility of the contamination of materials and the two bacteria, and therefore this could lead to anomalous data. Also, the bacteria used were non-pathogenic but were used as models for pathogenic bacteria. This creates uncertainty to if the cosmetic or therapeutic mouthwashes would produce the same result if the experiment is repeated with pathogenic bacteria. In future experiments, I could use the initial bacteria I intended to use: *S. salivarius, P. gingivalis,* and *S. pyogenes*.

Conclusion

This experiment investigated the question: To what extent does mouthwash containing chlorhexidine gluconate affect the growth of beneficial microflora, using lab safe *Escherichia coli* and *Streptococcus salivarius* as models?

In general, the analysis of the collected data demonstrates that mouthwash containing CHX produces no significant difference in the inhibition of growth between *E. coli* and *S. salivarius*. It is reasonable to conclude that mouthwash containing CHX is not specialized to differentiate between beneficial and harmful bacteria. Based on the results, the therapeutic mouthwash in both concentrations caused a larger zone of inhibition in the trial averages when compared to the cosmetic mouthwash. The results from my experiment correspond with the results of a journal published in *BMC Microbiology*. The study investigated the CHX resistance of oral bacteria in dental plaque, including *E. coli* and *S. salivarius*, and found similar inhibition of growth, measured by zones of inhibition in millimeters, to the results in this paper (Saleem, Seers, Sabri, & Reynolds, 2016). However, the researchers identified some strains of bacteria

that were resistant to CHX on some level. The study concluded that some bacteria that are resistant to CHX are also resistant to other antibiotics, which highlights the potential consequences of prescribing medications with CHX long-term.

I can expand on this experiment in the future by using bacteria, like *S. Pyogenes,* that I was unable to use due to being in a high school setting. Additionally, because I found there to be a small difference in the average between the 2 concentrations of CHX, another variation of this experiment could be to test at which concentrations of CHX the mouthwash decreases in its effect on bacteria growth. This experiment would be relevant for mouthwash companies to decide what concentration is needed in order to save resources and avoid unnecessary higher concentrations that could potentially harm patients.

Therefore, it is relevant to consider the positive and negative aspects of using therapeutic mouthwash. A new question that arises from the results of this experiment is: is it possible to counter the effect or prevent the inhibition of the growth of oral bacteria with a beneficial role?

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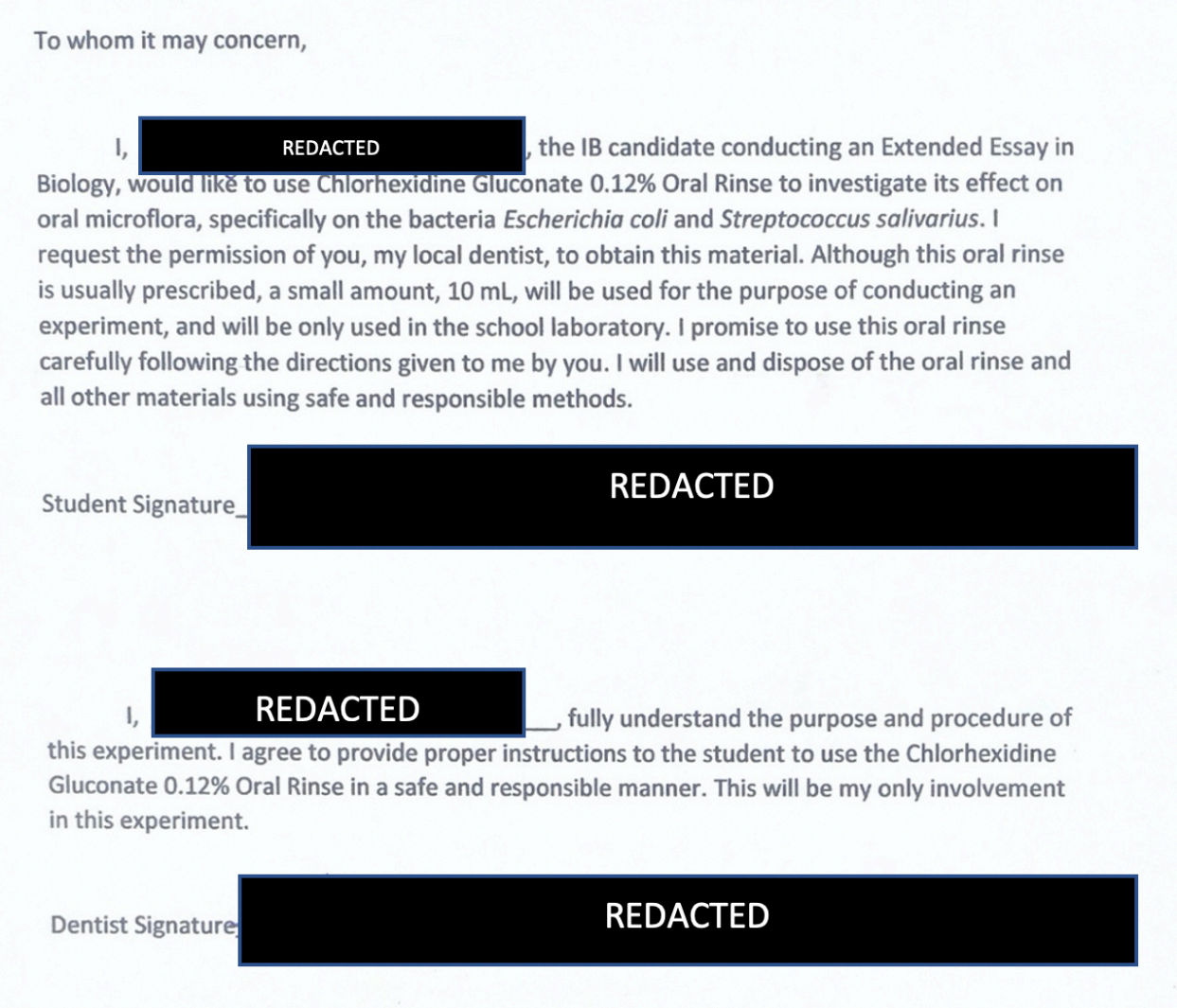
Appendices

