ANTIMICROBIAL PROPERTIES OF VARIOUS GREEN TEA PRODUCTS WITH AND WITHOUT THE ADDITION OF SUGAR

Samantha Acevedo, Conan Chen, Ethan France, Brian Goldspiel, Anna Kim, Amanda Li, Florence Ma, Sana Siddiqui, Stephanie Tarlowe, Anthony Yakely, Leanna Zhan

Advisor: Mrs. Rachel Sandler
Assistant: Gillian Bradley

ABSTRACT

Recent research indicates that green tea has numerous health benefits, including the ability to kill oral bacteria and prevent cavities. This lab tests the antimicrobial properties of green tea (with and without sugar) both in vitro simulation and in paper disk diffusion. It was hypothesized that mouthwash would be the most effective bacteria inhibitor, followed by the least processed loose sencha tea, and that the addition of sugar would impair tea’s germ-fighting abilities. The experimental results indicate that mouthwash and sencha tea were indeed the most effective growth inhibitors, but there was little evidence that sugar either helps or hinders bacterial colony formation.

INTRODUCTION

Green tea, a beverage consumed around the world, has progressed from casual beverage to medicinal powerhouse through the centuries. Green tea appears in Chinese literature and legend as early as 3000 BCE. According to legend, tea was discovered accidentally by either a man named Shien Non Shei or the Emperor Shen Nung. Either way, green tea soon became popular among wealthy Chinese nobles. After thousands of years, the many preparations of this beverage have become common worldwide. Each day, approximately 165 million cups of tea are consumed in Great Britain alone. Tea is popular not only for its good taste but for its high levels of antioxidants, resulting in its being tested for a variety of health benefits.

There are several different types of tea available on the market, including green, black, white, herbal, and oolong. Green tea, made from the leaves of the Camellia sinensis plant, is unfermented; the freshly plucked tea-leaf is steam blasted in perforated drums or cooked in iron pans, denaturing its oxidizing enzymes. On the other hand, black tea is completely fermented. Other types of tea, such as oolong and longjing, are partially fermented teas which vary in their degree of processing. White tea differs from green tea in its stage of harvesting, as white tea is picked before its leaves are fully opened. Herbal tea is not actually tea at all; rather, it consists of an herbal infusion of fresh or dried flowers, leaves, seeds, or roots made by pouring hot water over the plant components and letting them steep.

The leaves of the Camellia sinensis plant may be processed in several ways, depending on the type of tea desired. Tea can be found in bags, powders or pre-prepared bottles in addition to its natural leaf form. Whatever the case, the leaves and buds are usually harvested by hand to prevent damage caused by machine collection. In natural or bagged green tea, the leaves are then laid out to dry before being steamed to neutralize enzymes found in the leaves. These enzymes, if left in place, can result in the oxidation of the tea leaves and reduce its worth as a...
beverage. After steaming, the leaves are rolled, dried again, and chopped up for later use. They may then be placed immediately in hot water for consumption or packaged, bagged, and stored if they are to be used in tea bags\(^5\). Commercial tea brands typically mix leaves from a wide variety of tea plants. Lipton®, for example, grows its own leaves, which ship from over 35 different countries\(^6\).

Some higher-quality variants of traditional green tea include matcha and sencha. Matcha is produced primarily the Uji plantations of Japan, the country’s most famous tea-growing area. The leaves used in its preparation are grown in darkened conditions that enrich them with amino acids, and are dried flat rather than rolled after picking. They are then ground into an extremely fine powder; a process which takes a substantial amount of time. Matcha is highly valued for its health benefits, which include antioxidant properties. Like matcha, sencha is primarily harvested in Japan, usually during the early summer months. sencha is produced from unground tea leaves, and is valued for its texture\(^7\).

Tea can also be found in an instant powdered form or in bottles. Bottled tea is typically prepared from concentrate and does not include actual tea leaves; instead, a variety of sweeteners and artificial ingredients are often added. Snapple® green tea includes sugar, while other brands use corn syrup or honey as a sweetener. Other common additives include citric acid for flavoring, vitamins and minerals, and unspecified “natural flavors,” which can include a wide variety of chemicals extracted from a biological source\(^8\). Powdered tea mixes include similar artificial ingredients, especially citric acid and other flavorings or chemical preservatives such as silicon dioxide\(^6,8\). The preparation of instant tea mixes is similar to that of instant coffee, as the tea is first made and then dehydrated for later use. In this experiment, we will be using several types of tea, including Lipton® and Yamamotoyama tea bags, loose tea, matcha, sencha, bottled Snapple® (both diet and non-diet) and Snapple® instant powder.

