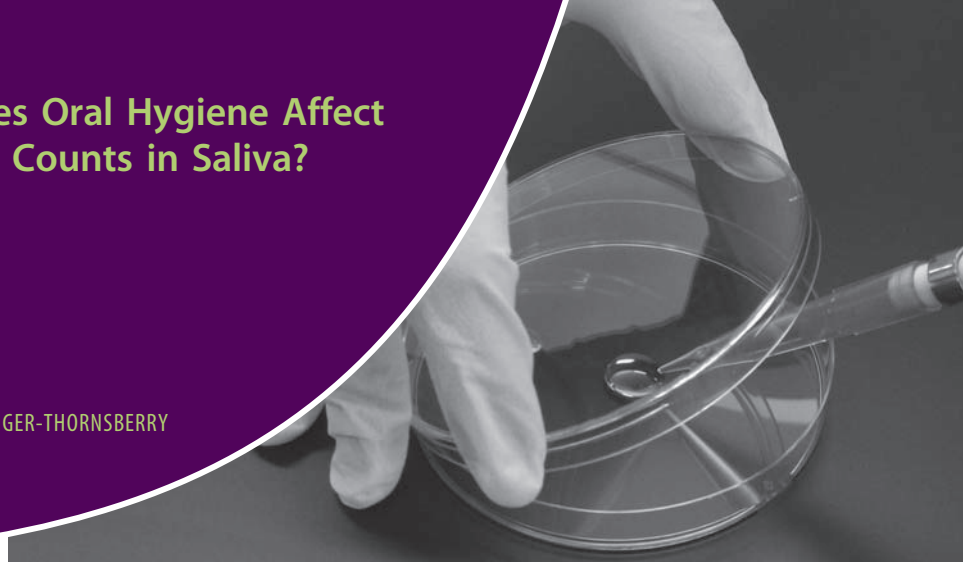
TIPS, TRICKS & TECHNIQUES

How Does Oral Hygiene Affect Bacterial Counts in Saliva?



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ABSTRACT

Oral health is often in the news lately as researchers explore the link of oral health with general health – and, particularly, with cardiac health (Shetty et al., 2012; Parkar et al., 2013). In this inquiry-based experiment, students explore microbes present in saliva and how the numbers are affected by using oral hygiene products such as toothpaste and mouthwash.

Key Words: *Oral health; microbiology; oral hygiene.*

Introduction

This activity introduces oral microbiology in a lower-division (100-level) college microbiology course but is suitable for general biology or high school microbiology. My students' interest is sparked when culturing microorganisms from themselves. To use their interest in their own microbiota to explore oral microbiology, students dilute and plate their own saliva, complete an oral hygiene treatment of their choosing (rinsing with water, rinsing with mouthwash with or without alcohol, brushing teeth with either water or toothpaste, or a combination of two treatments), and then repeat the dilution and plating procedure to look at the effect of the treatment on the number of bacteria present in their saliva. I don't use handouts for this experiment, preferring that students draw out their own dilution scheme, but I've included my introductory comments in the procedure section.

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Materials

Per student:

- 200 mL melted trypticase soy agar or nutrient agar
- 2 sterile test tubes
- 10 sterile (1.5 mL) microtubes
- 10 Petri dishes
- 15 mL of sterile deionized water

Per class:

- Adjustable micropipetters and tips to measure 100 and 900 µL
- Small plastic cups
- Toothpaste
- Toothbrushes
- Mouthwashes

Procedure

Determining the Number of Microbes in Saliva = Control Samples

Research has shown a direct association between periodontitis caused by oral bacteria and an increased risk of heart attack (Parker et al., 2003). Having good oral hygiene may reduce the number of oral microbes, resulting in not just better oral health, but perhaps better cardiovascular health. To determine whether oral hygiene reduces the number of microbes in saliva, it is necessary to first determine how many bacteria are in saliva before brushing teeth, rinsing with water, or rinsing with mouthwash. Can you predict what will happen when mouthwash is used? What about rinsing with water? Brushing?

1. Collect ~0.5 mL of saliva in a sterile test tube.
2. Pipet 900 µL of sterile water into 5 microtubes, labeled 1–5.
3. Slowly remove 100 µL of saliva from the test tube and transfer to microtube 1. Mix vigorously. This dilution is 1:10; there is one part saliva of 10 parts total. Each part is 100 µL.

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4. Using another pipette tip, transfer 100 µL from tube 1 into tube 2. Mix vigorously. This dilution is 1:100. One part of 100 parts is saliva; each part is now 10 µL.
5. Repeat this process with the remaining tubes. Tube 3 is 1:1000; tube 4 is 1:10,000; and tube 5 is 1:100,000.
6. Label five Petri dishes with your name and the date, and number them 1–5.
7. Pipette 100 µL from tube 1 into plate 1. Repeat with the remaining tubes and plates, using a new pipette tip for each transfer. Because only 1/10th of a milliliter is plated, the dilution factor changes: plate 1 = 1:100, plate 2 = 1:1000, and so on.
8. Add ~20 mL of melted and cooled (45°C) agar into each dish. Swirl gently but thoroughly to mix the sample into the agar. Let the plates solidify.

