Bacteria in the Oral Flora: A Study of Antimicrobial Agents in Toothpastes on Toothbrushes

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Abstract

This study was undertaken to determine which toothpaste (Aquafresh, Colgate, or Crest) represses bacterial growth most effectively. Toothpastes and toothbrushes were given to participants and were used for one week before being returned for analyses. Samples from toothbrushes were incubated in nutrient agar for 48 hours at 37°C. Three tests were performed in this study: gram staining, catalase testing, and optical density. The bacteria present on the toothbrushes were Gram-positive and catalase-negative. Before measuring optical density, toothbrushes were incubated in nutrient broth for 19 hours and 45 hours at 37°C. Optical density was measured at 686nm in a spectrophotometer. After performing a one-way ANOVA test, there was no statistically significant difference among the three brands of toothpaste. Thus, no toothpaste was more effective in repressing bacterial growth than the others.

Introduction

Bacteria form an important group of micro-organisms found in both healthy and diseased mouths (Roberts, 2005). There have been more than 300 types of bacteria found in the mouth (Roberts, 2005). Commensal bacteria are regarded as beneficial by defending against the colonization of invading pathogens (Kononen, 2000). One might think this suggests that the oral cavity is a relatively easy environment for bacteria to colonize. However, relatively few of these oral bacteria are found further along the gastrointestinal tract. This illustrates the unique ecology of the oral cavity and the specialized nature of the bacteria that reside in it (Roberts, 2005). Moreover, bacterial accumulation on oral surfaces is a major factor in the development of most of the common dental diseases such as dental caries and periodontal disease (Williams and Cummins, 2003). Streptococcus mutans, a bacterium in the mouth, is the chief bacterium that causes plaque and may also cause dental caries. Plaque is a complex biofilm found on the tooth surface that is a major cause of the development of dental caries (Benson et al., 2004). The accumulation and development of plaque depends upon the outcome of the interactions between the adhesiveness of plaque to the tooth surface and the physical shear forces which serve to dislodge and remove the plaque (Roberts, 2005). Toothpastes and toothbrushes are among the physical forces that remove plaque.

Fluoride-containing toothpastes have a significant effect on the initiation and progression of caries (Brailsford et al., 2005). Within these fluoride-containing toothpastes is a cationic agent called chlorhexidine (Williams and Cummins, 2003). Chlorhexidine has been documented for its ability to inhibit the formation of dental plaque (Williams and Cummins, 2003). However, the use of chlorhexidine has a few drawbacks. The first drawback is the staining of teeth and tongue (Williams and Cummins, 2003). These stains can be removed by having teeth professionally
cleaned. Another drawback is their unpleasant taste (Williams and Cummins, 2003). When eating or drinking something after the use of chlorhexidine, one can experience a change in taste perception. An example of this is brushing one’s teeth and then drinking orange juice. Many have done this and have experienced an aftertaste.

Triclosan, a compound commonly used for disinfection, is another broad-spectrum antibacterial agent manufactured specifically for use in oral care (Williams and Cummins, 2003). It has been shown in vitro to be active against many of the organisms associated with plaque, gingivitis, and bad breath. Triclosan does not irritate the oral soft tissue or cause staining like chlorhexidine does (Williams and Cummins, 2003). Triclosan works by disrupting the bacterial cytoplasmic membrane, resulting in the leakage of cellular contents and the death of the bacteria (Williams and Cummins, 2003). It is used in most oral-care ingredients and has a long history of use in consumer products.

Fluoride in drinking water and toothpastes do not appear to have demonstrable effects on the composition on dental plaque, although it inhibits the growth of oral Streptococci (Brailsford et al., 2005). Fluoride inhibits plaque fluid pH change and reduces lactate production following consumption of sugars (Brailsford et al., 2005). In vitro, fluoride also inhibits bacterial growth at concentrations less than dental plaque (Brailsford et al., 2005). The exact mechanism underlying this inhibition is not known, but fluoride has been shown to inhibit a variety of bacterial processes that are mediated by enzyme binding (Brailsford et al., 2005).

On occasion, many people experience stale or unpleasant breath upon waking in the morning. However, some people, about twenty-five percent of the population, experience halitosis, or bad breath, on a regular basis (Williams and Cummins, 2003). These people can be sensitive about it and may avoid social situations. Halitosis tends to worsen and become more frequent with age and is evenly distributed between men and women (Williams and Cummins, 2003). The problem results from the anaerobic breakdown of proteins from food and salivary debris by gram-negative bacteria, which generate amino acids such as cysteine and methionine (Williams and Cummins, 2003).