**Chemical Composition**

Tea contains many different compounds that grant it health-promoting properties, including a group of polyphenolic compounds called flavonoids. The most abundant class of these flavonoids is the flavonols. Flavonols are made up of monomers called catechins, natural plant antioxidants commonly found in tea (Fig. 1; note that Theaflavins are the result of fermentation and appear only in black tea)\(^9\). Catechins are minimally affected by processing and are present in comparable quantities in all teas\(^10\). They constitute about 30% of tea’s dry leaf weight\(^11\). Green tea extract is comprised of mainly epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin galate (EGCG)\(^12\). EGCG is the most common polyphenol found in green tea, making up over 10% of its dry weight\(^9\) and comprising 60-70% of its total catechins\(^11\). Many of green tea’s health-promoting abilities are attributed to EGCG\(^13\).

EGCG and other catechins exhibit strong antioxidant activities due to their single electron reduction potential. Free radicals are harmful, and reactive molecules are made unstable by their unpaired electrons. They are involved in diseases from blood clots to cancer. Catechins search out free radicals and bind them to produce stable, nonreactive products. They can scavenge reactive oxygen species such as superoxide anions (\(O_2^-\)) and singlet oxygen (\(\text{^1}O_2\))\(^14\), bind metal ions by chelation or scavenge lipid peroxyl radicals\(^15\). It has been suggested that EGCG
and GCG have greater scavenging abilities than other tea catechins due to the presence of their gallate group on the third carbon of the C ring. Among the latter four catechins, the hydroxyl group at the 5’ B ring position gives higher scavenging abilities to EGC and GC than EC and C\textsuperscript{14}.

Other compounds found in tea include caffeic, quinic, and gallic acids. Tea also contains caffeine (although only a third the amount in coffee), the amino acid theanine, and fluoride\textsuperscript{9}. According to dentist recommendations, the amount of fluoride found in tea is adequate for cavity prevention but not high enough to cause a health risk\textsuperscript{10}.

Addition of Sugar

Due to the bitterness of green tea, many of its drinkers choose to add sugar. Sugar contributes calories to a drink that otherwise has none, and is blamed for a variety of health problems from obesity to tooth decay. Previous studies have found that one teaspoon of sugar in a 200 mL cup of tea has no effect on the extraction of theanine, an amino acid found in tea\textsuperscript{16}.

However, the catechin EGCG is an effective antimicrobial in milligram amounts, whereas grams of sugar must be added in order to elicit the drinker’s desired sweetness. This fact accounts for bottled Snapple ® green tea’s listing of sugar before green tea extract in its ingredient list, as the tea is more potent than the added sugar\textsuperscript{17}.

A recent study suggested that the addition of sucrose along with ascorbic acid to tea may increase catechin availability to the body by enhancing intestinal uptake from tea\textsuperscript{18}. Researchers in that study found EGC, EGCG and ECG to be taken up by Caco-2 cells in significantly higher amounts than in other preparations. Within the mouth, however, the fermentation of sugar reduces pH and thus creates an environment more suited to caries-active plaque. An experiment conducted a test on the uptake of sugar by bacteria found that Gram-positive bacteria used 100% of added glucose within 4 hours, and that the Gram-positive bacterial cultures had a greater decrease of pH. Glucose is readily available in human diets and serves as an important food source for \textit{S. mutans} \textsuperscript{19}. The evidence seems to suggest that sugar both improves catechin update and increases plaque in the mouth. The planned experiment seeks to find out whether the drinking green tea has health advantages and if the addition of sugar will negate the oral health advantages of drinking green tea by promoting bacterial growth.
Health Benefits

The health benefits of green tea are purported to be vast. Tea is commonly believed to improve everything from cardiovascular to dental health, and research indicates that many of these claims may be grounded in fact. Limited studies in the Netherlands and Japan have shown that green tea lowers low-density lipoproteins (LDLs), also referred to as “bad” cholesterol. A study in Japan confirms that tea also reduces obesity. The 240 men and women of the study were given different amounts of green tea extract, and after three months, the scientists found a positive correlation between the amount of extract given and the amount of weight lost. Those who had higher amounts of green tea also had lower blood pressure and lower LDL cholesterol.

In a 2004 study at the Cedars-Sinai Medical Center, researchers explored the effects of green tea on the cardiovascular disease atherosclerosis. In this condition, fatty deposits form along the sides of arteries, leading to clotting. Studies have shown that green tea hampers atherosclerosis in experimental animals and humans; however, clinical studies were less promising. Unfortunately, treatment for human subjects does not start until well after atherosclerosis has developed, whereas with animals, treatment can begin in the disease’s early stages.
The flavonoids in green tea may also have benefits for diabetes patients, as they have been shown to enhance insulin activity. Though it differs from insulin in its methods, the EGCG in green tea can serve as an anti-diabetic agent due to its slower kinetics and its regulatory effects on certain genes. Japanese and Taiwanese investigators have found that green tea has an ameliorating effect on insulin resistance, but more research is still needed.

