Effect of Stevia on Glucose Tolerance and the Gut Microbiome

by

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Abstract:

Non-caloric artificial sweeteners have been correlated with reduced glucose tolerance and obesity in both mice and humans, however the effect of the non-caloric natural sweetener stevia has shown mixed results, with some indication it may act as an anti-hyperglycemic agent. Furthermore, the effect of stevia on the gut microbiota has yet to be characterized. Other non-caloric sweeteners are known to induce dysbiosis, which is correlated with changes in glucose tolerance, most pronouncedly saccharin [1]. We tested the hypothesis that stevia would reduce high fat diet-induced glucose intolerance which would be correlated with a significant change in the microbiome. We evaluated physiologic markers including caloric intake, liquid consumption, and body weight as well as glucose tolerance tests at the initiation and end of treatment. Fecal samples were collected at start of treatment, at the midpoint, and at the end of treatment.

Male and female C57BL/6J mice (n=40) were randomly assigned (unblinded) to one of four treatment groups: low fat diet and water (LF), high fat diet and water (HF), high fat diet and saccharin (saccharin), or high fat diet and stevia (stevia). Over 10 weeks of sweetener treatment, stevia mice ate an average of 1,244±44 kcalories per animal, however this was not significantly less than other animals on a HF diet, with HF animals eating on average 1,312±140 kcalories and saccharin eating 1,309±203. Liquid consumption was similar between groups on a HF diet, while the LF diet group consumed significantly more water (males p=0.017, females p=0.002). Stevia supplementation also did not prevent weight gain, though mice on LF diet weighed significantly less than HF, saccharin, and stevia (males p=0.017, females p=0.003). Sweetener treatment did not affect glucose tolerance, with no difference between HF, saccharin, and stevia in area under the glucose response curve (AUC) (81,300 ±3300 mg/dL, 76,200 ±2800 mg/dL, and 85,800 ±3400 mg/dL, respectively).

While all groups on HF diet had similar changes in operational taxonomic unit (OTU) counts over the course of treatment, stevia showed a significant increase in genera *Lactobacillus* (p=0.029), *Lactoccoccus* (p=0.034) compared to saccharin. Saccharin showed a significant increase in genus *Streptococcus* (p=0.034) which was not observed in HF or stevia. Saccharin did not display the changes observed in several *Ruminoccoccae* and *Clostridiales* families observed in both stevia and HF, suggesting there are distinct microbiome profiles for each sweetener beyond changes due simply to a HF diet.

Stevia had a significant effect on alpha diversity as measured by the Shannon index which was significantly different than both saccharin and HF groups (p=0.002 HF, 0.008 saccharin). Two measures of beta-diversity did not show a significant effect of treatment

(weighted UniFrac and Bray-Curtis). These data position stevia as a potentially beneficial non-caloric sweetener as it does not appear to alter the microbiome beyond the effects exerted by the stress of a HF diet though more research is needed to evaluate the extent of its functional consequences on the metabolome.

Introduction

More than one third of Americans are overweight or obese [2], leading the United States Surgeon general to declare obesity as the most important public health epidemic of our time [3]. Afflicting both developed and developing countries, obesity and its associated health risks lead it to be considered one of the leading causes of morbidity and mortality worldwide [4]. Incentive to find a solution for this public health epidemic is great considering the burden management of these conditions places on health care systems and the significant economic toll such conditions exert. The estimate annual cost of obesity in the U.S. in 2008 was $147 billion, $1,429 higher per person than health care costs associated with individuals at a healthy body weight. With the number of people with diabetes mellitus (DM) worldwide expected to exceed 300 million by the year 2025 the costs of obesity-related health care costs will only continue to rise [5]. Beyond the economic toll, quality of life is severely diminished for these populations as obesity has been linked to some of the leading causes of preventable death such as heart disease, DM, and some types of cancer [2]. Obesity is implicated in a variety of cascading health conditions, including DM, hypertension, hepatic steatosis, dyslipidemia, glucose intolerance, and impaired insulin sensitivity and chronic inflammation [4] [6] among many others.

The primary pathological contributor to these diseases is attributed to obesity [7]. These conditions have become known under the broad umbrella of metabolic syndrome, which is defined as a combination of risk factors for cardiovascular disease in addition to glucose intolerance [8]. Glucose intolerance is a key stage is the progression of DM, often predicating the development of formal DM with about 40% of glucose intolerant individuals developing DM

within 5-10 years [9]. While a variety of factors are known to contribute to metabolic syndrome, the specific etiology of obesity-associated metabolic syndrome (OAMS) remains unknown, though several lifestyle factors have been proposed as likely contributors. For example, dietary fat intake in particular is known to contribute to impaired glucose tolerance and insulin sensitivity, with high fat diets often used in laboratory settings to induce obese states [10]. Insulin is a hormone produced by the pancreas which serves as the primary regulator of blood glucose concentration while also initiating storage of substrates in fat, liver, and muscle by stimulating lipogenesis, glycogen production, and protein synthesis while inhibiting lipolysis, glycogenolysis, and protein degradation [4]. Insulin insensitivity, otherwise known as insulin resistance, is associated with both obesity and DM [11] [12]. However, most obese individuals with insulin resistance do not develop glucose intolerance as normally pancreatic islet ß-cells can increase insulin release to accommodate its reduced efficiency [7] [13]. ß-cell dysfunction dismantles this accommodating response, leaving the individual unable to maintain normal glucose levels and rendering them both insulin resistant and glucose intolerant [14], [15]. As such, insulin regulation serves a crucial role in managing glucose intolerance and DM, and the regulation of which suggests how integral dietary choices are the to the development and management of obesity-related conditions.