Appendix A: permission slip signed by a dentist allowing the appropriate use of 0.12% CHX Oral Rinse as a part of this experiment



To whom it may concern,

I, REDACTED, the IB candidate conducting an Extended Essay in Biology, would like to use Chlorhexidine Gluconate 0.12% Oral Rinse to investigate its effect on oral microflora, specifically on the bacteria *Escherichia coli* and *Streptococcus salivarius*. I request the permission of you, my local dentist, to obtain this material. Although this oral rinse is usually prescribed, a small amount, 10 mL, will be used for the purpose of conducting an experiment, and will be only used in the school laboratory. I promise to use this oral rinse carefully following the directions given to me by you. I will use and dispose of the oral rinse and all other materials using safe and responsible methods.

Student Signature_ REDACTED

I, REDACTED, fully understand the purpose and procedure of this experiment. I agree to provide proper instructions to the student to use the Chlorhexidine Gluconate 0.12% Oral Rinse in a safe and responsible manner. This will be my only involvement in this experiment.

Dentist Signature REDACTED

Appendix B: email from my IB Coordinator to the Biology Subject Manager ensuring it is allowed to use lab safe *Escherichia coli* and *Streptococcus salivarius* as a part of this experiment

Hello REDACTED

Yes, students should use sterile technique and should only work with cultures of bacteria which are safe strains for use by students (attenuated strains such as *Escherichia coli* K12 for instance) and are obtained from a reliable source (for example, from a commercial microbiology company or a university microbiology laboratory). As you know, plates and broths must be incubated at a temperature well below human body temperature (at or around 25 degrees Celsius) to avoid the inadvertent culturing of human pathogenic contaminants. K12 will grow successfully at this temperature, just more slowly than at a higher temperature.

Best wishes
Alison Davies
Biology subject manager

---------- Original Message ----------
From: support@ibo.org [support@ibo.org]
Sent: 1/23/2020 6:46 AM
To: REDACTED
Subject: Biology [ ]

Dear REDACTED
Thank you for your email regarding Biology EE.

I have forwarded your enquiry to the Assessment team, who will gladly assist you further and you should receive a response within 3 business days. If you do not hear back from them after this time please contact us quoting your case number REDACTED and we will happily follow it up for you.

Thank you for contacting IB Answers and I hope you have a lovely day!

Kind Regards,
Emily Oaten
Associate - IB Answers

Appendix C: procedure detailing Nutrient and Tryptic Soy Agar preparations

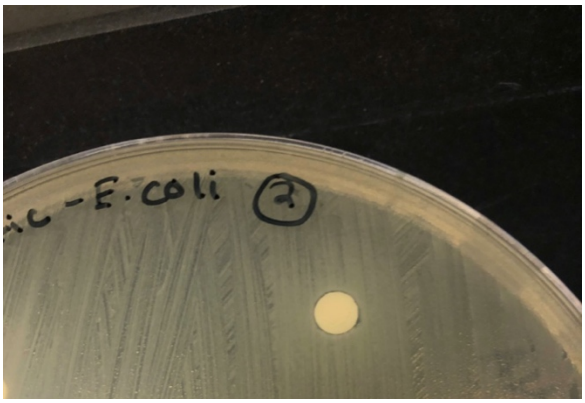
Agar Preparation: Nutrient Agar

1. Mix 2.00 g beef extract, 17.50 g acid hydrolysate of casein, 1.50 g of starch, 17.00 g of agar into approximately 300mL of warm, distilled water until dissolved, then bring the solution to 1000 mL with water in a 1 L flask.
2. Obtain another 1 L flask and divide the solution from step one evenly between the two flasks.
3. Cover both flasks with foil and place into the hydroclave to sterilize.
4. Allow the mixture to cool to 65°C and then pour agar into agar plates.
5. Once the agar has solidified, turn the plates upside down onto the benchtop and allow them to cure overnight.

