The Effects of Stevia on Species of Bacteria Common to the Human Gut

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The biome of bacteria in the human gut is highly variable in response to environmental changes. This study sought to determine how the increasingly common sweetener stevia interacts with several species of bacteria which have been shown to have important roles in the human gut. Because the common sweetener sucralose has been shown to harm populations of gut bacteria, it was used as a negative control so that the stevia had a standard with which to be compared. Specifically, *Lactobacillus plantarum, Lactobacillus reuteri,* and *Escherichia coli* were the bacterial species examined in this study. Each of these species was grown in three different environments: control MRS broth, a 20% w/v solution of sucralose and MRS broth, and a 20% w/v solution of stevia and MRS broth. In all cases, sucralose allowed for nearly no bacterial growth and stevia allowed for increased bacterial growth over the control. These results were repeated in the *Lactobacillus plantarum* and *Lactobacillus reuteri* cultures when concentrations of sweeteners were reduced to 10% w/v.

The human gut microbiome has been a rapidly expanding area of interest in the field of health and medicine. While the colonies of bacteria in the human gut were previously thought only to aid in digestion, this microbiome is now being shown to affect nearly every aspect of human health. Perhaps the area of most interest to the general public is that of the relationship between the human gut microbiome and obesity, where a favorable modulation of the bacterial species inhabiting the gut is shown to decrease rates of obesity in individuals by increasing satiety and decreasing inflammation and lipogenesis (1). In a similar vein, the human gut microbiome is also shown to play an important role in nutrient absorption, and the modification of the microbes in the gut can change how efficiently a person is able to access nutrients in food (2). The microbes in the human gut have even been shown to produce hormones which then enter the bloodstream and are able to play a wide variety of roles in the body; the most understood hormone which is produced by the gut microbiome is ghrelin, a hormone which helps to regulate appetite and energy expenditure (3). While all of these functions of the microbes in the gut have a close relationship with food, the effects of the gut microbiome extend far beyond merely obtaining nutrients.

The microbes of the human gut have been shown not only to affect matters related to food, but also the mental health of an individual. In a mouse study, ingestion of *Lactobacillus rhamnosus* changed the emotional behavior of the mice by lowering the mice’s levels corticosterone, a hormone released in response to high levels of stress (4). Not only are certain microbes able to reduce stress in their hosts, but links are being discovered between certain microbes in the human gut and instances of depression in humans, with the implication that some of the hormones produced by the microbes in the gut help prevent depression in the host (5). While there is still much to be learned about the human gut microbiome, research suggests that there is good reason for those who are concerned with their health to consider the human gut microbiome.

The human gut microbiome is easily altered in response to the things which a person ingests. Evidence suggests that the gut microbiome can rapidly change in response to a change of diet in a matter of only a few days (6). Since research suggests that this microbiome is so important, learning how certain foods affect this microbiome is greatly important for human health applications. For the purposes of this study, that food in question is stevia. In an attempt to live a more healthy lifestyle, many people will cut out sugar from their diets and replace it with some form of artificial sweetener. While this may be useful for reducing calories in one’s diet, certain artificial sweeteners such as sucralose (often sold under the brand name Splenda™) have been shown to greatly reduce the number of microbes found in the gut (7). Since the microbes in the gut play such an important role in health, finding an artificial sweetener which does not harm the health of these microbes would allow for a much healthier alternative to sweeteners like sucralose.

Due to the fact that stevia is a plant easily found in nature, many people have turned to stevia as a supposedly safe alternative to artificial sweeteners. However, there is some evidence to suggest that even stevia may not be entirely safe. A study in which *Lactobacillus reuteri,* a bacterium often touted for its many beneficial effects, was grown in

the presence of stevia glycosides showed significantly less growth in the presence of these glycosides compared to when the microbe was grown in absence of these glycosides (8). This suggests that certain compounds in stevia may be harmful to the inhabitants of the human gut. This study will seek to investigate whether or not stevia is harmful to three of the microbes which are shown to be beneficial to the health of the human gut: *Lactobacillus reuteri, Lactobacillus plantarum, and Escherichia coli*.

In order to prepare gel plates for growing *Lactobacillus,* 13.75g of MRS broth powder and 5g of agar were added to 250 mL of e-pure water and autoclaved at 121℃ for 15 minutes. As this solution cooled, it was stirred constantly using a magnetic stir bar. When the solution became cool enough to handle with bare hands, it was transferred onto petri dishes, each receiving roughly 25 mL of the gel solution. These dishes were allowed to set for 48 hours.

