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The effects of the artificial sweetener sucralose on the gut bacteria *Escherichia coli* and *Enterobacter aerogenes*

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SUMMARY Recent evidence suggests that the gut microbiome may be altered by the intake of artificial sweeteners. The goal of this study was to examine the effects of sucralose, an artificial sweetener, on two gut bacteria; *Enterobacter aerogenes* and *Escherichia coli*. Strains of each bacterium were established by long term culturing in three different concentrations of sucralose in addition to maintaining a control wild-type strain cultured for the same amount of time in the absence of sucralose. The growth rate of each strain was determined in the presence and absence of 150mM sucralose and compared to the control strain. The growth of the control *E. coli* strain was completely inhibited in the presence of sucralose, while *E. coli* cells chronically adapted to 150mM sucralose showed a lack of growth inhibition. The growth of the control *E. aerogenes* strain was also inhibited in the presence of sucralose, but to a lesser degree than observed for *E. coli*. In addition to examining the growth of these two strains in isolation, *E. coli* and *E. aerogenes* were studied in co-culture. In the presence of any concentration of sucralose tested, *E. aerogenes* was able to rapidly and completely out-compete *E. coli,* while similar major shifts in the co-culture composition were not observed in the absence of sucralose. These findings suggest that observed alterations to the gut microbiome composition in response to sucralose exposure may be due to the way this compound differentially inhibits various species of bacteria.

INTRODUCTION

Artificial sweeteners have become a common feature of the American diet, with low or no calorie beverages acting as the major source for these compounds (1, 2). One such artificial sweetener is sucralose, a derivative of the disaccharide sucrose in which three alcohol groups are replaced with chlorine (2). Sucralose activates the TIR2/TIR3 sweet taste receptor that also recognizes naturally occurring sugars such as sucrose, fructose, and glucose (3). However, in terms of sweetness intensity, sucralose is far more potent, meaning that exceedingly small amounts of sucralose can result in a relatively sweet tasting beverage (2).

Radioactive tracing experiments with ^{14}C labeled sucralose have demonstrated that the vast majority of sucralose is not absorbed in the gastrointestinal (GI) tract and is excreted in the feces, while the minimal amount of absorbed sucralose is primarily excreted unchanged in the urine (4). In rat studies, long term exposure to sucralose did not alter the amounts of sucralose excreted from the body when compared to previously unexposed rats, suggesting that long term use of sucralose does not alter the ability of the body to absorb and metabolize this compound (5). Although sucralose cannot be directly metabolized by the human body, recent studies have suggested that artificial sweeteners, including sucralose, can affect the bacteria that colonize the digestive tract (6-8). This "gut microbiome" is becoming increasingly more prominent as a major player in human health and disease, and evidence suggests that it responds rapidly and dynamically to diet (9, 10). Changes in gut microbiome composition have been linked to human disease, including inflammatory bowel disease (IBD), obesity, diabetes, and cancer (11-14).

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Changes in the gut microbiome as a result of exposure to artificial sweeteners could explain some of the observational studies identifying negative health effects associated with artificial sweetener consumption (15). The impact of commercially available sucralose preparations on the gut bacteria of mice has been studied, with observed decreases in total numbers for all bacterial groups tested (7). In addition to the limited data available on the impact of sucralose to gut bacteria, environmental studies have shown that sucralose can inhibit the growth of a wide range of environmental bacteria including *Streptomyces*, *Citrobacter*, *Ensifer*, *Rhizobium*, *Microbacterium*, and *Stenotrophomonas* (16). A recent study examining the impact of sucralose on anti-microbial resistance reported that sucralose alone could inhibit the growth of *E. coli* at a minimum inhibitory concentration (MIC) of 157mM (17).

In this study, the direct quantitative effects of sucralose on two gut bacteria, *Enterobacter aerogenes* and *Escherichia coli* were investigated in isolation and in co-culture. Initial experiments comparing the growth of *E. aerogenes* and *E. coli* at the same concentration of sucralose showed that growth of *E. aerogenes* was only partially inhibited at a concentration of sucralose that fully inhibited the growth of *E.coli*. Based on the difference in inhibition observed, it was hypothesized that the presence of sucralose would alter the composition of a co-culture of the two bacteria, and at any concentration of sucralose tested, *E. aerogenes* was able to completely outcompete *E. coli*, something this bacterium was unable to do in the absence of sucralose. Finally, the effect of chronic exposure to sucralose on these bacteria was examined, and demonstrated that prolonged exposure to sucralose is capable of producing strains of both *E. coli* and *E. aerogenes* that are not inhibited by sucralose. These data provide evidence that sucralose may alter the gut microbiome by differentially inhibiting individual strains of bacteria found within the gut lumen.

