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Natural and Artificial Sweeteners' Impacts on Bacterial Gene Expression and Protein Synthesis

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Research Article

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ABSTRACT

Debate exists regarding the effects of different sweeteners on the human gut microbiome, which includes bacteria like *Escherichia* coli (*E. coli*). It was hypothesized that varying sweeteners would have different impacts on E. coli gene expression and therefore protein synthesis. In this project, E. coli was grown on solid Luria Broth media with added sucrose, Swerve®, or Splenda®. Minimal media (solid and liquid) with added sucrose (table sugar), Splenda®, Stevia®, or Swerve® as the only source of carbon was also used as alternative media. Following incubation with each sweetener,

E. coli cultures were plated and the resulting colonies were analyzed using protein electrophoresis. Intensity of all gel bands was analyzed and the results showed significant differences in protein synthesis when comparing several sweeteners to the control (sucrose). Out of 23 protein bands that were detected on the electrophoresis gels, only bands 16 and 21 showed no significant variation between sweeteners and generations. Sweeteners were also combined with solid Luria broth and *E. coli* was plated for five generations. Colonies from the first and fifth generation were used in protein electrophoresis. From the collected data, it is clear that the tested sweeteners have varying impacts on *E. coli* protein synthesis and may alter the human gut microbiome. Additional studies are needed to identify which protein products are specifically affected. Though it is clear that these sweeteners have varying impacts on *E. coli* protein synthesis, their effect on human health is still undetermined.

INTRODUCTION

Artificial sweeteners, also known as sugar substitutes, are food additives that are many times sweeter than natural sugars, like sucrose. These sweeteners contain no calories and are used to control weight, diabetes, and obesity [1]. People diagnosed with diabetes mellitus often use sugar substitutes instead of natural sugars, because most do not raise blood glucose levels. Currently, the Food and Drug Administration approves of six artificial sweeteners: acesulfame potassium, aspartame, neotame, saccharin, sucralose, and adventame. Many of these additives are relatively new and therefore their effects, especially on the human biome, have not been sufficiently studied.

Currently, 32% of American adults consume artificial sweeteners on a regular basis [2]. With the sharp rise in obesity rates, the use of artificial sweeteners has increased, because though sweet in flavor like sucrose, they do not have many calories and they do not affect blood glucose as well as insulin levels by much [2]. However, in at least one study, low-dose aspartame consumption made rats more susceptible to obesity and significantly altered their microbiome [3]. So, although the older sweeteners such as saccharin and aspartame have been studied in some capacity, the newer substitutes like splenda (artificially made in laboratories) and erythritol (naturally made by stevia plant), induced an insignificant insulin response (**Table 1 and Figure 1**).

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Table 1: shows how some of the artificial and natural sweeteners affect the glycemic and insulin responses. Glucose and sucrose, which are used most commonly, increase a person's glycemic index after a meal more than other sweeteners compared to them. Glucose and sucrose sugars also have a high insulin response that can lead overtime to obesity and diabetes mellitus. When examining other sweeteners, like erythritol, that barely increase insulin production and have no effect on a person's glycemic index, it is clear why more people are using this type of substitute more frequently.

Sweetener	Caloric Value (kcal/g)	Glycemic Responses (GGE//100 g)	Insuline Response (IGE/100 g)
Glucose	4	100	100
Fructose	4	19	9
Sucrose	4	68	45
Erythritol	0	0	2
Xylitol	2.4	12	11
Sorbitol	2.6	9	11
Maltitol	2.1	45	27
Note: GGE: Glycaemic Glucose Equivalent, IGE: Insulin Glucose Equivalent			

Artificial sweeteners have been added to various foods without consumers being aware of their presence. The food and beverage industry continues to use artificial sweeteners as substitutes for sugar, marketing them mostly to those who are diabetic and/or obese. The industry informs the public of the positive effects of artificial sweeteners,

such as their lack of negative effects on teeth and improvement to one's blood sugar control. However, the impact these chemicals have on our microbiome has not been sufficiently studied. For an example, cyclamate was one of the first artificial sweeteners that are now banned by the FDA due to the findings that it may promote bladder cancer in rats. Debate continues on the effects of these food additives as the full potential of artificial sweeteners has not yet been sufficiently scrutinized. The ultimate question is whether a sweetener that does not negatively affect human health could be developed using unprocessed natural ingredients. Because of reports of negative side-effects of artificial sweeteners, consumers have been looking for more natural alternatives like stevia and erythritol.