Cultures of *Lactobacillus plantarum* and *Lactobacillus reuteri* were obtained from the American Type Culture Collection (ATCC) in a freeze dried form. MRS broth powder was obtained from RPI Corp. 50 mL of MRS broth was prepared by mixing 2.75g of the MRS broth powder into e-pure water and autoclaved at 121℃ for 15 minutes. Once cooled, 5 mL of MRS broth was used to suspend each of the bacterial cultures, and these suspensions were placed in vials. These were then cultured aerobically at 37℃ for 24 hours.

After a 24 hour incubation period, the *Lactobacillus plantarum* and *Lactobacillus reuteri* liquid cultures were removed from the incubator. About half of a milliliter of each of these were transferred onto separate MRS plates in the corner of the plates, and spread using an inoculation loop using common streaking techniques in order to obtain individual colonies. These plates were then labeled and allowed to incubate at 37℃ for 24 hours.

After 24 hours, the plates were removed from the incubator. Individual colonies from the ends of the streaks were selected and suspended in around 5 mL of DI water. About 1 mL of each of these suspensions was placed on an MRS plate and spread evenly using a sterile inoculating loop. 4 solutions were then prepared, 10g stevia/L water, 100g stevia/L, 10g sucralose/L water, and 100g sucralose/L. These solutions were used for several Kirby Bauer tests throughout the experiment. 5 µl of each solution was added to an autoclaved paper disk, and these discs were transferred to each of the two MRS plates. Each plate was marked to show the concentration of the respective discs and placed in an incubator at 37℃ for 24 hours.

This process was repeated with *E. coli.* LB broth was obtained from the stockroom and 5 mL was transferred to a sterile vial. A sterile inoculating loop was used to swab a slant culture and was then placed in the LB broth. This broth was allowed to incubate at 37℃ for 24 hours. After these 24 hours, just as with the last Kirby-Bauer test, four discs were prepared by dropping 5 µl of the prepared 10g stevia/L, 100g stevia/L, 10g sucralose/L, and 100g sucralose/L solutions onto the paper discs. 1 mL of the *E. coli* broth was pipetted onto an LB plate and spread evenly using an inoculating loop. The prepared discs were then placed on the plate and the plate was labeled to distinguish the discs. The plate was then allowed to incubate for 24 hours. This exact process was repeated again for all three bacterial species.

Up until this point in the experiment, a new plate would be prepared every few days for each of the bacterial species to propagate. This was always done by using an inoculating loop to transfer a small amount of bacteria into a tube containing 5 mL of DI water. This tube would then be vortexed briefly in order to suspend the bacteria. 1 mL of this suspension would be pipetted onto a new plate and allowed to incubate at 37℃ until a new plate was prepared. After this point, the old plates would be parafilmed and stored at 4℃ .

MRS plates containing stevia and sucralose were then prepared. These were made following a normal ratio of 55g MRS powder and 20g agar per liter of e-pure water. Three 250mL solutions were made in this fashion. To one of these solutions, 2.75g of sucralose was added, and to another 2.75g stevia was added, so that the added sweeteners accounted for 20% the weight of the MRS powder. The third of these solutions was left as a control. All three of these solutions were then autoclaved at 121℃ and stirred using magnetic stir bars until cool enough to handle bare handedly. These were poured into petri dishes, each being filled with about 25 mL. These were labeled and allowed to set for 48 hours before use.

Liquid suspensions of *Lactobacillus plantarum* and *Lactobacillus reuteri* were prepared by swabbing prepared plates of these respective bacteria with an inoculating loop and transferring them into vials filled with 5 mL of DI water. These were then vortexed in order to suspend the bacteria. 100 µl of each bacteria suspension was pipetted onto one of each type of the types of plates discussed previously: control, stevia, and sucralose. Using basic streaking technique, the suspensions were spread over the plates. These plates were then labeled and allowed to incubate for 24 hours.