METHODS AND MATERIALS

Growth Media. *E. coli* K12 and *E. aerogenes* strains were purchased from Carolina Biological Supply. Sucralose was purchased from SigmaAldrich (Catalog #69293). Cells were grown in Tryptic Soy Broth (TSB) media supplemented with various concentrations of sucralose. All growth media was prepared by diluting a 1.32X stock of sterile TSB with appropriate amounts of filter sterilized 0.7M sucralose and sterilized water to achieve a 1X concentration of TSB and the desired concentration of sucralose.

Establishment of strains chronically exposed to sucralose. To study the effects of chronic exposure to sucralose on bacteria, cultures of *E. coli* and *E. aerogenes* were continuously cultured at varying levels of sucralose for 38 days. Test tubes containing 5mL of TSB with varying levels of sucralose were prepared, designated as follows: No sucralose (NS), 0.07mM sucralose (Low, LS), 0.7mM sucralose (Moderate, MS), and 150mM sucralose (High, HS). Each tube was inoculated with a single colony of *E. coli* or *E. aerogenes.* After overnight growth at 37°C, a loop of each strain was transferred to a test tube of fresh media with its corresponding sucralose concentration. Each week, all strains were streaked onto eosin methylene blue agar (EMB) plates, which can be used to distinguish *E. coli* and *E. aerogenes,* in order to monitor for possible cross contamination. Initial attempts to culture *E. coli* overnight at the highest concentration of sucralose (HS) were unsuccessful. This strain was therefore established by transferring a loop of overnight MS *E. coli* into HS TSB until growth in HS TSB was observed, and confirming that this observed growth was *E. coli* by streaking onto EMB plates. At the end of 38 days**,** glycerol stocks were prepared for all eight resulting cultures and maintained at -80°C.

Growth Assays. To assess the growth of *E. coli* and *E. aerogenes* strains in the presence of sucralose, single colonies of the indicated strain were transferred into test tubes with 5mL of the corresponding sucralose concentration and grown at 37°C and 120RPM overnight. Overnight cultures were diluted into 25mL of media with the appropriate sucralose concentration to an approximate initial OD_{600} of 0.1. Cultures were grown at 37°C and 120RPM for three hours and the OD_{600} was determined every 30 minutes within that time period as a measure of growth. Each growth experiment was repeated independently three

times, and an average relative OD_{600} for each time point was determined. Average relative OD_{600} values for strains were compared at each time point via t-test (p=0.05).

Co-culture Experiments. To determine the impact of sucralose on co-culture growth of *E. coli* and *E. aerogenes,* single colonies of each NS strain were transferred into separate test tubes with 5mL of TSB and grown at 37°C and 120RPM overnight. After measuring the OD_{600} of each overnight culture, the two strains were diluted into a single flask containing 25mL of TSB media and the desired concentration of sucralose according to the Agilent online cell calculator (http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp) such that each flask of media contained approximately 50,000 cells/mL of each bacterial strain (*E. coli* and *E. aerogenes*). Initial diluted aliquots corresponding to 250 total cells from each flask were plated onto three separate EMB plates and grown at 37°C overnight to determine the initial ratio of *E. coli* to *E. aerogenes*. Mixed cultures were grown at 37°C overnight before plating diluted aliquots on three EMB plates corresponding to approximately 250 total cells based on the measured OD_{600} of the overnight culture as described above for the initial dilution. The resulting number of white *E. aerogenes* and metallic green *E. coli* colonies on each EMB plate were counted to determine the ratio of *E. coli* to *E. aerogenes*.