Stevia is a plant native to South America that has been used as a sweetener for hundreds of years. Today, zero-calorie stevia, as a high-purity leaf extract is being used globally in foods and beverages to add sweet flavor while reducing caloric content. The stevia plant was described in 1899 as Eupatorium rebaudianum by Moises Santiago de Bertoni, in Paraguay. In 1905, it was renamed Stevia rebaudiana, a member of the sunflower (Asteraceae) family [4].

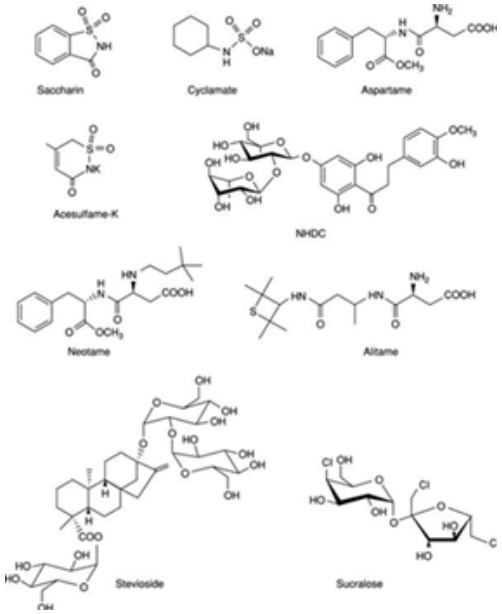

Figure 1: The chemical structures of various alternative sweeteners adapted.

Despite various studies into the effects of artificial sweeteners in model organisms, there is little known about how they alter bacterial gene expression and protein patterns. This is especially true now that we are becoming more aware of the importance of the composition of our microbiome and its link to health. Though some studies exist that suggest rats given aspartame actually resulted in higher rates of obesity, elevated liver triglycerides, increased blood glucose levels, and potential changes to the microbiomes in these mammals, most human studies have not been able to reliably replicate this data. Negative effects continue

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to be cited by ardent opponents of artificial sweeteners, but promoters of these food additives still staunchly defend them because of the positive effects especially in people with diabetes who can still enjoy the sweet flavor without elevating their blood sugar.

People either demonize or praise artificial sweeteners with most studies resulting in no clear conclusion about whether these food additives are dangerous when consumed by the general public. Even though the reports are sparse, it has been suggested that the human microbiome is affected by artificial sweeteners.

Unfortunately, even these studies demonstrate conflicting results. Some studies suggest that artificial sweeteners increase beneficial bacteria like lactobacillus in the intestine [5-7]. Others suggest that the microbiome is negatively impacted by the effects of artificial sweeteners when the tests are conducted in rats [2]. In this study, researchers gave rats aspartame and then measured the contents of their intestines and their physiology. The results showed that rats given aspartame became more obese than the control rats. The researchers correlated the presence of certain bacteria in the intestine to glucose intolerance in rats which eventually lead to obesity.

Research has also been conducted to measure the toxicity of six FDA approved artificial sweeteners using bioluminescent *E. coli* that detect toxic substances. The results revealed that the *E. coli* did luminesce in the presence of these food additives, but growth was consistent with controls. These results suggest that the negative

aspects of artificial sweeteners need to be studied further, especially since an increasing number of people are using artificial sweeteners.

With the increase in human consumption of artificial sweeteners and natural sugars and sugar alcohols, such as stevia and erythritol, further research needs to be carried out that more specifically determines the effects of these substitutes on human health. Emerging research is showing that a growing number of people around the world is making poor nutritional choices and often to counter these choices, they turn to alternative sweeteners to reduce calories and effects of exaggerated insulin responses. Although it is true that alternative sweeteners could lessen the health conditions associated with obesity and diabetes mellitus, we must be sure that they are not harming us in some other way. To continue to build a body of research necessary for the public to make educated choices about their food intake, this study examined the effects that alternate sweeteners may have on protein synthesis in bacteria; it specifically focused on *E. coli* as a model organism but additional studies in gut microbiome bacteria should also be carried out. The original study design utilized minimal media to force bacteria to only use these sweeteners as their carbon source; however, due to poor growth, LB media was ultimately supplemented with Stevia®, Swerve®, or Splenda®, and Sucrose was used as a control. After various generations of growth, the levels of bacterial proteins were analyzed in triplicate using protein electrophoresis and subsequent band intensity analysis.