A number of studies have been done on the relationship between green tea and cancer prevention. The chief polyphenols in green tea, including EGCG and EGC, target free radicals (which are believed to contribute to cancer) and can help prevent DNA damage. Some laboratory studies have shown that the polyphenols in green tea may even hinder the cell proliferation of tumors, promote apoptosis, protect against UVB radiation damage, and inhibit angiogenesis. Overall, the evidence supporting the link between green tea and cancer prevention is inconclusive. The results of clinical trials and epidemiologic studies vary, perhaps due to the differences in preparation and consumption of tea. Some epidemiologic studies have linked drinking tea to reducing the risk of breast, colon, ovary, prostate, and lung cancer.

Green tea has also been shown to improve oral health. Researchers published in the Journal of Periodontology have found that green tea may be beneficial for the teeth and gums. A study of 940 men found that green tea consumption often correlated to superior dental health. The examined factors were periodontal pocket depth, gum tissue detachment, and bleeding upon probing of the gum tissue; results indicated a decrease in all three factors per every one cup of green tea.

*Streptococcus mutans*

The human mouth plays home to hundreds of species of bacteria, most of which are harmless. These bacteria feed off of sugars and other chemical residues left on the teeth, breaking down these leftovers for use as food and fuel. The mouth maintains a system of defensive cells that prevent these bacterial colonies from causing problems, while the teeth themselves are layered with defensive enamel. This protection cannot prevent all damage to the teeth, however. Some species are able to destroy tooth enamel, dissolving their way into the core of the tooth and creating holes known as dental caries or cavities. These holes can cause structural damage to the tooth and serious pain.

*Streptococcus mutans* is a gram-positive bacteria species that comes in many different shapes. This species is known to inhabit the human oral cavity, making up 30 to 60% of the bacteria in human teeth, tongues, cheek, and saliva. It is an important cause of cavities and bad breath, producing lactic acid that breaks down the outer surfaces of the teeth. This lactic acid is produced by anaerobic respiration. Over the centuries, as human sugar consumption increased, *S. mutans* evolved to digest the sugar, using it to adhere to smooth surfaces like teeth and glass. The metabolism of this sugar leads to the production of the harmful lactic acid.

The teeth are largely made of enamel, which is composed of about 90% calcium salts. Enamel can effectively defend the teeth against most bacteria, but is easily broken down by the lactic acid secreted by *S. mutans*. Thus while more than 200 species of bacteria are associated with dental plaque, only *S. mutans* has been linked consistently to tooth decay. Previous...
studies show that the presence of \textit{S. mutans} has significant relation to both the prevalence and extent of caries. In sampled populations, the prevalence and proportion of \textit{S. mutans} in plaque seems to increase with the number of decayed teeth present, and the caries-active subjects proved more prone to new carious lesions than the caries-free subjects\textsuperscript{30}.

Recent studies even link oral-bacteria-caused disease to some serious cardiovascular diseases. After penetrating through teeth and gums, bacteria can find their way into the bloodstream, where they may migrate to coronary arteries and cause blood clots that restrict oxygen and blood flow. In response to the bacteria in the bloodstream, the liver releases C-reactive proteins that induce clot formation and thicken artery walls\textsuperscript{28}. The several hundred identified genes that appear unique to \textit{S. mutans} serve as potential drug targets, because disrupting them would help disable the pathogen without harming the other useful bacteria in the mouth\textsuperscript{29}.

Green Tea’s Antimicrobial Properties

Studies have been conducted testing the antimicrobial abilities of various teas, including green tea, on a variety of test organisms. Chou \textit{et. al.}, demonstrated that dry tea flush and green tea, in particular, are capable of killing bacteria. Dry tea proved most effective against \textit{Bacillus subtilis}, \textit{Proteus vulgaris} and \textit{Staphylococcus aureus}, but green tea demonstrated better performance against \textit{Escherichia coli} and \textit{Salmonella}.

Green tea’s effectiveness as an antimicrobial agent can in part be attributed to its low degree of fermentation. During the fermentation process, catechins such as EGCG are destroyed, reducing the tea’s antimicrobial properties. This can be clearly seen in the results of the study, as the highly fermented black tea killed the least bacteria in almost every case\textsuperscript{31}.

Hypothesis and Other Remarks

A variety of commercially available green teas will be tested in order to determine if they have antimicrobial properties against \textit{S. mutans}. In order to test such properties of green tea, both \textit{in vitro} simulation and paper disc diffusion assays will be performed. The \textit{in vitro} simulation will be done to replicate a possible method of drinking green tea, thereby testing how green tea would perform in human mouths. The paper disc diffusion test will be used in order to determine green tea’s antimicrobial properties without the other factors that might affect the \textit{in vitro} simulation. These tests will determine both if green tea does have antimicrobial properties in addition to its antimicrobial effect in human mouths during drinking.