While some degree of predisposition to development of obesity and related metabolic syndromes has been linked to genetic variance, the attribution to genetics in the etiology of obesity and related metabolic disease accounts for only about a few percent of the proportion of genetic variance linking loci to increased body mass index (BMI) [16] [17]. Rather, emerging evidence suggests these diseases may be modulated by humans' other genome, the

microbiome. A microbiota refers to a localized ecological community of bacteria, while microbiome refers to the collective genes which such a community encodes. The human microbiome is estimated to contain more than 10^{14} bacteria from over 1000 species [18], the collective genetic material of which exceeds the contents of the human genome by 100 fold [19]. These bacterial communities, known as microbiota, have been characterized all over the body, including in the mouth, gut and vaginal cavity, with the majority of research focusing on the gut microbiota as this bacterial population is rapidly emerging as a critical regulator of metabolic and immune functions, situated at the crossroads of genes, diet, and lifestyle [20]. The modern gut microbiota contains nine of the 70 described bacteria divisions, but is dominated by *Firmicutes* and *Bacteroidetes* [18]. These communities are described using alpha and beta diversity. Alpha-diversity refers to the microbial composition of a single sample which is measured as a factor of richness (the number of species present in a sample) and evenness (the distribution of these species within the animal). Beta diversity refers to differences in overall microbiota composition between groups.

While there is immense alpha-diversity between geographic locations and between individuals [21], conservation of alpha diversity is observed at a function level both within a site and between individuals, establishing a core microbiome at the level of shared genes involved in metabolic processes [22] [23] [24]. The microbiome is where genes, diet, lifestyle converge. Host genotype can affect the suite of microbial genes activated when microbial strains interact [25], creating a symbiotic system between gut microbiota and host which creates the enterotypes observed in humans [26] [23] [27]. A genome-wide association study (GWAS) based on shotgun sequencing of gut microbial DNA from 345 Chinese individuals characterized

60,000 DM associated genetic markers and developed a metagenomics linkage group with taxonomic species analysis to identify microbial markers that may help identify individuals at risk for DM [28]. Given the diversity of the human microbiome, the potential for microbe-mediated signal transduction is immense, with communication between microbes and metabolites, microbes and immune functions, and metabolites and immune functions [29]. The microbiota's production of metabolites initiates a signal cascade through the microbiome-host metabolic axis, defined as a multi-directional exchange and metabolism of transgenomically regulated secondary metabolites between host and the microbiome [26]. This allows for communication of signals originating in the gut lumen and disseminating throughout both brain and body.

Perhaps the most prominent source of such signaling are the by-products gut bacteria produce. As humans cannot digest complex polysaccharides, they are fermented in the gut by the microbiota to create metabolic by-products known as short-chain fatty acids (SCFA) which are able to be absorbed by the host. While patterns of SCFA fermentation depend on the amount and type of carbohydrates and the composition of the host gut microbiota [30] [31], three intermediary metabolites are predominantly produced: acetate, propionate, and butyrate [30]. SCFA have a wide range of effects on gut and overall health, serving a variety of functions including sources of energy, while also acting as potent vasodilators and affecting gut motility and wound healing. Such metabolites have been particularly implicated in the chronic inflammation associated with obesity and metabolic disease, directly affecting development and function of anti-inflammatory regulatory cells (T-regs) [29]. The microbiota is a source of inflammatory molecules such as lipopolysaccharide (LPS) and peptidoglycan, and plasma levels

of LPS have been shown to increase in mice [32], humans [33], and in DM patients [34] when following a high fat diet, an effect which was blunted following a high-carbohydrate diet [33].

A primary target of these signal molecules is the liver. For example, acetate and propionate are taken up by the liver and used as substrates for lipogenesis and gluconeogenesis [24]. However, it is likely metabolites affect host metabolism through other pathways as well, as SCFA dietary supplementation suppresses diet-induced obesity, reducing food intake, fasting insulin and leptin levels, and improving glucose tolerance [35]. Bile acids, synthesized in the liver from cholesterol, are primarily taken up by the distal ileum for transport to the liver, but some escape and are further metabolized into secondary bile acids by the microbiota and have been shown to influence glucose and lipid metabolism [36] [37]. Butyrate, acting as a histone deactylase inhibitor and a potent gene regulator through G-protein coupled receptors in the epithelium [20] is associated with lean phenotypes and healthier gut microbial profiles [38] [35] as well as improved insulin sensitivity [39].

Understanding the potential of the microbiome is essential as it appears to act as an driver controlling host physiology, and understanding its heterogeneous dynamics will be important in both achieving optimal health and in prevention of microbe-mediated disease, of which obesity has increasingly been implicated [26]. Obesity is transferable via microbiota transfer [40] [38], shown most elegantly in mouse models in which germ free mice colonized with microbiota samples from obese donors develop an obese phenotype, with the converse also true, in that an obese mouse can be made lean with microbial inoculation from a lean donor. Transfer of lean microbiota to obese patients improves insulin sensitivity [41] and microbiota diversity is associated with improved insulin resistance [41] [32]. Antibiotic

treatment, which essentially wipes the microbial slate clean, partially protects against Type 1 diabetes in a diabetes-prone rat model suggesting the contributions of the microbiome to impaired metabolic states may have a more direct on insulin regulation that previously thought [42]. It also suggests multiple pathways originating from the microbiome can contribute to a diabetic state DM has previously been linked to the microbiome [43]. Diabetes mellitus patients have been shown to have distinct compositional changes in their microbiota compared to non-diabetic controls, a state known as dysbosis characterized by unconventional composition of the gut microbiota [44]. This is supported by exciting research in which DM is reversed following gastric bypass surgery even before patients begin to lose weight [45] [46], positioning the gut microbiota as playing an active but still ambiguous role in the pathology of metabolic disease.