METHODS

Before experimentation with *E. coli* K-12 was started, the concentration of artificial sweeteners that would be used had to be determined. First, to determine solubility, three 250 mL beakers were obtained and 100 mL of distilled water was placed in each one. Artificial sweeteners, aspartame and sucralose, and sucrose were measured out in 1 g increments. Each sweetener was placed in a different beaker and the solution was stirred until the sweetener was dissolved. This process was repeated until the 100 mL of distilled water was saturated with each sweetener. It was concluded that the average standardized number of sweeteners for the media should be 5 g/L. A sterile M9 minimal media solution was then combined with sterile sweetener-water solutions (Splenda®, sucrose, Swerve®, and Stevia®). Following the manual and protocol provided by Gibco, 1.5 mL/L M9 Minimal Salts (2X) medium was aseptically added to a sterile container. Then, 2 mL of 1.0 M $MgSO_4$ solution and 0.1 mL of 1.0 M $CaCl_2$ solution were also added. The final volume was brought up to 1000 mL. Three culture tubes were then used for each sweetener, including sucrose. A new 10 mL sterile serological pipette was used to aliquot 2.5 mL of each sweetener solution and M9 media into their respective test tube. A starter tube of *E. coli* K-12 included 10 mL of M9 media and one colony from a starter plate. Starter tube was vortexed for even distribution. The tubes were then incubated at 37℃ for 48 hours. 12 more tubes were obtained and sweetener-water solution and M9 media were added the same as before but instead of culture from a plate, 100 µl of each tube from the previous generation was added to the new tube which then became generation 2. These were placed in the incubator at 37℃ for 48 hours. The described steps were repeated for five generations.

After viewing the results from the M9 media experiments, it was determined that using this liquid media limited growth too much and therefore would not be a viable method for the remainder of the experimentation. Another change to the methods was stopping the use of Stevia. After three generations, stevia did not support colony growth. The M9 liquid media method was stopped and replaced with a solid M9/agar media. This allowed for easy identification of contaminants on generational plates. The makeup of the solid M9/agar media includes an agar/water solution and the M9 media. The ratio of agar to distilled water was 30 g to 1000 mL. This solution was heated to dissolve the agar and autoclaved along with the M9 media for sterilization purposes.

The two liquids were then cooled to 50℃ and combined along with a 20 mL 20% sweetener solution, 1M 2mL MgSO4 solution, and a 0.1 mL 1M NaCl solution. This process was done three times in separate flasks while changing the sweetener solution to sucrose, swerve, or splenda. These three flasks were then poured into three groups of 18 plates that each included a

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different sweetener in the media. The 18 plates per sweetener media allowed for six generations.

E. coli was plated onto nine plates (three for each sweetener). After incubation at 37℃ for 48 hours, there was minimal growth on the plates. This observation leads to a change in the methods again. The use of M9 media was discontinued due to it creating an environment too harsh for the *E. coli* to grow. Using M9 media was supposed to force the bacteria to use the sweeteners as their only carbon source. However, it was clear that *E. coli* did not have pathways to sufficiently convert the chemical energy in these sweeteners to ATP. The choice of using LB instead of M9 was supported by the fact that bacteria in the human microbiome have more than a single carbon source. The method to create the solid media was carried out by adding 7 g of Luria Broth, 200 mL of distilled water, and 4 g of sweetener. This solution was made three times with a different sweetener (sucrose, swerve, or splenda) in each solution. These solutions were autoclaved and cooled to 50℃ and poured into three groups of 18 petri dishes specific to the sweetener included. Three groups, each containing triplets from each sweetener media, were inoculated with *E. coli* and incubated at 37℃ for 24 hours. After 24 hours, a second generation was created by inoculating with the *E. coli* from respective generation one. The *E. coli* from generation one was then used in protein electrophoresis analysis to determine protein synthesis levels. This was done for five generations.