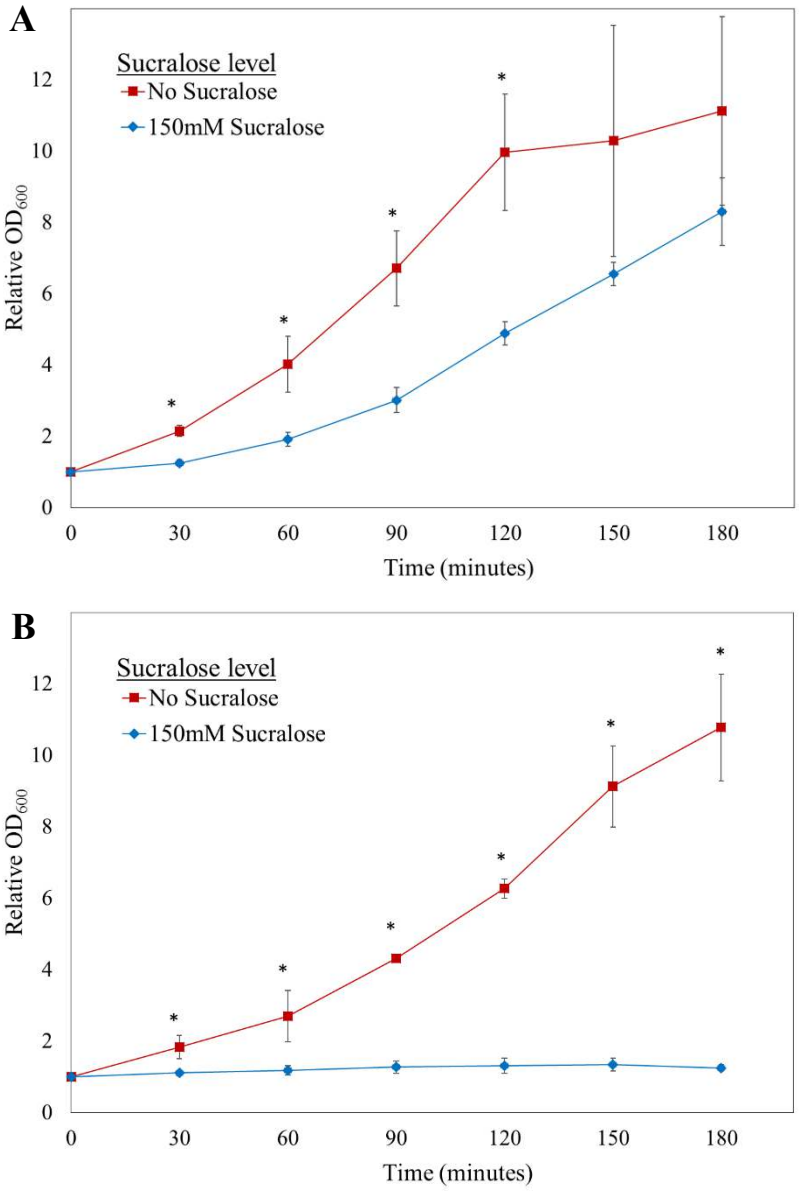

FIG. 1 At 150mM, sucralose partially inhibits the growth of *E. aerogenes* and completely inhibits the growth of *E. coli*. (A) *E. aerogenes* and **(B)** *E. coli* no sucralose (NS) strains were grown in the presence or absence of 150mM sucralose. The OD_{600} was determined after an initial dilution, as well as every 30 minutes for a total of 180 minutes. Graphs show the average OD_{600} relative to the initial OD_{600} for three experiments. Error bars represent the standard deviation. *p<0.05.

RESULTS

Sucralose can inhibit the growth of *E. aerogenes* and *E. coli*. To observe the effect of sucralose on the growth of *E. aerogenes* and *E. coli*, strains of each bacteria were established after continuous exposure to varying levels of sucralose present in Tryptic Soy Broth (TSB) over the course of 38 days, including 150mM sucralose (HS strains), 0.7mM sucralose (MS strains), 0.07mM sucralose (LS strains) and no sucralose (NS strains). Initial attempts to generate a HS strain of *E. coli* were unsuccessful as the cells were incapable of growth at 150mM sucralose. To generate a HS strain of *E. coli,* cells were taken from the MS *E. coli* strain each day and transferred to 150mM sucralose until overnight growth was observed, which occurred on day 29 of the 38-day experiment. The NS strains for each bacteria species, having been continuously cultured like all other strains, but never exposed to sucralose, were used as wild-type controls.