One more Kirby-Bauer test was performed using the exact same techniques as the previous Kirby-Bauer test, this time using much higher concentrations. A stevia solution was prepared by mixing 12.5g of stevia into 50 mL of DI water and stirring using a magnetic stir bar over a hot plate until dissolved. Likewise, a sucralose solution was prepared by mixing 12.5g sucralose into 50 mL of DI water and stirring with a magnetic stir bar until dissolved. 100 µl of each solution was combined in micropipette with 100 µl of DI water in order to obtain a solution of half the initial concentration. Four paper discs were prepared for the Kirby-Bauer test by pipetting 5µl the 250g/L stevia, 250g/L sucralose, 125g/L stevia, and 125g/L sucralose onto one disc each. A plate was prepared by transferring a small colony of *Lactobacillus plantarum* into 5 mL of DI water and vortexing to suspend the bacteria. 100 µl of this suspension was pipetted onto the plate and spread evenly using an inoculating loop. The four prepared discs were then added to the plate. The plate was labeled and allowed to incubate at 37℃ for 24 hours.

500 mL of MRS broth was prepared by mixing 22.5g of MRS powder into 500 mL of e-pure water and autoclaving at 121℃ for 15 minutes. 5 mL of this broth was transferred to a tube. A small colony of *Lactobacillus reuteri* was transferred to this tube using an inoculating loop. This tube was allowed to then incubate at 37℃ for 24 hours.

After incubation, the *Lactobacillus reuteri* culture was vortexed to suspend bacteria which had settled to the bottom of the tube. Three tubes were prepared by transferring 5 mL of MRS agar into each. Into one of these tubes was added 1g of sucralose, and into another was added 1g of stevia. These tubes were vortexed until the stevia and sucralose were dissolved. 5 µl of the *Lactobacillus reuteri* culture was added to each of the three tubes. All Three of these were allowed to incubate for 24 hours. This exact process was repeated to produce a liquid *Lactobacillus plantarum* culture and then to make three liquid cultures of *Lactobacillus plantarum* in stevia, sucralose, and plain MRS agar. After these trails, the same process was repeated again, cutting the amount of stevia and sucralose which was dissolved into 5 mL of MRS broth by half, so that only 0.5g of sucralose or stevia was added to each tube.

After the liquid the three *Lactobacillus reuteri* cultures (stevia, sucralose, and control) were incubated for 24 hours, they were removed from the incubator. A dilution series was prepared for each one by transferring 90 µl of DI water into 15 small vials, separated into 3 rows of 5. Into one vial, 10 µl of the control *Lactobacillus reuteri* culture was pipetted. 10 µl of this vial was transferred into the next vial, and so on down the row, so that there was a dilution series of 1/10, 1/100, 1/1,000, 1/10,000, and 1/100,000 the initial bacterial concentration. This same process was repeated with the cultures containing stevia and sucralose. 5 µl was then taken from each of these vials and pipetted onto an MRS agar plate in rows of descending order of concentration. Each row of dilutions was labeled and the plate was allowed to incubate for 24 hours. This process was repeated exactly for the control, stevia, and sucralose cultures for *Lactobacillus plantarum,* and was repeated again for the lower concentration stevia and sucralose cultures of both *Lactobacillus plantarum* and *reuteri,* so that dilution series were made for each of these separate cultures.

This dilution series was performed on *E. coli* as well. 5 mL of LB broth was pipetted into a test tube. A small colony of *E. Coli* was obtained from one of the *E. coli* plates that was grown earlier and had been stored at 4℃. This colony of *E. coli* was transferred into the LB broth and allowed to incubate for 24 hours. After this incubation period, three solutions were prepared. One consisted of 5 mL of LB broth and nothing else, one of 5 mL of LB broth and 1g of stevia, and one of 5 mL LB broth and 1g sucralose. Each of these was vortexed in order to dissolve the stevia and the sucralose. 5 µl of the initial *E. coli* culture was transferred into each tube and allowed to incubate for 24 hours. A dilution series was then performed, following the exact procedure as outlined for the previous dilution series.

My results for the various experiments depicted in the following figures, and are discussed afterwards.

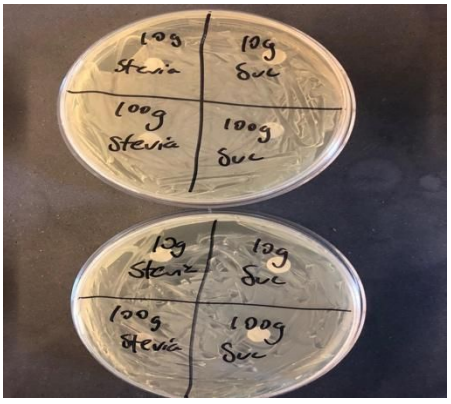

Fig.1: Kirby-Bauer test on *L. plantarum* and *L. reuteri*.

Concentrations given as g/L.