Agar Preparation: Tryptic Soy Agar

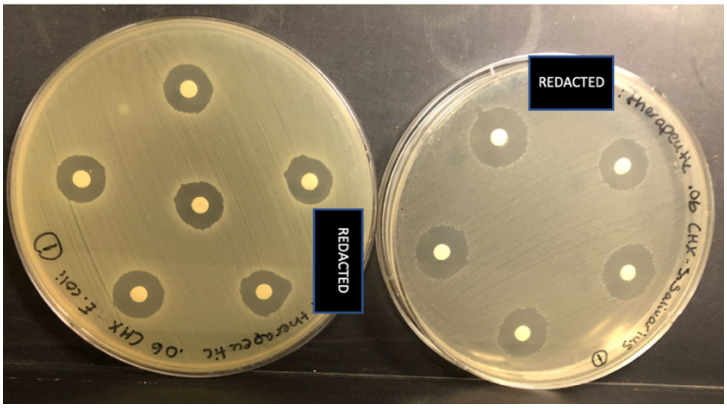
1. Mix 15.00 g of casein, 5.00 g soybean meal, 5.00 g of sodium chloride, and 15.00 g of agar into approximately 300L of warm, distilled water until dissolved, then bring the solution to 1000 mL with water in a 1 L flask.
2. Obtain another 1 L beaker and divide the solution from step one evenly between the two flasks.
3. Cover both beakers with foil and place into the hydroclave to sterilize.
4. Allow the mixture to cool to 65°C and then pour agar into agar plates.
5. Once the agar has solidified, turn the plates upside down onto the benchtop and allow them to cure overnight.

Appendix D: image displaying the zone of inhibition of *E. coli* ring around the disk

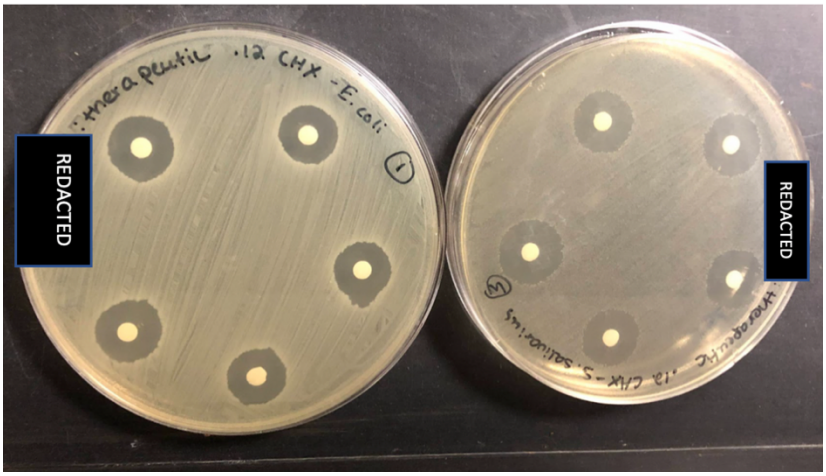


Appendix E: image displaying the zone of inhibition of *S. salivarius* ring around the disk

Appendix F: image displaying the zones of inhibition of *E. coli* and *S. salivarius* in 0.06% CHX (Trials 3 and 4)



Appendix G: image displaying the zones of inhibition of *E. coli* and *S. salivarius* in 0.12% CHX (Trials 5 and 6)



Appendix H: raw data of the measurement of the zones of inhibition in millimeters (± 0.5 mm)

Zone of Inhibition (± 1/2 mm)						
data no.	cosmetic - *E. coli*	cosmetic - *S. salivarius*	therapeutic 0.06% CHX - *E. coli*	therapeutic 0.06% CHX- *S. salivarius*	therapeutic 0.12% CHX- *E. coli*	therapeutic 0.12% CHX - *S. salivarius*
1	6.5	6.5	21	21	22	21.5
2	7	6	20	19.5	22.5	24
3	7.25	6.25	21	22	23.25	22.25
4	7	6	21.75	22.5	23	24
5	6.75	6.5	21.5	21.5	25	22
6	6.5	6	21	21	20	21.25
7	6.25	6.25	20.75	20.5	20	21.5
8	8	6.5	20.5	21	21.75	22
9	6.5	6.25	19.75	21	23	22
10	6.25	6.5	21.5	21.25	21	23
11	6.25	6.25	22.5	21.5	24.25	23.5
12	6.5	6.25	20	21	23.5	24
13	6.25	7	19.75	22	25	
14	6.25	7.25	20.25	21.5	21	
15	6.25	6.5	21.75	20.25	22	
16		8	20.5			
17		6.5	21.5			
Average	6.633333	6.5	20.88235	21.16667	22.48333	22.58333

Appendix I: chart displaying options for detecting live versus dead bacteria cells, taken from Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems (*Microbiome*, *5*(1), 86) by Emerson, J. B. et al., 2017