Many different products are currently marketed that promise to provide consumers with fresh breath. It is estimated that more than one billion dollars are spent annually worldwide on lozenges, chewing gum, mouth rinse, and dentifrices in an effort to resolve this condition (Williams and Cummins, 2003). The active agents that are incorporated into treatment forms include surfactants, antibacterial agents, baking soda, peroxide, metal salts, herbal and natural extracts, and chlorine dioxide (Williams and Cummins, 2003).

Because oral bacteria are the primary source of bad breath, the most effective way to control this problem is by controlling bacteria. The key to a product’s effectiveness in controlling breath odor is an effective level of an ingredient with antibacterial activity in the mouth for an extended period of time following toothbrushing (Williams and Cummins, 2003).

Contaminated toothbrushes can also be a source for bacteria. Toothbrushes which are used regularly become contaminated with micro-organisms that colonize the teeth and the oral cavity. Under the usual conditions of storage, a toothbrush can therefore serve as a vector for the reintroduction of potential pathogens into the oral cavity, and also for the introduction of other microbial species originating from...
the bathroom environment (Verran and Leahy-Gilmartin, 1996). It has been reported that toothbrushes could be a source of repeated oral infections (Warren et al., 2001).

Significant bacteria on toothbrushes have been reported after toothbrushing, especially in patients with severe periodontitis (Quirynen et al., 2003). This can be caused by simply leaving one’s toothbrush in a place that is not clean. In recent studies, toothbrushes kept in a moist environment, like that of a bathroom, retained up to fifty percent of herpes simplex virus Type I after one week (Warren et al., 2001). An in vitro study involving fifty-nine patients who had oral inflammatory disease found that thirty-four percent required no additional therapy after they changed their toothbrushes biweekly (Warren et al., 2001). Different options have been explored to reduce toothbrush contamination. Some studies suggest that the general population replace toothbrushes every month or after any illness (Quirynen et al., 2003). The use of disposable toothbrushes is also a good option. Another idea to prevent the contamination of brushes is to coat the brush with chlorhexidine after each use (Quirynen et al., 2003). One study found that soaking toothbrushes for twenty minutes in a mouthrinse containing essential oils killed one hundred percent of the bacteria present (Warren et al., 2001).

Bacteria in the mouth are an issue everyone has to deal with. Some of the bacteria can be helpful. However, most bacteria are harmful and cause plaque and bad breath (Williams and Cummins, 2003). There are toothpastes and other remedies that help kill and prevent bacteria in people’s mouths.

In this research study, I examined the relationship between toothpastes and their abilities to repress bacterial growth on toothbrushes after use. I evaluated this by giving participants toothbrushes and specific toothpaste, Aquafresh, Colgate, and Crest. My hypothesis is that there will be a different amount of bacterial growth on the toothbrushes among the toothpastes. I believe there will be less bacterial growth on the toothbrushes that had Colgate used on them. This is because the ingredient triclosan used in Colgate toothpastes seems to inhibit bacterial growth most effectively.

Methods

Participants

The participants for this study were 13 students at Saint Martin’s University. They all were of senior status. I handed out toothbrushes and specific toothpaste for each of the participants to use. I told the participants to brush their teeth like they would their own. The toothbrushes were the same for all the participants: Crest®. After one week, I collected the toothbrushes from the participants in plastic bags and analyzed the toothbrushes. I had three different categories that the participants fell under: Crest®, Colgate®, and Aquafresh®. The three categories were based upon what type of toothpaste they used. There were no qualities that a participant might have had that would have excluded them from this experiment.

Collection of Samples

I collected the toothbrushes from the participants after one week of use. I took a sample with an inoculation loop from each toothbrush for bacterial testing. I then streaked these samples onto a Petri dish from side to side and from top to bottom. The inoculates were then incubated at 37°C for 48 hours and I observed the changes that occurred.
**Culture medium**

A culture medium contains essential nutrients for the growth of a microbial culture. It must provide suitable surroundings for growth. These surroundings include nutrients, the proper pH, osmotic pressure, and atmospheric oxygen (Williams and Cummins, 2003).