While there appears to be a core microbiome based on genetics with shared microbial genes, diet appears to play a large role in individual variation [23]. High fat diets have been shown to alter the microbiota, skewing it towards an obesity-associated enterotype by inducing changes such as a reduction in *Bacteriodetes* and an increase in *Firmicutes* and *Proteobacteria* while decreasing diversity [23] [38] [47] [48] [40]. Furthermore, high-fat diets have been shown to increase the inflammatory signal molecule lipopolysaccharide (LPS) in plasma and in the gut microbiota, presenting LPS as a possible trigger for obesity as subcutaneous LPS injections lead to increased body and liver adiposity [32]. Diets high in saturated fats and low in fruits and vegetables may select against microbiota associated with leanness [38], and breakdown of dietary intake may alter the efficiency and composition of the microbiome. Germ-free (GF) mice, or mice without any micro-organisms living on them, are protected from obesity, insulin

resistance, dyslipidemia, and fatty liver disease even when fed a high fat diet, consuming fewer kcalories, excreting more kcalories, and weighing significantly less [49] [50]. However, the microbiome appears to be essential for fat gain independent of reduced food consumption, as conventionally raised mice weight more than GF mice despite eating fewer kcalories and also display increased leptin, glucose, and insulin levels [51].

Thus, one proposed pathology for the development of obesity is the increased energy harvest theory, which suggests the gut microbiome influences the harvest of energy from food sources and regulates how that extracted energy is processed and stored, such as upregulating genes which allow for increased capacity for fermenting otherwise indigestible polysaccharides [40] [40] [27] [49]. This theory is supported by the reduced energy content characteristic of obese fecal samples compared to lean counterparts [27]. While evolutionarily this would have given individuals a survival advantage in times of food scarcity, a highly efficient microbiota may actually pose a disadvantage in our modern society inundated with readily-available, calorie-rich food choices. For example, obese human gut microbiomes are upregulated for phosphotransferase systems, a class of transport systems involved in the uptake and regulation of carbohydrate [41]. Phosphotransferase, known to be present in Western diet-associated microbiomes, regulates microbial gene expression and may help the cell prefer simple sugars over more complex carbohydrates [52]. Diet then can alter the composition and function of the microbiome resulting in consequential metabolic derangement.

One such dietary additive prevalent in modern society are non-nutritive artificial sweeteners (NAS). Much more potent than sucrose, these sweeteners can be used in miniscule amounts in order to get a comparable amount of sweetness and are thus considered non-

caloric, often diluted with dextrose for ease of use by commercial and private consumers. NAS use has skyrocketed in the past decade, with more than 6,000 NAS-containing products launched between 1990-2000. In the U.S., national nutrition survey research from 2004 estimated that 15% of the U.S. population was using artificial sweeteners [53], and more recent estimates place this figure closer to 30% of adults and 15% of children ages 2-17 years [54]. While these products reduce the energy density of foods, they may not actually have the intended effect of leading to a decrease in energy consumption and a following weight loss. While high NAS consumption has long been associated with populations at-risk for or suffering from metabolic syndrome, the causal relationship of this correlation is unknown [55].

Long assumed that NAS consumption sought to manage weight and blood glucose, recent research suggests consumption of these products may actually contribute to development of obesity and metabolic disease, as the reverse causality does not fully explain the higher risks observed in humans after adjustment for confounding factors [56]. Regular consumption of NAS has been associated with an increase in body weight and adiposity [57] [58], impairment of glucose homeostasis [56] nearly doubling an individual's risk of developing DM, even if they are at a normal body weight [59]. Among adolescents, artificial sweetener use increases the odds of obesity by a whopping 70% completely independent of total energy intake [60]. The potent effects of artificial sweeteners may be transmissible via epigenetic changes in utero or through the placenta to affect a child's development, as mothers who consume high amounts of artificial sweeteners have an increased BMI [61].

Two primary mechanisms have been proposed to explain such observations. The sweet taste of artificial sweeteners may heighten motivation to eat, but as NAS do not provide caloric

density this desire is never satiated leading to an increase in overall energy intake, leading to eventual weight gain [62]. Such a theory suggests the detrimental health effects of NAS consumption are not directly due to NAS itself, but rather to the adiposity associated with these products. While this may be true to an extent, recent research suggests an alternative theory. NAS themselves may be exacerbating the effect of weight gain and even diet on metabolic health through interactions with the gut microbiota.

Research by Suez et. al localized the effect of artificial sweeteners to the gut microbiome in an elegant experiment that tested a variety of the most common artificial sweeteners' effect on glucose tolerance, insulin sensitivity, and body weight in mice fed a high-fat diet [1]. While all artificial sweeteners tested (saccharin, sucralose, aspartame) had a significant effect on glucose tolerance, saccharin exhibited the most pronounced effect on glucose tolerance [1]. This effect was shown to be specific to the gut microbiome, as four weeks of both Gram-negative and Gram-positive antibiotic treatment abrogated the observed difference in glucose tolerance between controls and saccharin-fed mice. Fecal transplantation from saccharin-fed mice into normal-chow-consuming mice resulted in impaired glucose tolerance in the recipient animals [1]. Subsequent 16S ribosomal RNA genomic sequencing showed significant gut dysbiosis in the saccharin group with more than 40 operational taxonomic units (OTUs) significantly different in abundance, higher levels of SCFA, and upregulated glycan degradation pathways. Direct culture of fecal matter from saccharin-fed mice was used to inoculate germ-free mice also resulting in impaired glucose tolerance compared to controls. These results were further corroborated by a pilot study in humans where individuals who did not normally consumer NAS were given the FDA's maximum

accepted daily intake (ADI) for saccharin and developed a worse glycemic response after only seven days [1]. While extensive research has been conducted to establish the ADI, defined by the FDA as "the amount of a food additive expressed over a body weight basis that can be consumed daily over a lifetime without appreciable health risk" a lifetime of saccharin consumption could have serious and potentially irreversible metabolic consequences, given the results obtained by Suez et. al in only 7 days. While ironic considering saccharin was first developed to manage DM, these results clearly demonstrate the enhanced risk of NAS consumption, especially precautioning against saccharin usage.