As an initial test of the effect of sucralose on these two gut bacteria, growth assays of the NS control lines for both *E. aerogenes* and *E. coli* in the absence and presence of 150mM sucralose were performed. Overnight cultures of each NS strain were diluted into fresh TSB or TSB+150mM sucralose and growth was monitored by measuring the OD_{600} of each culture over the course of three hours (Table S1). The NS *E. aerogenes* strain showed measurable growth in both the absence and presence of 150mM sucralose, but growth was markedly reduced in the presence of 150mM sucralose, resulting in significantly lower relative OD_{600} values during the exponential growth phase when sucralose was included in the medium (Figure 1A). While NS *E. aerogenes* showed measurable growth in the presence of 150mM sucralose, the growth of NS *E. coli* in the presence of 150mM sucralose was completely inhibited (Figure 1B). These data show that sucralose inhibits the growth rate of both *E. aerogenes* and *E. coli* but at the concentration tested, sucralose acted as a more potent inhibitor of *E. coli*.

Sucralose alters the competition dynamics of *E. aerogenes* and *E. coli.* The differential impact of sucralose on *E. aerogenes* and *E. coli* suggested that the presence of sucralose could impact the ratio of these two gut bacteria when grown together. Co-cultures of *E. aerogenes* and *E. coli* were examined in order to quantify the effects of sucralose on the amounts of these two bacteria over time. NS *E. coli* and NS *E. aerogenes* were mixed together in a single culture and grown overnight. Initial aliquots of each co-culture were plated onto eosin methylene blue (EMB) plates to determine the starting ratio of *E. coli* to *E. aerogenes*. This ratio was then compared to the ratio of *E. coli* to *E. aerogenes* after overnight growth (Figure 2, Figure S1). In the absence of sucralose, the composition of the *E. aerogenes* and *E. coli* co-culture shifted overnight, with an increase in the proportion of the culture that was *E. aerogenes* and a corresponding decrease in the proportion of the culture that was *E. coli* (Figure 2A). In three separate no sucralose experiments, the starting composition of the initial co-cultures varied from 41-56% *E. coli* and 44-59% *E. aerogenes,* while the overnight cultures were shifted to a composition of 15-23% *E. coli* and 77-85% *E. aerogenes*. This reflects an ability of *E. aerogenes* to grow faster than *E. coli* in the absence of sucralose, but not to a degree that allowed *E. aerogenes* to overtake the culture overnight. In the presence of any level of sucralose tested (LS, 0.07mM – HS, 150mM), however, *E. coli* was completely outcompeted by *E. aerogenes* overnight (Figure 2B-D). At the lowest concentration tested, the initial cultures actually skewed in favor of *E. coli* in three separate experiments, with initial co-cultures composed of 57-71% *E. coli* and 29-43% *E. aerogenes*. However, even when the initial cultures contained a majority of *E. coli* cells, after overnight growth in the presence of sucralose, only *E. aerogenes* bacteria could be detected. These results clearly demonstrate that the differential inhibition sucralose exerts on *E. aerogenes* and *E. coli* can produce rapid changes in population composition when these two organisms are cultured together. This provides direct evidence that sucralose can impact the ratio of bacteria in a mixed population.

Long term exposure to sucralose results in strains with lowered sucralose growth inhibition. In order to examine the long-term effects of sucralose exposure on both *E. aerogenes* and *E. coli*, the growth of LS, MS, and HS strains of each, which were chronically