To begin my bacterial culture, the inoculates were transferred from the toothbrushes into sterilized media. The inoculation process was done with an inoculation loop that had been sterilized by flaming immediately before and after the transfer. The transfers were made into sterile Petri dishes containing deMan, Rogosa, and Sharpe (MRS) broth (10 gm/liter peptone, 8 gm/liter ‘Lab-Lemco’ powder, 4 gm/liter yeast extract, 20 gm/liter glucose, 1 ml/g sorbitan mono-oleate, 2 gm/liter dipotassium hydrogen phosphate, 5 gm/liter sodium acetate, 2 gm/liter triammonium citrate, 0.2 gm/liter magnesium sulfate, 0.05 gm/liter manganese sulfate, and 10 gm/liter agar) (Benson *et al.*, 2004).

The inoculates were incubated at 37°C for 48 hours. Incubation was at 37°C, because this is the optimal temperature for the bacteria to grow (Williams and Cummins, 2003). Incubation was for 48 hours because this showed the minimum amount of mesophilic bacterial growth. Each dish contained roughly 5 ml of MRS agar. Once the colonies of bacteria appeared, the dishes were compared to see if there were different amounts of growth among the three groups.

**Catalase Testing**

This was a simple process that enabled me to identify if the catalase enzyme was present in the bacteria. To see if the bacteria were catalase positive or negative, I added a couple drops of 3% hydrogen peroxide to the culture. If the catalase enzyme was present (catalase-positive), bubbles appeared. If the catalase enzyme was not present (catalase-negative), the culture did not bubble. I did this test to learn more physiological characteristics of the bacteria.

**Gram Staining**

From the Petri dishes, I collected samples of bacteria using an inoculation loop. I placed the organisms onto slides. After air drying, I heat fixed each slide with a Bunsen burner. I then flooded the slide with Hucker’s Crystal Violet Stain (2g crystal violet (90% dye content), 20ml 95% ethyl alcohol, 0.8g (NH₄)₂C₂O₄·H₂O). I let it sit for 1 minute and then rinsed it with water for 5 seconds. I then flooded the slide with iodine. After 1 minute, I immersed the slide into 3 different Coplin jars containing ethyl alcohol. I left the slide in each jar for 20 seconds. Once this was completed, I rinsed the slide with water for 5 seconds and then added a counter stain of Safranin. I let the Safranin sit on the slide for 1 minute. I then rinsed the slide with water for 2 seconds. I blotted the slide with bibulous paper to dry. Once this process had been completed, I placed the slide under a microscope for observation at 100 times magnification.

**Streak Plate**

This test produced well-separated colonies of *S. mutans* from the toothbrushes in Petri dishes. The cells were closely packed together at the start of a streak and formed colonies that ran together. But, as streaking continued, fewer and fewer cells remained on the needle, making the colonies separated (Quirynen *et al.*, 2003). I first flamed the loop to sterilize it. Then, I took a sample from each toothbrush by rubbing it along and inside the bristles. Starting at the edge of the dish, I streaked the inoculation loop across the agar, while also holding the
dish cover over the dish to prevent contamination. I streaked the culture back and forth in the dish, making parallel lines. After each streak, I flamed the needle and repeated. This continued until the dish had been completely streaked. With this test, I was able to calculate the colony forming units. Petri dishes were observed every 24 hours for 5 days.

**Optical Density**

To record the optical density, a spectrophotometer was used. Test tubes were filled with 15 ml of broth. The heads of the toothbrushes were dropped into the tubes and were incubated for 48 hours.

Optical density was recorded after 19 hours and after 45 hours of incubation. MINITAB was used to statistical analyses. With these methods, I hoped to discover more about the morphology and the amount of the bacteria present on the toothbrushes. I hoped to identify which toothpaste is able to repress bacterial growth most effectively.

**Results**

**Catalase Testing**

After adding a couple drops of 3% hydrogen peroxide to the culture, I found that there was no bubbling that occurred. This indicates that the bacteria present on the Petri dishes did not contain the catalase enzyme; making them catalase-negative.

**Gram Stain**

After performing the gram stain techniques, I found that the bacteria accumulated on my Petri dishes were gram-positive.

**Optical Density**

After using the spectrophotometer, I found the optical density (O.D.) of the samples of the three different groups. Optical density was checked after 19 hours of incubation and then again after 45 hours of incubation. A one-way analysis of variance (ANOVA) test was performed via MINITAB. The test was done to evaluate the differences in bacterial growth after 19 hours of incubation and after 45 hours of incubation. After 19 hours of incubation, there was no statistically significant difference among the toothpastes (F = 0.64; D.F. = 2; P = 0.549). After 45 hours of incubation, there was also no statistically significant difference among the toothpastes (F = 1.44; D.F. = 2; P = 0.282).