The methods for protein electrophoresis was as follows. 100 µl from each test tube in generation 1 was used to inoculate LB plates for a contamination check and to grow samples to use in protein electrophoresis. The plates were then incubated for 48 hours at 37℃. After 48 hours, generation 1 tubes were taken out and checked for contaminants. These *E. coli* samples were used for protein electrophoresis. To begin protein electrophoresis, the Laemmli sample buffer was made: 0.3 g of DTT was added to 30 ml of Laemmli sample buffer andwirled to resuspend. The final concentration of DTT was 50 mM. Leftover solution was stored at –20℃, as DTT is labile. Prior to each use, the solution was warmed to room temperature to dissolve any SDS precipitates that would form upon freezing. Precision Plus Protein Kaleidoscope standard was loaded in the first lane of every gel (prior to each use, the solution was warmed to room temperature to dissolve any SDS precipitates that formed upon freezing). To make the TGS Running Buffer 100 ml of 10x Tris-glycine-SDS running buffer was mixed with 900 ml of distilled water. Twelve screw-cap microtubes were labeled corresponding to each bacterial plate. 300 µl of room temperature Laemmli sample buffer was added to each microtube. Using a new inoculation loop each time, an equal number of colonies was scraped from each plate and transferred to the corresponding tube. It was then thoroughly mixed by spinning the loop with the thumb and forefinger to ensure that there were no visible clumps of bacteria in the tube. Pipetting up and down with a 100 µl setting on a pipet aided in the dispersion. The tubes were heated to 95℃ for 5 minutes in a water bath and cooled to room temperature. Mini-PROTEAN TGX Precast Gel was prepared for electrophoresis in the Mini-PROTEAN Tetra cell (Bio-Rad). 10 µl of the Kaleidoscope standard was loaded into the first lane of each gel. 20 µl of each sample was loaded into subsequent wells. Each sweetener was represented in triplicate on each gel. The gels were electrophoresed at 200 V for 30 minutes. After opening the casing to retrieve the gels, they were rinsed three times for 5 minute each with water. Water was poured out and 50 ml of Coomassie stain was added. The gel was stained overnight on a shaker while covered to avoid evaporation. While the gel was staining the next generation were made and incubated. The next day, the stain was poured out and the destain process began by doing another three, five-minute washes. The gel was then placed in the gel doc and analyzed. The protein electrophoresis was completed for all five generations.

RESULTS

The results from the bacterial growth and electrophoresis for the M9 liquid media onto LB are shown in Figures below.

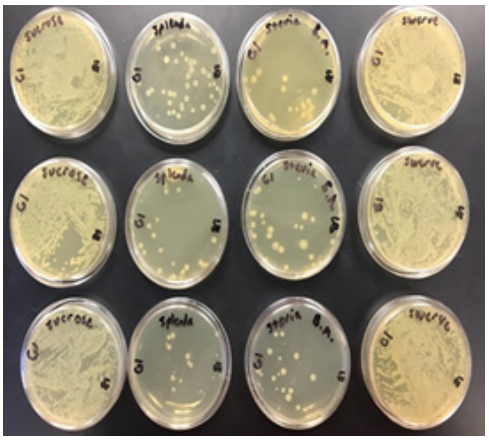

Figure 2: Growth from generation 1 of *E. coli* growth on LB plates.

Generation 1 showed significant difference in growth as seen in **Figure 2.** These colonies were picked and analyzed using protein electrophoresis (**Figures 3 and 4**).