However, more natural non-caloric sweetener options exist. While only recently incorporated in the diets of developed countries, the plant *Stevia rebaudiana* Bertoni has been by indigenous South American populations for centuries as a food sweetener and medicine, touted for its antibacterial and antifungal properties [63] [64]. Naturally non-caloric, the leaves of the plant are extracted for commercial use, and while four major and six less prevalent steviol glycosides have been isolated, the most abundant in commercial use are stevioside and rebaudioside A [65] [66]. However all glycosides contain a common chemical core, diterpene steviol, which is the final product of their eventual metabolism in the colon [67]. Beyond its use a sweetener, research has shown stevia glycosides may stimulate pancreatic ß-cell activity [68] and regulate glycemic control through improving glucose tolerance [69] [70] [71] [72] [73] [74] and insulin sensitivity [75] [76] [77] potentially by acting as an exogenous insulin mimetic [78]. Stevia may then be primed to both actively reduce the metabolic syndrome, which other NAS have contributed to while also filling the role of a non-caloric sweetener to assist with weight management. However, the effect of stevia on the microbiome remains unknown. Given the

effect of both diet and NAS consumption on shaping the microbiome, and the deleterious effects such alterations can pose to an individual's health, further investigation into the effect of stevia on the microbiome is warranted to characterize the effect of chronic stevia consumption on the gut microbiome and to characterize these changes with corresponding shifts in glycemic response.

This study seeks to characterize the changes in the microbiome and corresponding physiological changes of chronic stevia exposure in C57BL/6J mice to determine if a commercially-available steviol glycoside product can correct high-fat diet induced glucose intolerance and impaired insulin sensitivity through demonstrable shifts in the microbiome.

Materials and Methods

We created four treatment groups, which were fed a mixture of diets and NAS for 10 weeks, a period of time during which we monitored body weight, liquid consumption, caloric consumption, and glucose tolerance were measured. We also collected fecal samples and analyzed them for microbiome composition.

Animals

We randomly assigned male C57BL/6J (n=20) and female (n=20) mice (unblinded) to one of four treatment groups with 10 animals per treatment group (5 males, 5 females). Of these four treatment groups, three served as controls. These included a group consuming LF food and drinking water (LF), a group consuming HF food and drinking water (HF), a group consuming HF diet and drinking a saccharin solution (saccharin). Our experimental group consumed a HF diet and a stevia solution.

Mice were individually housed at 23˚C on a 12 hour light/dark cycle. The high fat food (HF) was from Research Diets, formula D12492. We measured food intake, body weight, and liquid consumption tri-weekly and changed cages weekly. We monitored baseline data (water, BW, and food intake) for eight days prior to day 0, when the HF diet was initiated. After six days on the HF diet, the sweetener protocol began. One female mouse from the stevia group did not adjust to the HF diet and refused food, dying in the third week of treatment. We do not believe this death was caused by the stevia supplementation and this animal was not included in statistical analysis.

Artificial sweeteners

Saccharin (98% purity) obtained from Sigma Aldrich (product code: 1002359797, lot # MKCB3758). Powdered stevia was bought commercially (GreenLeaf brand, lot #6060417). Sweetener solutions were changed weekly and dosage was recalculated by group to reflect changes in body weight. Control water was measured to account for liquid lost due to handling and was factored into the dosage. Five empty cages were set up and the water was weighed as though there was an animal in the cage each time the true treatment group's water was measured. Therefore, we were able to account for water lost solely due to handling as any difference in the weight of these bottles could be attributed to evaporation and handling. This amount was averaged between the empty cages to calculate the average water loss, which was subtracted from the liquid weight of the treatment groups to determine the amount lost solely to drinking. The dosage was calculated to reflect the United States Federal Drug Administration's (FDA) allowable daily intake (ADI), which was used by the Suez et al. protocol [79]. These dosages were calculated by group and were recalculated each week to reflect changes in weight gain and liquid consumption. The ADI is 5mg/kg per day so this was scaled to a mouse weight by multiplying 5mg/kg*day by the average mouse weight of each group, which was divided by the average daily liquid intake of the group.

Glucose tolerance

Glucose tolerance tests were conducted before and after treatment. 10 animals were measured initially to establish a baseline area under the curve (AUC) and all animals were tested post-treatment. AUC was calculated by adding two consecutive data points, dividing by two and multiplying by the difference in time in Excel per the formula ((B1+B2)/2)*(A2-A1), where column A contains the time in minutes and column B contains the blood glucose

measurement at that time. On the day of the test, a fresh solution of glucose was prepared using 2 g glucose dissolved in a final volume of 10mL of sterile saline. Animals were weighed and then fasted 6 hours during their light phase in a fresh cage with water (or sweetener solution) ad libitum. Following the fast, animals tails were nipped with a surgical blade and a drop of blood placed on a glucometer test strip [Novamax, product code #3] to obtain the fasting blood glucose concentration prior to injection. Animals were then injected intraperitoneally with 1mg glucose per kg body weight with a typical volume of 0.3cc in a 30 gram mouse. Blood glucose was measured with the glucometer at 0, 15, 30, 60, 90, 120 minutes in the initial group and at 0, 20, 40, 60, 90, 120 minutes post-treatment as the high volume of animals during the second testing made testing take longer so a longer time interval was needed to get through all the animals before the next round could begin. Mice were returned to their treatment diet following testing. Serum blood glucose was measured on week 4 and 8 to ensure treatment was having a noticeable effect by increasing blood glucose concentrations [Table 1].