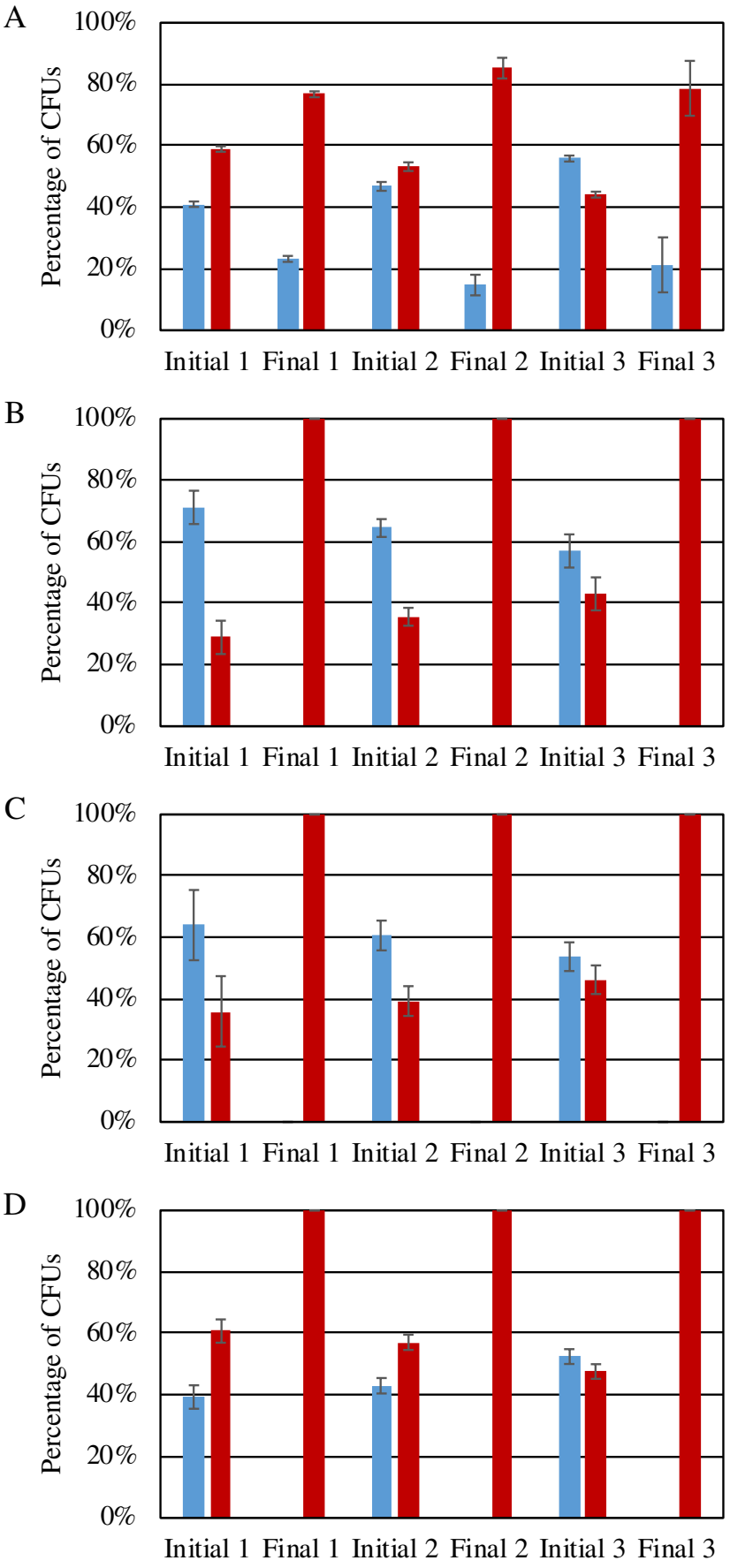

FIG. 2 In the presence of sucralose, *E. aerogenes* outcompetes *E. coli*. Initial and final percent of total colony forming units (CFUs) for *E. aerogenes* (red) and *E. coli* (blue) grown in co-culture in (A) the absence of sucralose, (B) 0.07mM sucralose, (C) 0.7mM sucralose, and (D) 150mM sucralose. Graphs show three independent experiments (initial and final numbered correspondingly). Percentage of CFUs is plotted as the average of three technical replicates for that experiment. Error bars represent the standard deviation.

exposed to varying levels of sucralose for 38 days, were studied and compared to that of the corresponding NS strains in the presence and absence of sucralose, as described above. In the absence of sucralose, the four strains of *E. aerogenes* (NS, LS, MS, and HS) all grew equally well (Figure 3A), with no observable differences in their growth. However, in the presence of 150mM sucralose, the NS, LS, and MS showed measurable but significantly slower growth when compared to the HS strain (Figure 3B). In addition, the inhibition observed for the LS and MS strains, which had been previously exposed to low concentrations of sucralose (0.07mM and 0.7mM, respectively) was similar to the NS line.

When the growth of NS, LS, MS, and HS *E. coli* strains was compared in the absence of sucralose, differences in growth could be detected, particularly between the NS and HS strains of *E. coli.* The HS *E. coli* strain showed significantly higher growth when compared to the NS strain, but all four strains showed robust, measurable growth (Figure 4A). In stark contrast, the growth of NS, LS, and MS strains of *E. coli* were strongly inhibited in 150mM sucralose, while the HS strain showed no such inhibition (Figure 4B). It should be noted that the HS strain reached an average relative OD_{600} of 9.7 after three hours in 150mM sucralose, which is similar to the average relative OD_{600} of 10.8 achieved by the NS *E. coli* strain in the absence of sucralose. These data combine to show that both *E. aerogenes* and *E. coli* were capable of adapting to an inhibitory concentration of sucralose.