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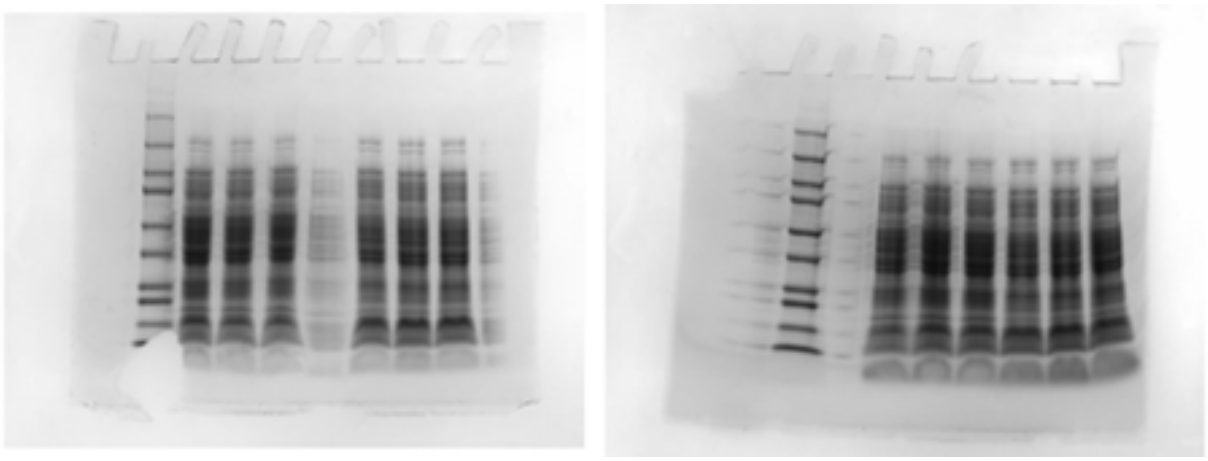

Figure 3: These gels show protein levelsof four different sweeteners in triplicates. The left gel shows Stevia® in lanes 2-4 and Sucrose in lanes 6 -8. The right figure shows Swerve® in lanes 3-5 and Splenda® in lanes 6-8. The first lane is the standard.

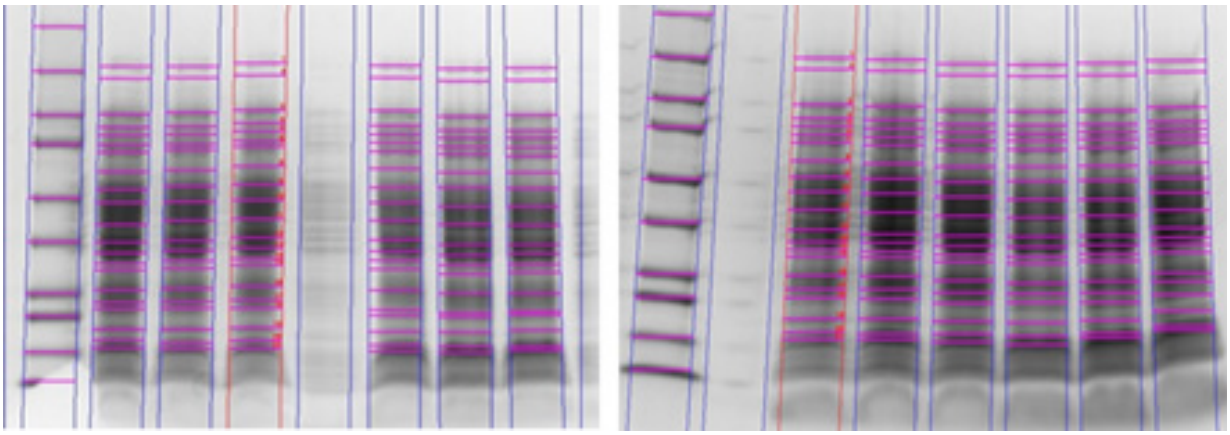

Figure 4: These are the same gels as in **Figure 2**, but with the main 21 bands indicated in pink.