Two Snapple® bottled teas will be tested along with bagged and loose tea in this experiment. These teas contain some chemicals not found in any other tea varieties. Diet Snapple® contains acesulfame potassium, an artificial sweetener. At the moment, no clinical study has tested acesulfame potassium as either an antibacterial agent or a compound that promotes bacterial growth. Diet Snapple® also contains honey flavor, though the company does not specify if this is an artificial flavoring or real honey\textsuperscript{17}. Recent tests seem to say that honey can be used as oral bactericide, specifically on \textit{S. mutans}\textsuperscript{32}. This could affect results of this experiment, as neither natural honey nor honey flavoring will be tested in the other sets of green
Both diet and original Snapple® contain citric acid, which has been known to kill bacteria during root canal irrigation\(^3\). Finally, ascorbic acid, an ingredient of both Snapple teas, has been known to kill *Staphylococcus aureus*, a bacterium found in the human mouth\(^34\).

The final pre-prepared Snapple® product is the Snapple® On-the-Go green tea powder mix. Each packet contains several ingredients not found in the other Snapple® varieties being tested. One such ingredient is potassium citrate; although no tests have been done on potassium citrate, citrate on its own is capable of acting as a bactericide\(^3\). Snapple® powder also contains the artificial sweetener aspartame. Aspartame actually enhances bacterial growth, even at concentrations ten times smaller than those used here\(^3\). The last unique ingredient of the powdered mix is calcium silicate, an anti-caking agent at quantities less than 2%, which will therefore not serve as either an inhibitor or a catalyst of bacterial growth.

The effects of green tea on *S. mutans* will be tested through the use of paper disk diffusion and an *in vitro* simulation of drinking cooled tea. Many different factors will play into the effectiveness of the tea. It is hypothesized that the loose leaf sencha tea will be most effective in inhibiting the growth of *S. mutans*. More natural and concentrated teas are speculated to be more efficient than more processed and diluted teas. In addition, it is conjectured that the addition of sugar will counteract the beneficial dental hygiene properties of green tea by promoting the growth of bacteria.

**MATERIALS AND METHODS**

**Tea Samples**

Green tea samples were tested for antimicrobial properties using the paper disk diffusion method and an *in vitro* method was developed to simulate the effect of drinking tea. Boiled and cooled deionized water served as a control. In addition to this control, Original Mint Scope® Mouthwash was utilized in order to observe how a commercially marketed antiseptic can effectively curb microbial growth. Beverages not necessitating lab preparation were bottled Snapple® Green Tea and Diet Snapple® Green Tea, which were kept at room temperature before and during the time of testing. One packet of Diet Snapple® On-the-Go Original Green Tea powdered drink mix was also tested with minimal lab preparation.

Samples of several leaf teas were brewed in the lab and prepared both with and without the addition of Domino® sugar for comparison. The most expensive of the bag teas tested was the Yamamotoyama brand of sencha green tea, made with 100% Japanese green tea and prepared in pyramid-shaped bags. The same brand was also used for standard bagged green tea, which is made with a blend of young leaves. The third tea bag tested was Lipton® 100% Natural Green Tea, which is made of leaves from around the world and touts the benefits of its 150 mg flavonoids per serving.

Loose sencha tea leaves and matcha tea were also tested. Organic sencha Uji Cha leaves from the Eden® Organic food company were prepared. The tea powder used in this experiment was Maeda-en’s “Culinary Quality” matcha Green Tea Powder.
Preparation of the *S. mutans* Culture

A *Streptococcus mutans* culture was obtained from WARD’S Natural Science and prepared for use in tryptic soy broth. The culture was incubated for 48 hours at 37º C. It was estimated that the culture contained $3.7 \times 10^8$ CFU/mL through dilution.

Preparation of Teas

Tea was prepared by boiling 240 mL (one cup) of deionized water in a covered flask and then steeping the tea in tea bags or loosely, as per package directions, for 15 minutes once the water cooled to 90º C. In addition, samples were prepared with sugar by adding 15 grams of Domino® brand sugar following completion of the tea’s diffusion. Water was boiled as a control sample with and without sugar. The instant powdered Snapple® was prepared by allowing 507 mL (the recommended 16.9 fl. oz. bottle) of boiled water to cool before adding the powder and mixing well. Ten milliliter samples of the prepared tea, bottled drinks, mouthwash, and water were pipeted into sterilized test tubes, and additional tea was poured into a small sterilized beaker for use in the paper disk diffusion.

Paper Disk Diffusion

Agar plates were prepared with a bacterial lawn of *S. mutans*. Two plates were not prepared with a lawn to serve as a control, with half of the samples on each disk. The other plates were prepared so that tests were done in triplicate with eight disks per plate. Filter-paper disks were soaked in each sample for approximately five minutes and then removed from the liquid and placed onto the prepared lawns and the control plate using forceps sterilized in Bunsen burners. The zones of inhibition were measured after approximately 48 hours of incubation at 37ºC. A gram stain was performed on larger bacterial colonies.