<table>
<thead>
<tr>
<th>Toothpaste</th>
<th>No. Samples</th>
<th>Mean O.D.</th>
<th>St Error Mean</th>
<th>Standard Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquafresh</td>
<td>4</td>
<td>0.1775</td>
<td>0.0682</td>
<td>0.1365</td>
</tr>
<tr>
<td>Colgate</td>
<td>5</td>
<td>0.2848</td>
<td>0.0774</td>
<td>0.1731</td>
</tr>
<tr>
<td>Crest</td>
<td>4</td>
<td>0.2120</td>
<td>0.0571</td>
<td>0.1142</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Table 1. Shows the number of samples, mean optical density, standard error mean, and standard deviation of the three toothpastes after 19 hours of incubation.

<table>
<thead>
<tr>
<th>Toothpaste</th>
<th>No. Samples</th>
<th>Mean O.D.</th>
<th>St Error Mean</th>
<th>Standard Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquafresh</td>
<td>4</td>
<td>0.5013</td>
<td>0.0363</td>
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</tr>
<tr>
<td>Colgate</td>
<td>5</td>
<td>0.6570</td>
<td>0.0433</td>
<td>0.0969</td>
</tr>
<tr>
<td>Crest</td>
<td>4</td>
<td>0.5520</td>
<td>0.1100</td>
<td>0.2210</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Table 2. Shows the number of samples, mean optical density, standard error, and standard deviation of the three toothpastes after 45 hours of incubation.
Figure 1. Shows the three types of toothpastes and their mean optical density after 19 and 45 hours of incubation. Error bars show standard error. Aquafresh appears to have the most effective antimicrobial agents.

Discussion

After completing statistical analyses, I must accept my alternative hypothesis that no toothpaste represses bacterial growth better than the others. There is insufficient evidence to accurately conclude that one toothpaste works better than another. I believe that there are many factors contributing to why I was unable to determine which toothpaste has the best bacterial inhibiting power.

The major reason is that I did not have enough samples. I had a total number of 13 participants for my study. This number would have to be much higher in order to find results that would tell me which toothpaste performs best. I believe that in order to find the most effective toothpaste, I would need many more samples for each of the toothpastes.

Another factor that may have contributed to varied results is the number of times people brushed their teeth per day. I gave the participants the toothpastes and toothbrushes and asked them to use them for a week and return them to me. I did not assign a certain number of times per day that the participants were supposed to use the toothpastes and toothbrushes. I did not think I would be able to assign a number of times to brush per day, because some people brush their teeth many times per day and some people might only do it one time per day, depending upon their oral hygiene. If I were to do this study again, I would need a large enough sample size that I could include the number of times the participants brush their teeth per day in the analyses of data.

Composition of the participants’ diet could also be a determining factor on bacterial growth. Some people eat foods with more sugar than others. This would cause more bacterial growth and plaque in the oral flora than someone who eats healthier foods (Williams and Cummins, 2003). Also, the participants might have been using other mouth cleansing products
during the week of use of the toothpaste, like mouthwash and dental floss. These products could eliminate some bacteria that would have accumulated on the toothbrushes that they were giving back to me. In order to remove this variable I would have to set a specific diet for all of the participants.

Another factor that may have complicated my results would be where the participants put their toothbrushes after using them. Some participants might have put them back into the bag which I gave to them. Others may have put them in a toothbrush holder where they store their own toothbrush. Some might have simply put the toothbrush on a dirty bathroom counter. The differences in storage places could result in the accumulation of different amounts of bacteria. When I gave the participants the toothpastes and toothbrushes, I did not specify where to put the toothbrushes when they were not being used. In order to remove this variable, I could have told the participants to put the toothbrush back into the zip-lock bag when they were not using it.

These factors, along with others, may have contributed to why I am unable to conclude which toothpaste represses bacterial growth most effectively in this study. If I were to go about doing a similar study, I would have to have more control over how often the participants used the toothbrushes, what the participants were eating, and where the toothbrushes were stored when they were not being used.

It is important for people to have good oral hygiene because bacteria can accumulate in the oral flora. Toothbrushing is the most effective way to repress bacterial growth (Roberts, 2005). The toothpaste that is being used during brushing will also help determine the accumulation of bacteria in the oral flora. The key is to find the toothpaste that represses the growth of bacteria most effectively on toothbrushes.

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Literature Cited