Taxonomic microbiota analysis

Fecal samples were collected from cages at day 0 of treatment (14 days after animals had been established in our facility), at the midpoint (week 6) and at end of treatment (10 weeks of treatment) using forceps to remove pellets from the bottom of cages. Samples were stored in 1.5mL microcentrifuge tubes in a -80˚C freezer until processing. Only initial and final samples were sequenced due to cost. Sample were processed frozen using the QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen). DNA concentrations were confirmed with a NanoDrop which measures the absorbance of the sample at 260nm and

280nm. These absorbances create a ratio which serves as a proxy for DNA purity, as DNA is considered pure with a 260/280 ratio between 1.8-2.0. Twenty-microliter aliquots of purified DNA were shipped to Edna Chiang at University of Wisconsin-Madison. Samples were diluted to a concentration of 5ng/uL and 5uL of sample were used for PCR for a total of 25ng DNA used in PCR amplification of the 16s V4 region (~380bp amplicons) and subsequent Illumina MiSeq sequencing of paired 250bp. Reads were normalized to 1907 reads per sample. Three samples failed to amplify in PCR and were excluded from analysis (SC4C, TV2C, and HF8A which correspond to the end point samples of Sacc4 and Stev2 and the initial sample for HF8). Reads were processed using the mother pipeline using an OTU-based approach to generate OTU and taxonomy tables. Samples were grouped by time and treatment so each group had an initial sample and a final sample.

Samples were analyzed for differences in alpha and beta diversity. There are two main metrics typically employed to analyze alpha-diversity: Chao and Shannon indices. Chao is an index of the predicted number of taxa in a sample and measures sample richness while also attempting to take into account absent species which may have been missed in the actual sample. At lower read counts, the absence of a taxa is often due to the fact that the taxa was not picked up by the OTU clustering, rather than that the species itself wasn't actually present in the sample. The Chao index attempts to normalize for this discrepancy.

To examine differences in microbiota composition between treatment groups, we measured beta-diversity which compares similarity of samples between samples. Each animal can be compared to every other animal based on a set distance in order to generate an ordination plot which compares the percent variation between samples. This allowed us to

quantify the similarity between samples. Each symbol on the plots represents the total microbial community of that animal. Symbols closer together have more similarly microbiota composition than those farther apart. Beta diversity was measured using two methods which employ different metrics. The Bray-Curtis index is a principal coordinate analysis system which compares pairwise dissimilarity between two points using a rank-based approach to measure beta-diversity.

Statistics

Physiologic data was evaluated using SPSS with a 1x4 ANOVA followed by LSD alpha post-hoc test. Error bars represent standard error and p-values are reported in the figure legend. Analysis and statistics for microbiome data was conducted in R (v. 3.4.4). Mann-Whitney Wilcoxon tests were used to compare correlations of treatment and overall OTU count at a genus level within and between treatment groups to calculate significance levels and difference in means. ANOVA was used to evaluate Chao and PERMANOVA (a non-parametric version of ANOVA) was performed to evaluate interactions between group and time for Bray-Curtis and UniFrac. Subsequent paired or unpaired Student's *t*-tests were conducted to determine which group and time interactions could be attributed to which treatments. Hotelling's multivariate *t*-test was used to do post-hoc analysis on PERMANOVA results from Bray-Curtis indices. $P < 0.05$ was considered significant in all analyses.

Results

Physiology

Physiologic indicators were measured over the course of treatment to track weight gain, caloric consumption, and liquid consumption. Diet was the strongest driver of weight gain, with all mice on a HF diet gaining similar amounts of weight [Figure 1a, b]. LF animals had significantly lower body weight ($p<0.0001$, 0.003 for males and females respectively).