*In vitro* Simulation

Sterilized loops were prepared for the *in vitro* simulation by inoculating the end portion of the loop with the *S. mutans* culture. The loop was then swabbed and inoculated onto a quarter of a tryptic soy agar plate in a straight line. The inoculated loop was dipped into a test tube containing the tea samples for a total of two minutes. During this time, the loop was dipped in, stirred slightly for 5 seconds and then removed for 5 seconds to imitate the movement of liquid upon swallowing. This process was repeated for the allotted time, to represent “sipping” from a cup of tea. After two minutes, the loop was removed from the liquid, and was swabbed by a sterilized swab. This swab was then used to draw a line on another quarter of the agar plate. The test was then repeated in duplicate with each of the 15 solutions tested. (Tbl. 1) The swabbed agar plates were then incubated at 37ºC for 48 hours. The process was finally repeated using the Scope® Mouthwash, this time for 30 seconds with continuous stirring in order to mimic the directions on the bottle.
RESULTS

Table 1: Paper Disk Diffusion Assay

<table>
<thead>
<tr>
<th>TEST SOLUTION</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>No Zone</td>
</tr>
<tr>
<td>Water with Sugar</td>
<td>No Zone</td>
</tr>
<tr>
<td>Green Tea Powder</td>
<td>No Zone</td>
</tr>
<tr>
<td>Green Tea Powder with Sugar</td>
<td>No Zone</td>
</tr>
<tr>
<td>Snapple Powder</td>
<td>No Zone</td>
</tr>
<tr>
<td>Bottled Snapple ®</td>
<td>No Zone</td>
</tr>
<tr>
<td>Bottled Diet Snapple ®</td>
<td>No Zone</td>
</tr>
<tr>
<td>Lipton ® Tea Bag</td>
<td>No Zone</td>
</tr>
<tr>
<td>Lipton ® Tea Bag with Sugar</td>
<td>Zone of Growth: 0.95 ± 0.023cm</td>
</tr>
<tr>
<td>Sencha Tea Bag</td>
<td>No Zone</td>
</tr>
<tr>
<td>Sencha Tea Bag with Sugar</td>
<td>No Zone</td>
</tr>
<tr>
<td>Loose Tea</td>
<td>No Zone</td>
</tr>
<tr>
<td>Loose Tea with Sugar</td>
<td>No Zone</td>
</tr>
<tr>
<td>Yama moto yama Tea Bag</td>
<td>No Zone</td>
</tr>
<tr>
<td>Yama moto yama Tea Bag with Sugar</td>
<td>No Zone</td>
</tr>
<tr>
<td>Scope® Mouthwash</td>
<td>Zone of Inhibition: 1.77 ± 0.087 cm</td>
</tr>
</tbody>
</table>

Paper Disk Diffusion

The paper disk diffusion demonstrated that Scope® mouthwash had the greatest inhibitory effect on bacteria with an average zone of inhibition of 1.77 cm, with a standard deviation of 0.087 cm (Fig. 2). Secondly, it was found that the Lipton ® tea with sugar actually had the opposite effect, resulting in an average zone of growth of 0.95 cm, with a standard deviation of 0.23, around the disk (Fig. 3). There was no zone of growth around the Lipton ® tea with sugar on the control plate. The other disk tests showed no inhibition of bacteria, in addition to some primarily fungal contamination.
In vitro Simulation

All samples, including the control (Fig. 4a) in the in vitro simulation demonstrated reduced bacterial growth: there was a higher concentration of bacteria colonizing in the before swabs than in the after swabs. All observations and collected data were visual, since there was no quantitative data to be collected. The Scope® plate (Fig. 4c) had the least growth, while the loose tea sample was second (Fig. 4b). It was also found that the expensive Yama tea bag had an intermediate effect on the growth of bacteria. Although it was not nearly as effective as both the loose tea and the Scope®, it is clear that the tea inhibited some bacteria when compared to the control.
DISCUSSION

In this experiment, two methods of study were used to examine the antimicrobial effects of various green tea samples: an in vitro simulation and paper disk diffusion. The paper disk diffusion method allowed for comparison of bacterial inhibition between the different samples. Measurements of growth and inhibition zones were made to demonstrate the teas’ effectiveness in restricting bacterial growth. Additionally, a more subjective in vitro study was conducted. Based solely on visual comparison of bacterial growth, this test was more qualitative than the quantitative paper disk diffusion method.

Paper Disk Diffusion Test

Paper disk diffusion allows for comparison of the antimicrobial properties of tea to those of a control sample (water). A bacterial lawn is grown on petri dishes while paper disks are immersed in the liquid samples being tested (tea, water or Scope mouthwash). The disks are then placed atop the bacterial cultures and left in place to wait for results. While the disks sit atop the bacterial lawn, the liquid they contain naturally diffuses outwards. If the liquid contains antimicrobial properties, bacterial growth in the area is inhibited and a circular “zone of inhibition” forms around the disk. No bacteria are found in this zone, as the antimicrobial agent prevents them from growing. It is possible to compare the bactericidal properties of the different liquids by measuring and comparing their zones of inhibition.