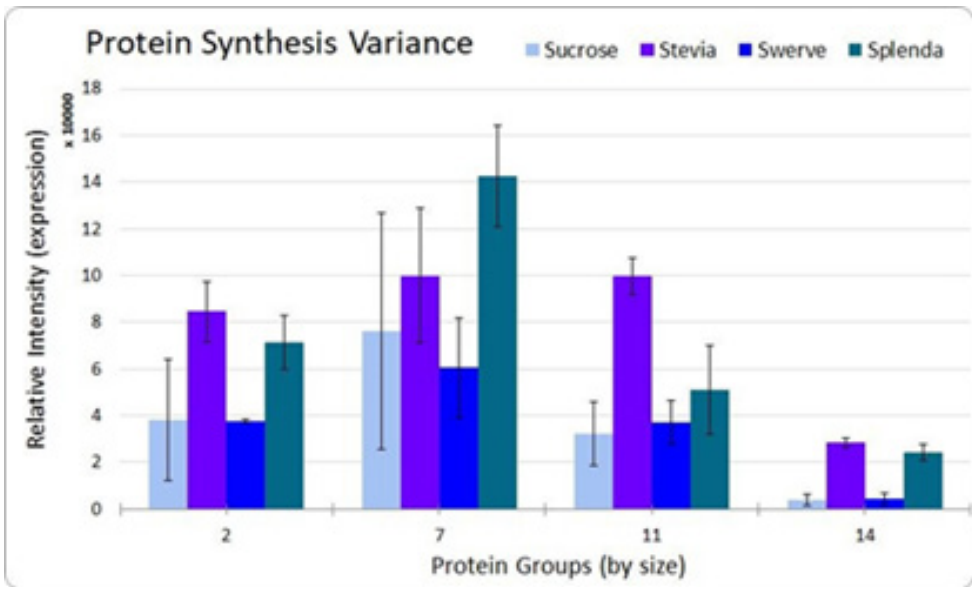

Figure 5: t Bands which showed different intensity between treatments.

Graph in **Figure 5** shows the average band intensity of each band that differed for at least one sweetener. The following band intensities differed significantly between the listed sweetener and sucrose as determined by two-sample t test: protein band 2, Stevia ($p=0.0125$);protein group 11, Stevia ($p=0.0181$);protein group 14, Stevia ($p<0.001$), and Splenda ($p<0.001$).There were 21 bands but only these 4 showed statistically significant differences in gene expression. The results from the M9 methods are very interesting but were not continued due to low bacterial growth an inability to check for contaminants.

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After experimentation with the M9/agar solid media, analysis was not completed due to a lack of cell cultures. The results from the LB/sweetener method are shown below.

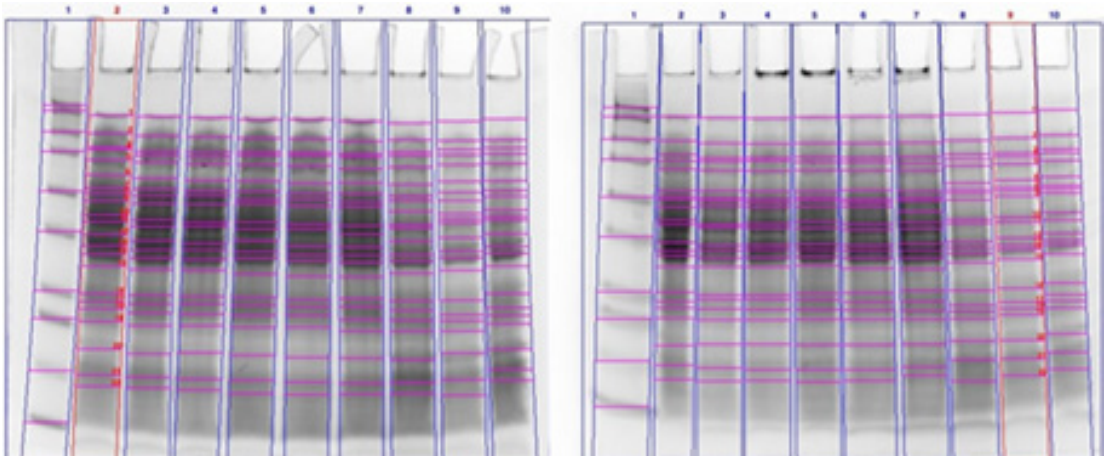

Figure 6: The gel on the left is generation 1 and the gel on the right is generation 5. The first lane of each gel is the kaleidoscope standard. These gels include sucrose (lanes 2-4 in both gels), Swerve® (lanes 5-7 in both gels), and Splenda® (lanes 8-10 in both gels). The pink bands are markers used for analyzing protein intensities in the *E. coli* samples.