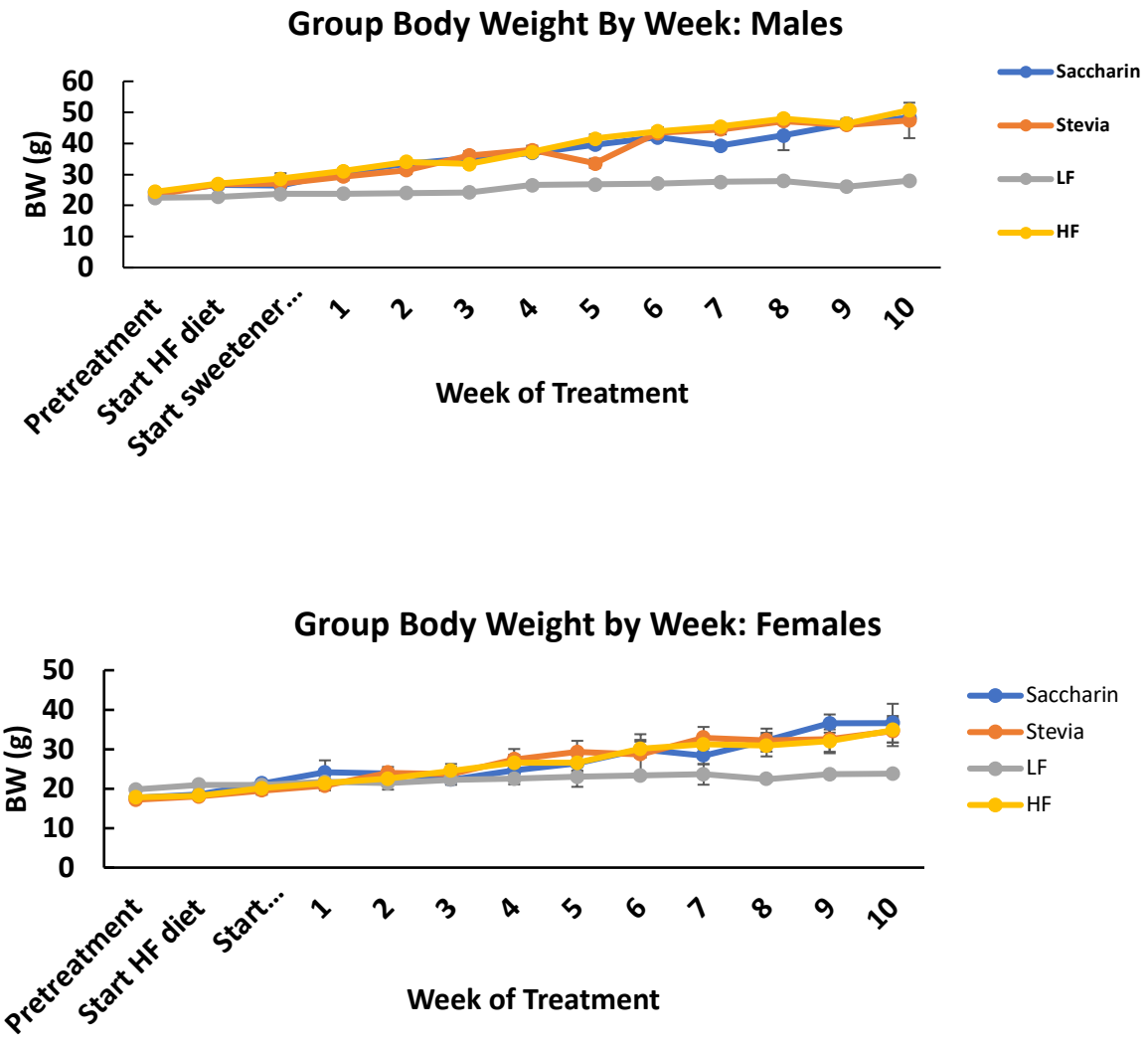

Figure 1a, b. *Both LF males and females had significantly lower BW ($p<0.000$, 0.003). No significant difference observed between HF groups.*

There was also no difference in liquid consumption between groups on HF diet, though the LF diet group consumed significantly more water ($p<0001$). The increase in water consumption by the LF group is likely due to the higher fiber content of their chow. Similar liquid consumption by groups on HF diet suggests sweeteners do not influence drinking behavior to make sweetened solutions more or less palatable as both saccharin and stevia drank similar amounts as the HF group.

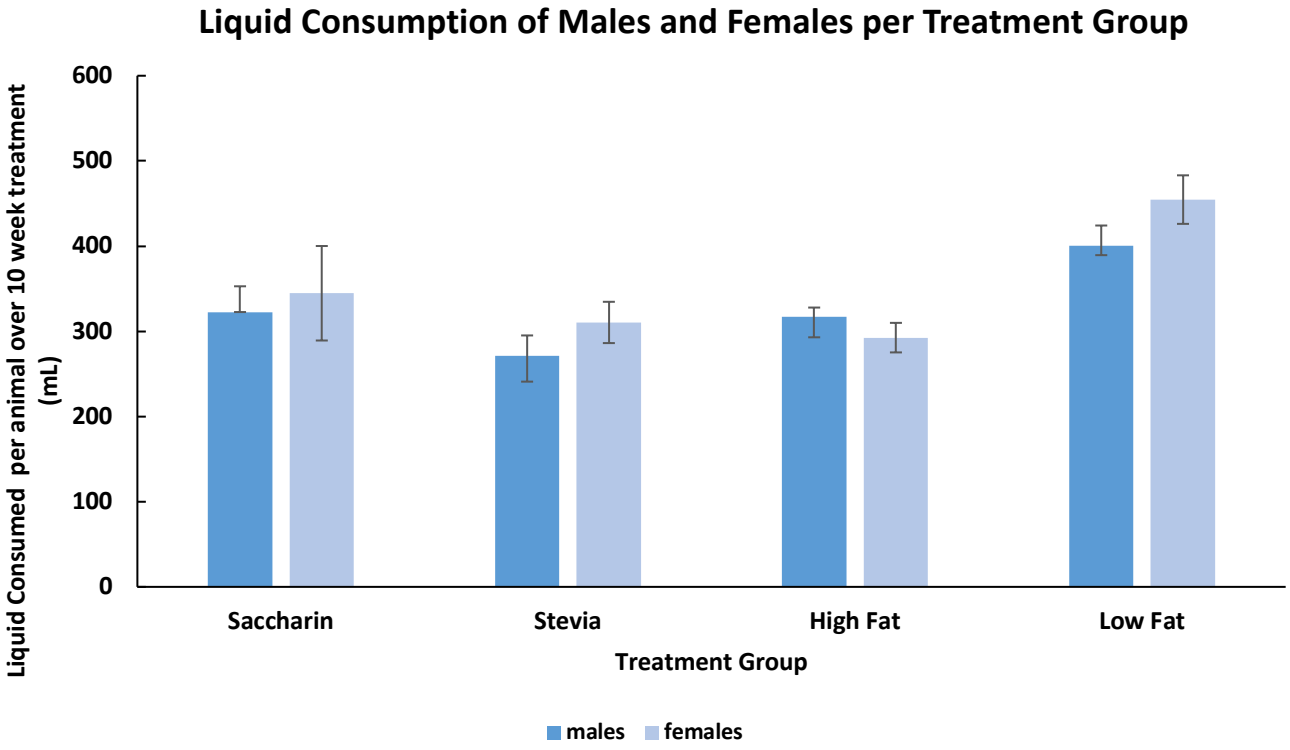

Figure 2. *Sweetener supplementation did not change liquid consumption. LF animals consumed significantly more water ($p<0.0001$) over the course of the 10 week treatment period than the HF groups.*

There was no difference in total caloric intake between animals on HF diet [Figure 3]. Over 10 weeks of sweetener treatment, stevia mice ate an average of 1,244±44 kcalories per animal, however this was not significantly less than other animals on a HF diet, with HF animals

eating on average 1,312±140 kcalories and saccharin eating 1,309±203 kcalories. There was also no difference between males and females in caloric intake between groups on HF diet. The LF animals consumed significantly fewer kcalories than all groups on HF diet, consuming an average of 857± 45 kcalories per animal over the course of treatment ($p<0.000$).