Oral Hygiene Treatment & Preparing the Experimental Samples

You may choose which oral hygiene treatment to investigate. Toothbrushes, toothpaste, mouthwashes, and bathroom cups are available. Complete your treatment and repeat the saliva dilution process with your remaining supplies. If you use mouthwash, wait 10 minutes to collect the saliva sample to reduce residual mouthwash, which could alter your results. Label the experimental sample supplies 6–10 to differentiate them from the control samples. When all 10 plates have solidified, invert them and incubate at 37°C for 24–48 hours.

Counting Plates

Today you will determine the number of colonies on the plates. Wear gloves and a lab coat, and be careful when handling your plates. We started with just saliva, and now the bacteria are growing under optimal conditions and are much more numerous. While it is unlikely, these organisms may cause infection if not handled correctly. If you contaminate yourself or your work space, notify the teacher immediately so that decontamination procedures can be started as quickly as possible.

Everyone has a different starting number of oral microbes. To eliminate this difference, compare the samples by looking at the reduction from the control to the experimental sample. First determine the number of colony-forming units (CFU) per milliliter of saliva by finding one plate from each set that has 25–250 colonies. Plates with <25 may have sampling error that negatively affects the colony number; these are referred to as Too Few to Count (TFTC). Plates with >250 may cause underestimation because growth from one colony can inhibit another; these plates are called Too Numerous to Count (TNTC). Start with the most diluted control plate, which is 1:1,000,000. Are there fewer than 25 colonies (TFTC) or can you use this plate? Repeat with the next plate, which is 10× more concentrated (1:100,000), and move through the plates until you find a plate that is TNTC. Repeat this procedure with the experimental plates.

When performing a standard plate count, the data are reported in CFU/mL ("colony-forming units" are used because a single colony may be founded by more than one cell, and there is no way to determine how many cells started the colony). To put the plate count data into a usable form, multiply the CFU by the dilution factor of the plate, convert to scientific notation, and use a single significant figure. For example, if there are 43 colonies on plate 4 (1:100,000), then CFU/mL = 43 × 100,000 = 4,300,000 = 4.3×10^6, which is reported as 4×10^6 CFU/mL. Only one significant figure is reported because of the amount of error in the dilution, plating, and counting procedure. Determine the CFU/mL for your control sample set and experimental sample set. Only one number is needed per set of plates.

To compare data from experiment to experiment, the numbers must be converted into the percent reduction of bacteria due to the oral hygiene treatment. Use the following equation: [(control count – experimental count)/control count] × 100. If the control sample was 4×10^6 CFU/mL and the experimental sample was 5×10^5 CFU/mL, then $[(4 \times 10^6 - 5 \times 10^5)/ 4 \times 10^6] \times 100 = 87.5\%$.

Safety

Oral microbes have the potential to cause disease, especially when in high numbers. The majority of what students will recover in this experiment are *Streptococcus* species, which may be biosafety level 1 or 2. These are facultative anaerobes, and growing them in the agar rather than on top slightly reduces the chances of contamination. You could also seal the plates with tape or Parafilm before the class performs the plate counts to reduce the chances of contamination. The American Society for Microbiology biosafety guidelines are a great resource for keeping our students safe.

Guiding Students through the Post-lab Discussion

Following the calculations, students list their experimental treatment and percent reduction on the board to help them make simple comparisons. Depending on the data generated, the following are questions that I may ask to guide discussion. Which technique was the most effective at reducing bacteria? Which was the least effective? Is there a difference in alcohol-based versus non-alcohol-based mouthwash? What does rinsing with water do? Is brushing followed by mouthwash more effective than brushing alone? Give them some time to come up with conclusions. Lastly, shift the emphasis back to the relationship between bacterial counts, oral hygiene, and overall health by introducing them to one of the first two websites listed in the Resources section below.

○ Conclusion

The experiment presented above is one way to introduce oral microbiology as well as reinforce fundamental laboratory techniques (pipetting, dilutions, cultivating bacteria, experimental design). Students are typically enthusiastic, if a little disgusted, by studying microbes collected from their own bodies. Furthermore, this experiment can be used to introduce the importance of oral microbiota to cardiac and general health.

○ Resources

- **Delta Dental**
  http://www.deltadentalins.com/oral_health/heart.html
  The heart and mouth connection: How heart disease and oral health link?
- **American Heart Association**
  http://www.heart.org/HEARTORG/GettingHealthy/Dental-Health-and-Heart-Health_UCM_459358_Article.jsp
  Dental health and heart health

- **Micro eGuide**
  http://www.microeguide.com/lab_skills/plate_count_examples.asp
  Methods for determining CFU/mL
- **American Society for Microbiology**
  http://www.asm.org/images/asm_biosafety_guidelines-FINAL.pdf
  Biosafety guidelines for teaching laboratories

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