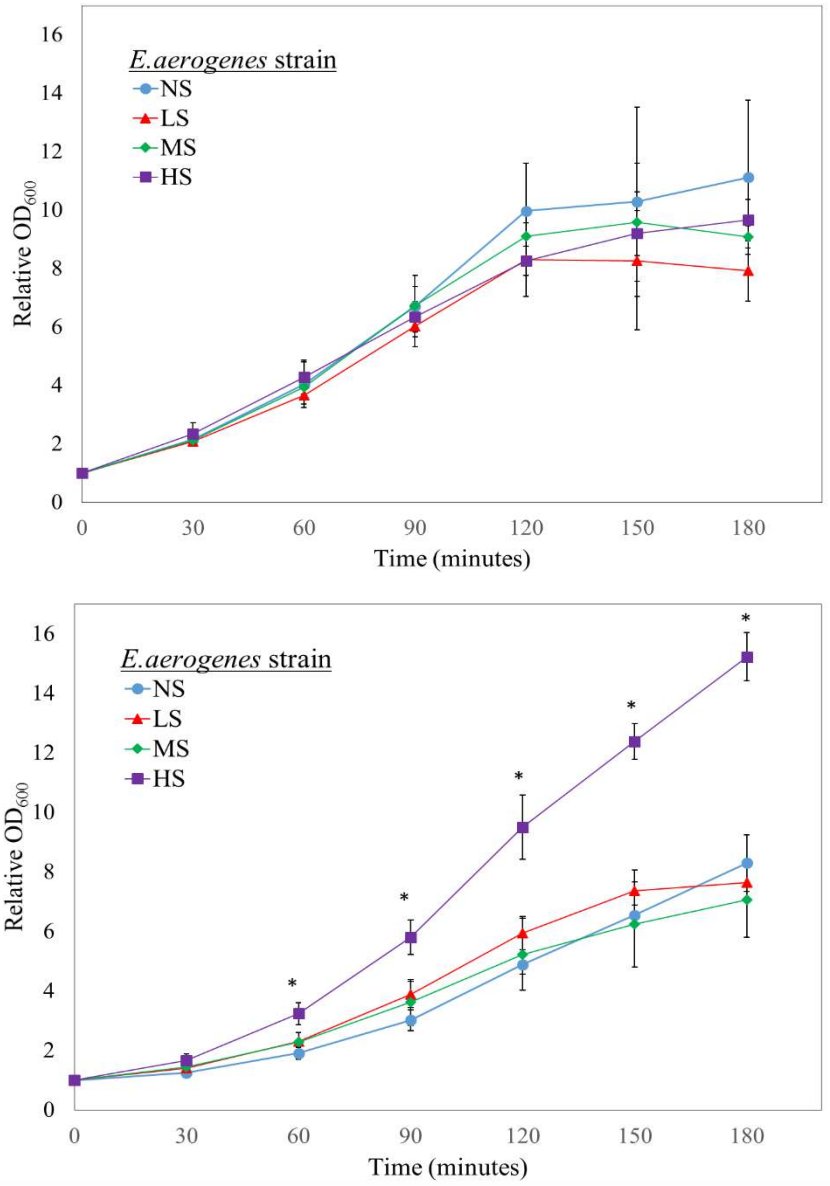

FIG. 3 *E. aerogenes* adapted long term to 150mM sucralose shows a lack of growth inhibition. No sucralose (NS), 0.07mM sucralose (LS), 0.7mM sucralose (MS), and 150mM sucralose **(**HS) *E. aerogenes* long term sucralose adapted strains cultured in the (A**)** absence or (B) presence of 150mM (High) sucralose. The OD_{600} was determined after an initial dilution, as well as every 30 minutes for a total of 180 minutes. Graphs show the average OD_{600} relative to the initial OD_{600} for three experiments. Error bars represent the standard deviation. *$p<0.05$ compared to the NS strain.