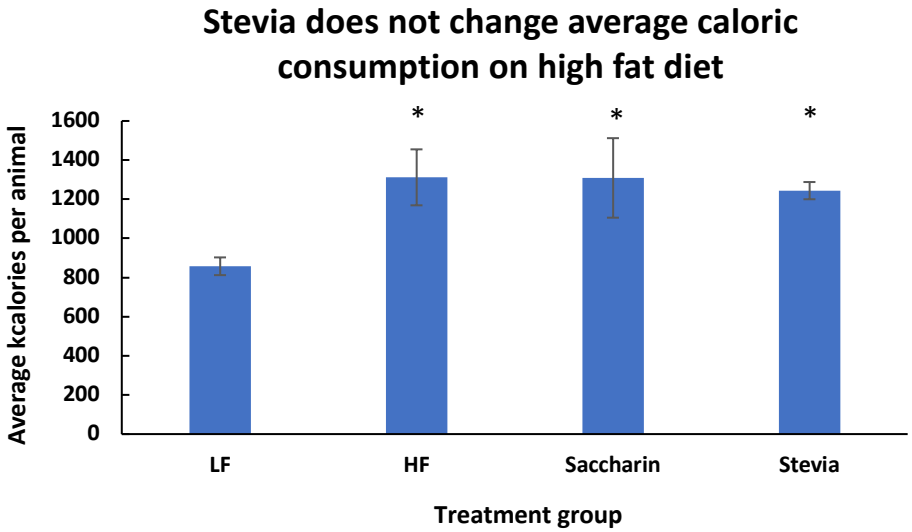

Figure 3. *The average caloric intake over 10 week treatment indicates sweetener addition did not significantly effect kcalorie consumption vs. HF diet. Graph displays aggregate data of both males and females.*
** indicates $p<0.05$ vs LF.*

To generate the initial glucose tolerance curve and establish a baseline to be compared to post-treatment, glucose tolerance tests were conducted before sweetener additions. Two animals from each treatment group were selected initially when all animals were still on a LF diet and glucose tolerance tests were conducted to generate a baseline curve.

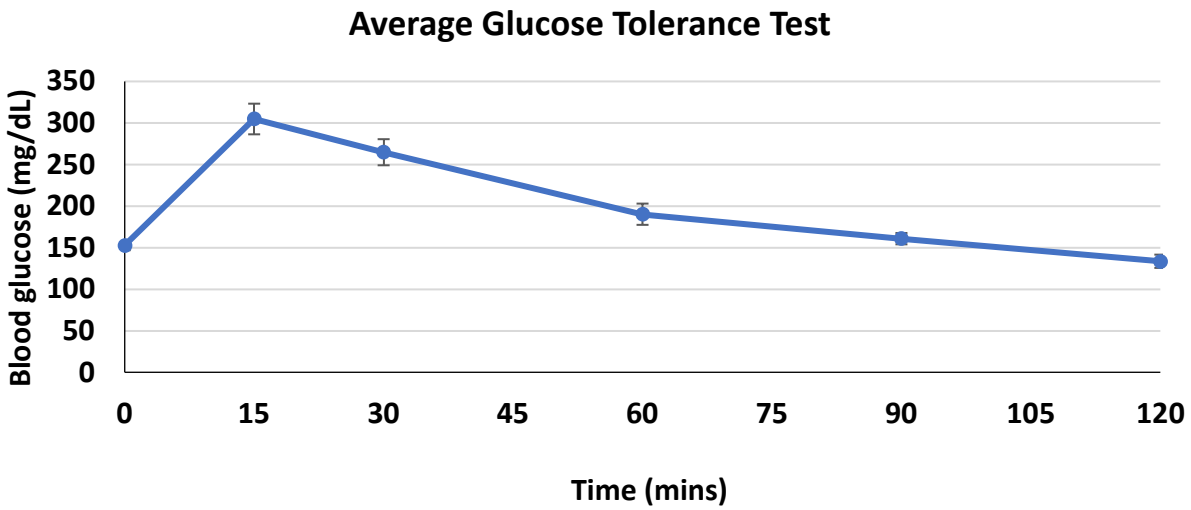

Figure 4. *Glucose tolerance test of initial animals (males and females), all on LF diet. Representative of a standard glucose tolerance test. Used to calculate area under the curve (AUC).*

Blood glucose measurements were also taken at week 4 and week 8 to monitor response to the HF diet and to ensure serum glucose levels were increasing due to treatment. The HF diet increased serum glucose concentration compared to LF animals [Table 1].

Table 1. *Blood glucose concentrations (mg/dL) over course of treatment. LF does not change, while there is an increase in all groups on HF diet. The difference between the groups was not significant. All animals were on LF diet at start, and have been consolidated into one data point.*

	Week 0	Week 4	Week 8	Week 10
LF	140 +/- 5.7	137 +/- 9.0	146 +/- 4.9	152 +/- 7.9
HF		189 +/- 14	170 +/- 6.4	201 +/- 9.0
Sacc		176 +/- 14	181 +/- 10	205 +/- 11
Stevia		170 +/- 10	191 +/- 9.0	216 +/- 11

Following treatment, glucose tolerance tests were conducted on all animals. Area under the curve (AUC) analysis was used to quantify the glucose response. Stevia did not have a

significant effect on glucose response as measured by the area under the curve of a glucose tolerance test.