In this experiment, each liquid sample was tested with four paper disks. One of the disks were placed into a S. mutans-free control dish. This would give indication if a liquid sample itself carried bacteria. The other three disks were experimental, placed into petri dishes that were cultured with the S. mutans bacteria.

Analysis by Comparison to Control

The water disk control test produced no zone of inhibition, as expected since it lacks any chemicals such as catechins that would inhibit bacterial growth. The water disk provided a
baseline comparison against which the tea sample disk could be compared. However, none of the tea samples tested produced any observable zone of inhibition either. The paper disks of the matcha tea, Snapple® powder, bottled Snapple®, bottled diet Snapple®, sencha tea, loose tea, and Yamamoto bagged tea all did not produce any observable effect on the nearby bacteria culture. Since these tea samples produced the identical result as that of the water disk control test, it was determined that these tea samples had comparable antimicrobial properties as that of water. Thus, this suggests that the matcha, Snapple® powder, bottled Snapple®, bottled Diet Snapple®, Lipton®, sencha, loose, and Yamamoto bagged teas are not effective in combating *S. mutans* bacterial growth (Table 1). Of the disk samples tested, only the Scope® mouthwash produced a zone of inhibition with an average measurement of 1.77 cm (Fig. 3).

**The Observed Effect of Sugar**

This paper disk diffusion also tested the effect of sugar on the tea samples’ antimicrobial properties. Separate tea samples of matcha, Lipton® bag, sencha bag, loose, and Yamamoto bag were also prepared with 15 grams of sugar. These sweetened samples were subjected to the same paper disk diffusion method. Their zones of inhibition were compared with that of a control paper disk, this one of sweetened water which did not produce any zone of inhibition. As with the unsweetened tea samples, the paper disks of the sweetened tea samples did not produce any observable zones of inhibition. Since the zones of inhibition of these disks were comparable to that of the sweetened water control, it was concluded that those tea samples also lacked any real antimicrobial properties. Also, since neither the sweetened nor the unsweetened samples inhibited bacterial growth, it was concluded that the addition of sugar does not act against tea’s antimicrobial properties, if any.

**Increased Bacteria Growth Associated with Addition of Sugar**

While none of the sweetened samples inhibited bacterial growth, one disk sample, the Lipton® tea with 15 grams of added sugar, appeared to have actually promoted bacterial growth. There was no zone of growth on the control plate for Lipton® tea with sugar, which was free of *S. mutans*, but all three experimental plates demonstrated bacterial growth for the Lipton® with sugar. The lack of bacterial growth in the control plate and the presence of it in the experimental plates indicates that the ring of growth was not caused by any bacteria from the tea itself; such contamination would be revealed by growth in the control plate. The paper disks of the unsweetened Lipton® tea also did not produce any zone if inhibition. Since the only difference between the sweetened and unsweetened samples is the sugar, it can be concluded that the sugar does in fact promote the growth of *S. mutans*. However, a gram stain test of the growth around the Lipton® with sugar produced inconclusive results. Thus, it is not completely confirmed that the observed growth was due to *S. mutans*. Although it is likely that the bacteria was indeed *S. mutans*, contamination still could be the reason for the zone of growth surrounding the Lipton® with sugar disk as there was no comparable growth on the control.

The similarity in a antimicrobial capabilities between teas and sweetened counterparts should not be taken as an excuse to add more sugar to tea. The considerations of adding more sugar to tea should extend beyond the influences it may or may not have on *S. mutans* bacteria in
the mouth. More research should be taken before deciding to add sugar to a regular tea-drinking diet.

Possible Sources of Variation and Error

There may be some error in the results due to the procedure of the paper disk diffusion. Error is seen in the presence of fungal growth on many of the petri dishes used in the paper disk diffusion. This indicates contamination either in the preparation of the dishes or in the placement of the disks. It is possible that fungal spores present in the lab were able to get into the petri dishes while they were exposed to the open air, as some petri dishes were open to the air longer than others. The fact that there may have been lapses in proper aseptic technique during the experiment can also explain the contamination present in the results. Forceps may not have been properly sterilized between usage.

Another possible explanation for the presence of contamination in the petri dishes used for the paper disk diffusion is that when placing disks, excess liquid was not entirely removed before the disks were placed on the dishes. While there is no indication that this had any effect on the zones of inhibition, contamination in the teas could have been spread around the dish.