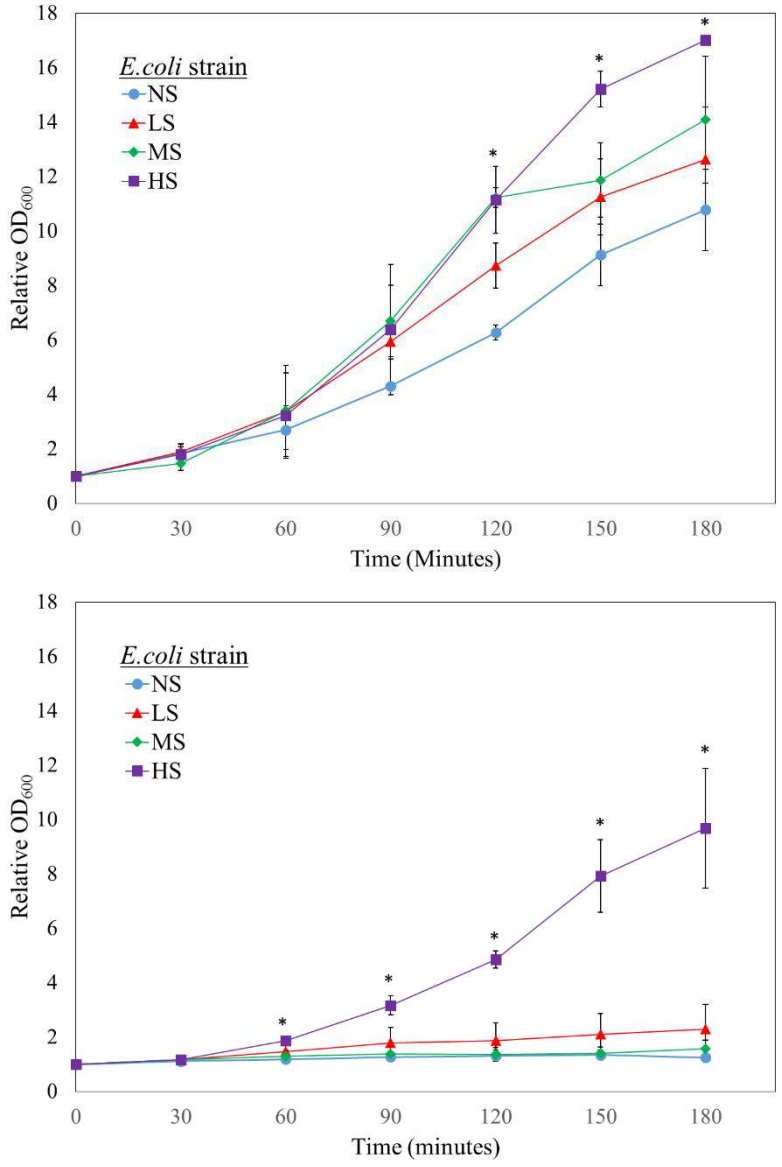

FIG. 4 *E. coli* cells chronically exposed to no or low levels of sucralose are completely inhibited by sucralose, while those exposed to higher levels of sucralose show adaptations that relieve inhibition. No sucralose (NS), 0.07mM sucralose (LS), 0.7mM sucralose (MS), and 150mM sucralose **(**HS) *E. coli* long term sucralose adapted strains were grown in the (A) absence or (B) presence of 150mM (High) sucralose. The OD_{600} was determined after an initial dilution, as well as every 30 minutes for a total of 180 minutes. Graphs show the average OD_{600} relative to the initial OD_{600} for three experiments. Error bars represent the standard deviation. *p<0.05 when compared to the NS strain.

DISCUSSION

It has been previously shown that artificial sweeteners, including sucralose, can alter the composition of the gut microbiome (7, 18). However, the precise mechanism by which this alteration occurs is not clear. For some compounds that alter the composition of the gut microbiome, the changes that occur are likely the result of certain bacteria preferentially utilizing that compound as an energy source. The bacteria that can metabolize a given compound more readily will flourish when the concentration of that compound increases in the gut lumen. This is thought to explain the changes in the gut microbiome linked to meat vs. plant-based diets (9).

An alternative mechanism would involve a compound that inhibited the growth of certain bacteria, allowing others either unaffected or affected to a lesser degree, to grow more readily. The ability of sucralose to inhibit the growth of bacteria has previously been demonstrated in soil bacteria with environmental isolates showing varying degrees of inhibition in the presence of sucralose (16). In addition, it was suggested that the effect of sucralose on these bacteria was bacteriostatic, not bactericidal, possibly acting by inhibiting both the uptake of sucrose as well as the enzyme invertase (16). A recent study examining the effect of sucralose on antimicrobial resistance in *E. coli* reported a minimum inhibitory concentration (MIC) for sucralose of 157mM (17), similar to the 150mM used here to demonstrate partial or complete

inhibition of *E. aerogenes* and *E. coli,* respectively (Figure 1). These results combined suggest that sucralose can act as an inhibitor for a wide range of bacterial types and could therefore differentially inhibit the bacterial strains that make up the human gut microbiome.