In order to obtain the images seen in **Figure 6,** ImageLab software (Bio-Rad) was used to add the pink bars. 23 bars were adding to each lane even if the band on the gel was not clearly present (the software was able to account for this with a much lower intensity calculation). This ensures that every band has a comparable intensity measurement across lanes, sweetener types, and generations. The Image Lab software was also used to obtain raw data charts. The gels were standardized to each other to account for uneven loading and exposure in the Gel Doc Image Lab. The standardization was done by finding the ratio of band intensity to average band intensity in the standards. A ratio was found that was stable across all gels used in the methods. Band 6 in the Kaleidoscope standard was used for standardization of all bands. The ratio of Band 6 in generation 5 to band 6 in generation 1 was 0.831945. All band measurements in the generation 1 gel were therefore multiplied by 0.831945 to normalize the results. After normalization, the ratios of band intensities to average band intensity were still the same as before. The analyzed data is shown below in **Table 2** and **Figures 7 and 8.**

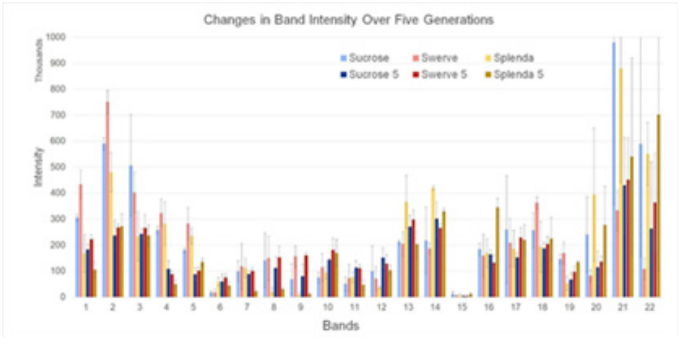

Figure 7: Band intensities of all bands in both gels. The light colors show results from generation 1 and correlate with the darker colors from generation 5.

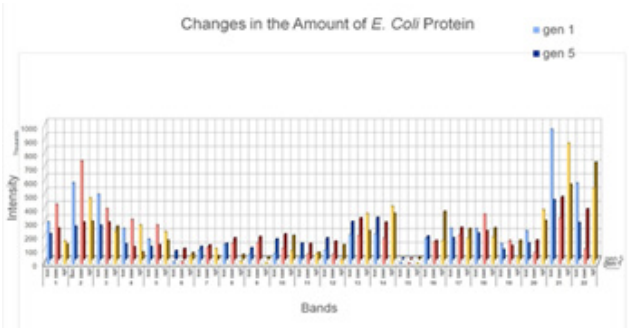

Figure 8: A 3D graph showing the differences in band/protein intensity of all bands and both generations. Blue is sucrose, red is Swerve®, and yellow is Splenda®. The lighter color in the front represents generation 1, while the darker correlating color behind represents generation 5.

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Table 2: All of the two-sample t tests between generations and across generations comparing sweeteners. The numbers highlighted in yellow show p-values <0.05 that reveal statistically significant changes.