In Vitro Test

The *in vitro* tests offered additional insight into the antimicrobial effects of the teas. The *in vitro* testing first involved swabbing *S. mutans* bacteria from a metal ring onto an agar-covered petri dish. Then, the remaining bacteria on the metal ring were immersed in a tea sample, taken out, and then swabbed onto another section of the petri dish. Next, the petri dishes were incubated. This method allows the experimenter to observe the number of colony-forming units before and after the *S. mutans* were immersed in a tea sample. To determine whether or not the tea sample itself had any bacteria-killing effects, the before-after swabs for all the samples were compared to those of the control plate. The control swabs did indeed display a decrease in bacteria between the before and after swabs. Since the water does not have any antimicrobial properties, the difference between the before and after swabs was due to the swabbing and dipping procedure. If there was a greater difference between the before-after swabs of the sample than there was between the before-after swabs of the control, then it was concluded that the tea demonstrated antimicrobial effects. If the difference was equal or less, then it was assumed that the decrease in bacterial growth was due to simply the dipping of the metal rings in liquid.

Analysis by Comparison to Control

The *in vitro* test showed little evidence of green tea’s effectiveness against *S. mutans*. In all trials, including the control, there was reduced bacterial growth between swabs. However the only tea sample that showed a greater difference between the before and after swabs than the water control was the unsweetened loose tea sample. Thus, this tea was the only sample that demonstrated antimicrobial properties. While its greater antimicrobial abilities relative to those of the other teas could be due to the loose tea’s composition, it could have also had the most antimicrobial powers of all of the teas due to the preparation methods used. Perhaps the method
used in this experiment was more suited for this type of tea than for the others. The optimal brewing used may have enhanced the levels of catechins released from the loose leaves. Since catechins are the chemical compounds believed to cause green tea’s antimicrobial properties, such a difference may have resulted in the loose tea’s greater antimicrobial capabilities.

The Scope® sample demonstrated the greatest antimicrobial capabilities out of all of the samples tested. The difference between the before and after swabs of Scope® was the greatest out of all samples. The mouthwash had known antimicrobial properties due to the alcohol, poloxamer (a wetting agent for antibiotics), polysorbate (a pharmaceutical dispersing agent), and sodium benzoate (an antifungal preservative) present in the liquid\textsuperscript{36}. It has been demonstrated to reduce bacterial levels in the mouth. However, the Scope® was conducted using a different method. For all of the tea tests, the metal loops were periodically dipped in and out of the tea samples. The Scope® test involved simply agitating Scope® with the metal loop for thirty seconds. This difference in procedure may have also contributed to the large difference in effectiveness between the teas and the mouthwash. The 30-second procedure was chosen in compliance with directions on the mouthwash bottle, rather than to mimic the slow sipping of tea over a longer time frame.

On its website, Scope® lab tests claim to have killed 99% of germs that cause bad breath. Both the \textit{in vitro} and paper disk diffusion methods reveal Scope®’s capability to kill \textit{S. mutans}, but without a colony count for the before and after swabs, it is unsure whether the Scope sample killed off 99% of the \textit{S. mutans}. However, based on visual observation, a good percentage of the bacteria was killed off, and any discrepancy could have been due to errors present throughout the experiment and the possible differences in experimental procedure between this particular experiment and the one used to obtain the data on Scope’s website. Also, even though \textit{S. mutans} is one of the main bacteria responsible for bad breath, there are other bacteria in the mouth that Scope® could have tested to obtain a 99% yield. About 75 to 100 different germs live in an individual’s mouth\textsuperscript{37}.

\textit{Observed Effects of Sugar}

The only tea sample that showed any antimicrobial properties for this test was the loose tea sample without sugar. None of the other tea tests, including the sweetened tea samples, indicated any antimicrobial properties. Since the loose tea with sugar also did not show any antimicrobial properties, it seems that the addition of sugar to the loose tea decreased its antimicrobial properties. However, all of the other tea samples with sweetened counterparts showed no such reduction in antimicrobial properties. Thus, the overall experiment shows that the addition of sugar generally does not act against green tea’s antimicrobial properties.

\textit{Possible Sources of Variation}

The fact that no other tea samples besides the loose leaves showed any anti-microbial properties could be due to the preparation method used. Instead of following the individual preparation directions on the packages of each tea, all tea samples were uniformly prepared by letting the tea steep for fifteen minutes in 90º C water. The uniform preparation method was intended to control the amount of variability across the tea tests. However, there is no single
preparation method that is best for all tea types and the uniform preparation method was not optimal for many of the teas. Thus, the preparation probably did not allow all teas to release their maximum chemicals during tea preparation. A different tea preparation method may have produced different results.

Also, a longer length of exposure to the tea may have been necessary for an observable antimicrobial effect. The experiment simulated only two minutes of sipping, but one would be likely to drink tea for longer periods of time. The presence of sugar seems to have no net effect on the antimicrobial capabilities of the other teas.