The differential inhibition of sucralose on *E. aerogenes* and *E. coli* resulted in sucralose impacting the growth of these two gut bacteria in co-culture. Analysis of co-cultures revealed that at any concentration of sucralose tested, *E. aerogenes* was able to completely outcompete *E. coli* overnight, which it was not capable of doing in the absence of sucralose (Figure 2). It should be noted that this impact on competition in co-culture occurred even at the lowest concentration tested of 0.07mM sucralose, a 1:10 dilution of the 0.71mM concentration of sucralose found in some artificially sweetened beverages (19) and could be a reasonable concentration of sucralose in the gut of someone consuming large quantities of artificially sweetened beverages.

Changes in these two gut bacteria as a result of chronic exposure to sucralose was monitored by growth in varying levels of sucralose for 38 days, and then re-assessing growth in the presence of 150mM sucralose. NS, LS, and MS *E. aerogenes* strains, which were previously exposed to no or low levels (0.07-0.7mM) of sucralose all showed a similar decrease in growth at 150mM when compared to the HS *E. aerogenes* strain (Figure 3B).

When *E. coli* chronically exposed to two lower concentrations of sucralose (LS and MS *E. coli*) were challenged to grow at 150mM sucralose, both showed the same level of inhibition that wild-type (NS *E. coli*) showed, resulting in a lack of measurable growth. At the same time, the HS *E. coli* strain showed no such similar level of inhibition, demonstrating robust growth in the presence of 150mM sucralose (Figure 4B). This data demonstrates that sucralose is a potent inhibitor for *E. coli*, but also that *E. coli* is capable of adapting to sucralose inhibition over time. It should be noted, that initial attempts to establish an HS *E. coli* strain were unsuccessful as no growth was observed in a 150mM sucralose tube overnight when initial chronic exposure was attempted. Eventually an HS *E. coli* strain was established by pulling from the MS *E. coli* strain into fresh 150mM sucralose until overnight growth was observed. This suggests that even the lower level of sucralose in the MS cultures (0.7mM) exerted selective pressure on *E. coli* and resulted in a very small proportion of cells in that culture that could eventually grow at the inhibitory concentration of 150mM sucralose. These results are consistent with our competition data (Figure 2) that showed an impact of sucralose in co-culture at both 0.07mM and 0.7mM sucralose.

It has been proposed that inhibition of environmental bacteria by sucralose was due in part to inhibition of sucrose uptake and competitive inhibition of the enzyme invertase, which catalyzes the hydrolysis of sucrose (16). However, the inhibition observed in that study was detectable in TSB media, which lacks sucrose, and instead includes glucose as a carbohydrate source (16). Similarly, the inhibition detected here was identified when cells were grown in TSB, and was observed in two strains of bacteria, *E. coli* and *E. aerogenes,* that do not readily metabolize sucrose (20, 21). This suggests that the inhibition observed here for both *E. coli* and *E. aerogenes* occurs via a currently unknown mechanism. Similarly, at this time the specific changes in the HS strains that allowed them to overcome growth inhibition by sucralose are unknown. The HS strain of *E. coli* developed here showed not only a lack of inhibition in the presence of sucralose, but higher growth when compared to the NS, LS, and MS strains in the absence of sucralose. This suggests that the changes that occurred produced a strain with higher fitness even in the absence of sucralose.

The data presented here suggests that the alteration in gut microbiome composition by the artificial sweetener sucralose is likely due to differential inhibition of the various bacteria within the gut by this compound. This study also demonstrates that over time, with chronic exposure to sucralose, bacteria adapt to inhibition, allowing individual strains to increase their growth rate in the presence of sucralose. This too would impact the overall gut microbiome composition as different organisms are each uniquely inhibited by sucralose upon initial exposure, and then adapt in different ways and to different degrees of success upon prolonged exposure. One limitation to the work presented here is that the inhibition was observed under aerobic conditions, while in the gut lumen, the environment is anaerobic. Future work is needed to determine the precise mechanism by which sucralose inhibits these two gut bacteria, as well as to expand the species of gut bacteria known to be directly inhibited by this compound.

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