	Sweetener								
				Glucose			Generation 1 vs Generation 5		
Band	**Sucrose vs. Swerve®**	**Sucrose vs. Splenda®**	**Swerve® vs. Splenda®**	**Sucrose vs. Swerve®**	**Sucrose vs. Splenda®**	**Swerve® vs. Splenda®**	**Sucrose**	**Swerve®**	**Splenda®**
1	0.01067	0.02088	0.00438	0.04113	0.01472	0.00281	0.00059	0.00183	0.19528
2	0.00311	0.04739	0.00342	0.37705	0.39639	0.8206	0.00035	0.00003	0.00958
3	0.37781	0.06625	0.05211	0.44094	0.77383	0.41295	0.05528	0.04316	0.97021
4	0.0768	0.58977	0.46033	0.30659	0.03006	0.03015	0.00101	0.00103	0.00531
5	0.03051	0.02722	0.2436	0.37872	0.03375	0.03581	0.00223	0.00465	0.00428
6	0.84936	0.00836	0.01619	0.42396	0.40649	0.04413	0.04642	0.00457	0.26261
7	0.70988	0.67355	0.89062	0.34077	0.00075	0.00052	0.66568	0.69668	0.00854
8	0.90599	0.08708	0.04193	0.25903	0.02234	0.00554	0.62793	0.96237	0.27753
9	0.07029	0.1201	0.00209	0.02944	0.04436	0.00002	0.77946	0.85385	0.77577
10	0.25075	0.2995	0.54486	0.19235	0.3838	0.77697	0.0036	0.13388	0.05981
11	0.50242	0.21405	0.90619	0.84929	0.01567	0.0073	0.03065	0.24291	0.10948
12	0.64292	0.26654	0.19514	0.39829	0.083	0.27558	0.37277	0.12478	0.00329
13	0.78162	0.0392	0.04475	0.40553	0.11564	0.03949	0.06122	0.0353	0.04536
14	0.65899	0.03496	0.00004	0.35161	0.42933	0.00294	0.31121	0.00372	0.00031
15	0.3783	0.42402	0.98111	0.79629	0.14049	0.12771	0.24939	0.33941	0.34898
16	0.59211	0.65068	0.85162	0.13978	0.00084	0.0008	0.26015	0.5685	0.00623
17	0.6836	0.54291	0.71274	0.02298	0.0933	0.81786	0.36683	0.72115	0.50418
18	0.04309	0.34672	0.0288	0.42244	0.42841	0.66376	0.12196	0.00123	0.65225
19	0.37298	0.00438	0.00895	0.15526	0.00713	0.02741	0.00767	0.03526	0.0037
20	0.09717	0.35538	0.07217	0.54461	0.11162	0.13187	0.18422	0.0273	0.4747
21	0.10821	0.8037	0.06992	0.86418	0.62393	0.68589	0.16521	0.25549	0.3079
22	0.09304	0.87634	0.00221	0.5645	0.24921	0.34166	0.26686	0.05875	0.64761

DISCUSSION

The hypothesis that there would be differences in expression patterns when *E. coli* were grown in the presence of different artificial and natural sweeteners was supported by the collected data as seen in **Figure 7** and **Table 2**. The difference in protein synthesis between sucrose, Swerve®, and Splenda® could have an impact on what we know about how the human gut microbiome responds to these chemicals. If the same changes are happening in the human gut, there could either be a cause for concern or these changes could actually mean that the good gut bacteria could be thriving better than before. With altered protein synthesis, there could be varied chemical products being excreted into the human gut or being absorbed by the bacteria.

CONCLUSION

To make this study more inclusive, additional types of bacteria and sweeteners should be tested and perhaps most importantly, the next step should focus on identifying the types of specific proteins that are up and down regulated. This analysis would require either Western blotting using specific antibodies or RNA sequencing to identify gene expression variation of various, specific genes. The results presented in this paper suggest a correlation but do not explain the mechanism for the variations in protein expression due to exposure of bacteria to various sweeteners. However, it is critical to continue these types of studies so that the general public can be aware of both the potential benefits and problems that alternative b sweeteners can cause within our gut.

REFERENCES

1. Qurrat-ul-Ain, et al. Artificial sweeteners: Safe or unsafe? J Pak Med Assoc. 2015; 65: 225-227.
2. Rojas E, et al. Stevia rebaudiana Bertoni and Its Effects in Human Disease: Emphasizing Its Role in Inflammation, Atherosclerosis and Metabolic Syndrome. Cur Nutr Rep. 2018. 7: 161-170.

e-ISSN: 2320-3528
p-ISSN: 2347-2286

3. Suez J, et al. Non-caloric artificial sweeteners and the microbiome: Findings and challenges. Gut Microbes. 6: 149-155.
4. Palmnäs MS, et al. Low-dose aspartame consumption differentially affects gut microbiota-host metabolic interactions in the diet-induced obese rat. PLoS One. 2014; 9.
5. Ashwell M. Stevia, Nature's zero-calorie sustainable sweetener. Nut Today. 2015; 50: 129-134.
6. Harpaz D, et al. Measuring artificial sweeteners toxicity using a bioluminescent bacterial panel.
    a. Molecules. 2018; 23: 2454.
7. Gu W, et al. Recombinant expressions of sweet plant protein mabinlin II in *Escherichia coli* and food-grade Lactococcus lactis. World J Microb Biotechnol. 2015; 31: 557-567.