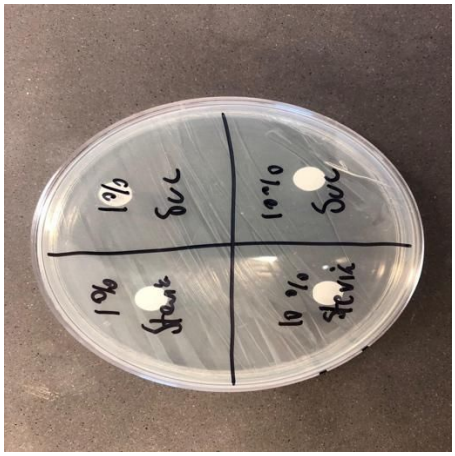

Fig.2: Kirby-Bauer test on *E. Coli.* Concentrations given as % w/v.

Fig. 3: Left to right: *L. reuteri* grown on sucralose plate, stevia plate, and control plate. All MRS agar.

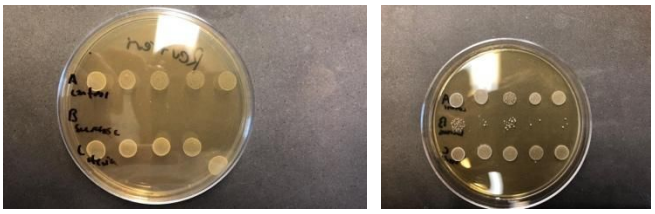

Fig. 4: Dilution series of *L. reuteri*. Left: 20% w/v concentrations of sucralose and stevia. Right, 10% concentrations. Top to bottom rows: control, sucralose, stevia.

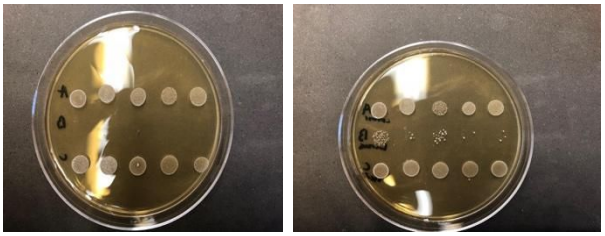

Fig. 5: Dilution series of *L. plantarum.* Left: 20% w/v concentrations of sucralose and stevia. Right: 10% w/v concentrations. Top to bottom rows: control, sucralose, stevia.

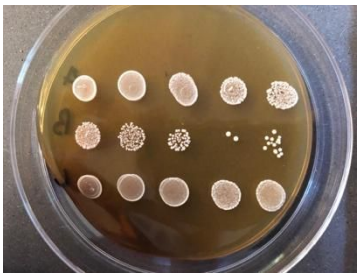

Fig. 6: Dilution series of *E. coli.* 20% w/v solutions of stevia and sucralose. Top to bottom rows: control, sucralose, stevia.

Much of the work done in this project is preliminary work for further research. Being unsure of the best method to observe the effects of stevia on these bacterial species, several methods were employed until one worked. The method which produced the most clear results seems to have been the liquid culture growth with a dilution series. Going forward, this is the method that will be employed. Unfortunately, as this experiment took place, the bacterial cultures were never diluted enough to accurately count the number of colony forming units. Because of this, no statistical analysis can be performed on these results. Even so, this is an easy enough matter to fix. In future experiments, dilution series will be significantly more dilute in order to find exact colony forming units so that proper statistical analysis can be performed.

Even without the ability to do statistical analysis properly, the visual difference in the amount of bacteria in both the control and stevia groups versus the sucralose group is striking. At high concentrations of sucralose, no growth was seen at all. Meanwhile at the same concentrations of stevia, there appears to have been equal or greater amounts of growth than is observed in the control. From these results it is clear that stevia is less damaging to bacteria than sucralose, but further dilution series are required in order to obtain an exact number of colony forming units in order to determine how well bacteria

grow in the presence of stevia versus in a control environment.

None of the other experiments were useful for learning about the interaction of stevia with these bacteria. Each of the Kirby-Bauer tests, even when concentrations were brought up near the maximum solubility of stevia and sucralose, failed to produce any zones of inhibition. When growing the bacteria on plates inoculated with stevia or sucralose, each plate had no visible difference in growth. Going forward, there will be no more experiments of those types.

As stated, everything that was done so far was preliminary work. Going forward, there will be many more dilution series in order to be able to perform statistical analysis comparing stevia, control, and sucralose growth conditions. Several more bacterial species will be tested in order to understand the effects of these sweeteners on a wide array of the inhabitants of the human gut.

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