Some error may have arisen from the procedure itself. Metal loops were swabbed once with $S. mutans$ culture before a new sterile swab transferred some of the bacteria from the metal ring onto a quarter section of the petri dish. A new sterile swab was used for the second transfer (after the bacteria was immersed in the samples). No additional bacteria was added to the loop to replace those that were swabbed off. While this test procedure for the effect of green tea on the bacteria was consistent for every green tea sample tested, the amount of bacteria transferred onto the petri dish was not tightly regulated. For example, if the first transfer took a large fraction of the bacteria from the metal ring to the petri dish, then the second transfer would naturally contain less bacteria regardless of the effects of the green tea. Thus, even something such as the way the swab is rubbed against the metal ring could affect the amount of bacteria put onto the petri dish and ultimately the observations.

There also exists the possibility that some bacteria was washed off the metal ring during the tea-dipping process. Although this possibility was accounted for by the water control plate to which we compared all the experimental tea samples, perhaps the slight differences with which the metal loops were stirred affected the amount of bacteria washed off by liquid. The stirring and dipping of the metal loops in tea was done in a uniform manner, but each tea sample was stirred and dipped by a different person. Even with a control liquid, errors can be significant given the qualitative nature of the observations.

The number of tests performed for each tea sample also presents some limitations to the validity of the results. Each tea test included one control and two experimental trials. In general, performing trials in duplicate still leaves room for error. Even a single erroneous trial for a tea would greatly affect the interpretation of the tea’s antimicrobial properties. Many more trials are required to obtain more conclusive information.

There could have been human error with the qualitative observation for the in vitro experiment. There was much subjectivity in comparing the before-after bacterial growths of the various tea samples to the control plate to determine whether or not the tea inhibited the bacterial growth more than the water itself. In some cases, as in the Lipton® with sugar sample, one trial revealed a significant difference between before and after swabs, showing the tea sample to have antimicrobial effects, while the other trial showed little difference between the before and after swabs relative to the control. However, this discrepancy could have been due to the swabbing differences between the two trials. Unlike its paper disk diffusion counterpart, the Lipton® with sugar sample did not exhibit any more growth upon the addition of tea. Perhaps the paper disk diffusion method enhanced microbial growth while the in vitro method did not, because the tea-
soaked disks helped to concentrate the tea in one small area, enhancing any growth-inducing properties that Lipton® with sugar might have. The paper disk diffusion allowed the disk soaked in the Lipton® tea with sugar to be in contact with the bacteria for a longer period of time than the in vitro test, in which the bacteria was only in contact with the tea for a short period of time. This longer period of contact allowed the sugar to promote growth in the paper disk diffusion.

The results of this experiment reveal that with the preparation method used in these tests, loose green tea leaves are the most effective antimicrobial for S. mutans, while the other tea samples have no particular effect on this strain of bacteria. Therefore, tea drinkers should not depend heavily on tea consumption for dental hygiene, but instead on more effective means such as commercial mouthwash. Additionally, although it is not completely clear why, Lipton® tea with sugar seems to have the potential to promote bacterial growth, causing possible detrimental effects on those who drink it. In conclusion, green tea does not have significant antimicrobial properties at concentrations normally consumed based on the results of this experiment. Future experiments should be done to confirm these results, as there were multiple sources of error present throughout the experiment and the procedure itself.

Conclusion

In conclusion, this experiment has shown that green tea in general does not have antimicrobial properties. Almost all of the tea samples tested do not seem to inhibit bacterial growth. However, since the loose tea does show some effectiveness in inhibiting bacterial growth, the results do support our hypothesis that the loose tea would be the most effective inhibitor.

The experiment in general does not support the hypothesis that the addition of sugar would act against tea’s antimicrobial properties, especially given that many of them showed none of those characteristics at all. The only instance that does support this hypothesis is the example of the loose tea in comparison with the sweetened loose tea. The Lipton® tea with sugar was inconclusive without clear gram stain results and thus cannot be used to support the hypothesis.

Future Experimentation

This experiment has potential for future testing. As the lack of antimicrobial properties for most of the tea samples may have been due to preparation method, future experiments could include testing different preparation methods to see which method produces tea with the greatest antimicrobial effects. Such testing could provide insight into the correlation between preparation method and release of the chemicals in tea.

The effect of tea on different microbes could also be examined, as S. mutans is not the only bacteria that green tea has the potential to inhibit. Since there are many more types of bacteria in the mouth, a future study could examine green tea’s effects upon a wider range of species.

Future research could also test the chemical compositions of the teas. Since the loose leaf tea in this experiment demonstrated the greatest antimicrobial effects, such tests could be
performed to see which compositional properties account for its antimicrobial abilities on the molecular level. The effects of sugar on the antimicrobial powers of tea should also be tested further. Due to the uncertainty of the Lipton® with sugar sample in one of the paper disk diffusions, it cannot be determined if this growth was caused by sugar. A more focused experiment must be used to retest this result to confirm that it is not just caused by contamination.

REFERENCES