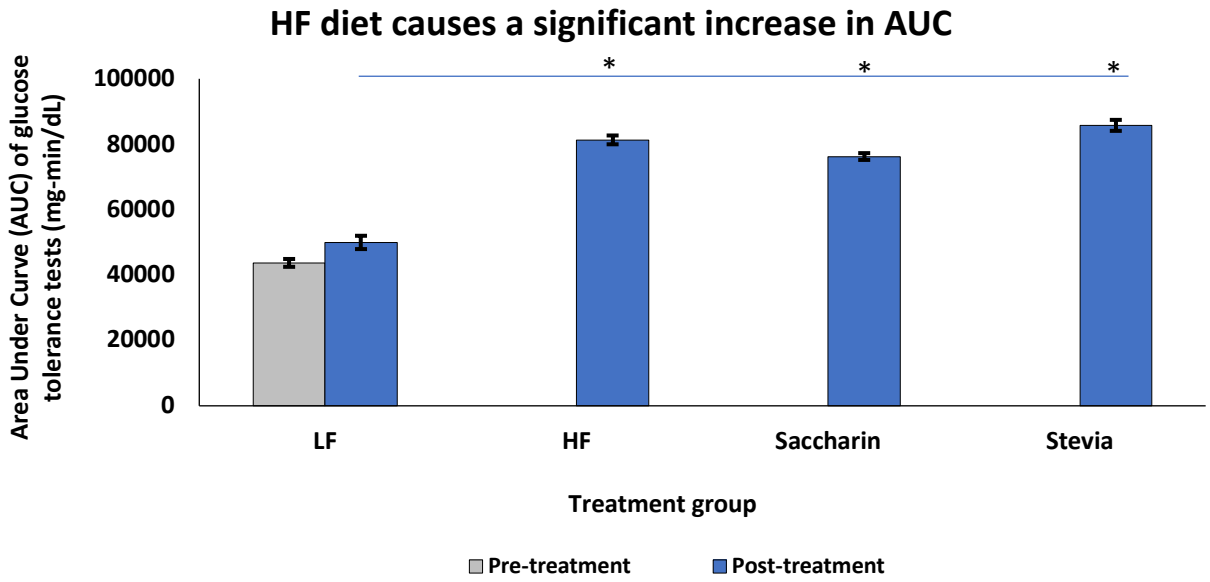

Figure 5. *Area under the curve (AUC) analysis of GTTs. Stevia did not significantly affect AUC vs HF, though LF was significantly lower than all other groups (p<0.000 males, 0.003 females).*

There was no difference in AUC between baseline and the LF group post-treatment, however there was a significant difference between LF and all groups on HF diet (p<0.000 males, 0.003 females). Sweetener treatment did not affect glucose tolerance, with no difference between HF, Saccharin, and Stevia in AUC following (81,300 ±3300, 76,200 ±2800, and 85,800 ±3400, respectively). Therefore, stevia did not rescue high fat diet-induced glucose intolerance or reduce caloric intake, weight gain, or liquid consumption.

Microbiology

Overall differences in abundance

There are a variety of perspectives from which to view changes in microbial composition due to treatment, and several strategies were employed to evaluate the changes in the microbiome due to stevia consumption in this study. Marker gene sequences from 16S rRNA cluster based on their similarity to one another which comprise operational taxonomic units (OTU), a proxy for quantifying taxa at various levels in order to classify bacteria. OTU abundance was analyzed at several different taxonomic ranks to compare the effect of treatment. Several differences in mean abundance were observed between groups at the family level following treatment [Figure 6].

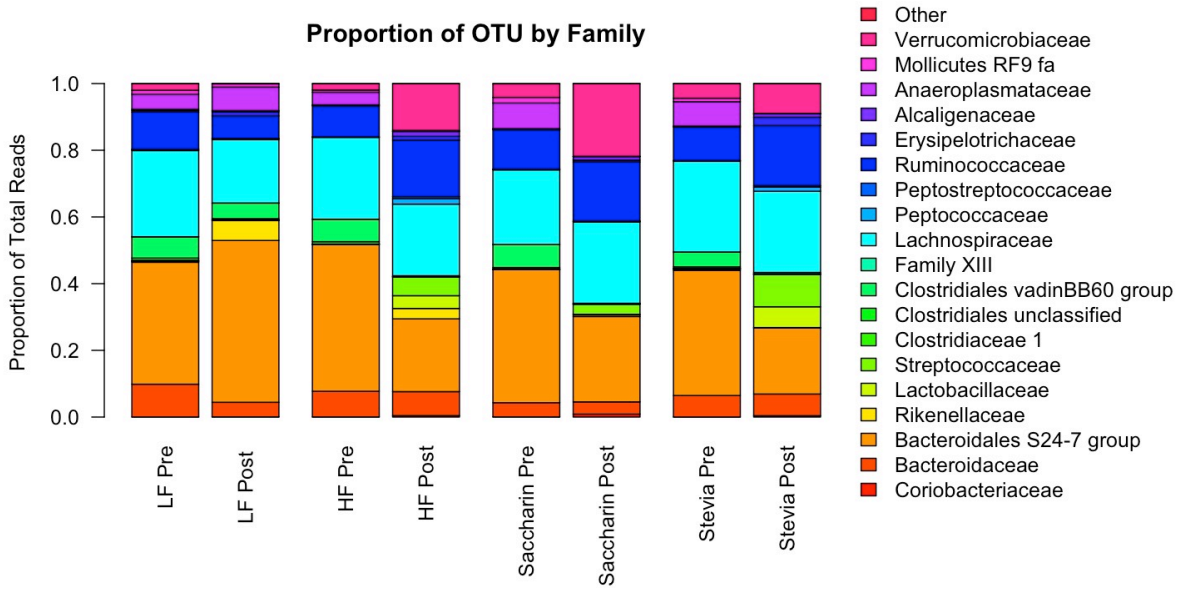

Figure 6. *Relative abundances based on OTU counts of experimental groups before and after treatment at the family level.*

These differences in OTU were further quantified at the genus level and several significant differences were found between groups [See supplemental materials for table with

family percentages and genus calculations]. While all groups on HF diet had similar changes in OTU counts over the course of treatment, saccharin showed a significant increase in genera *Lactobacillus* (p=0.029) and *Streptococcus* (p=0.034) over the course of treatment which was not observed in HF or stevia. Differences due to treatment were most common among *Ruminoccoccae* and *Clostridiales* families among all HF groups. Some changes in these families were specific to just the stevia and HF treatment groups including an increase in genera *Intestinimonas* (p=0.021 HF, 0.036 stevia), *Ruminococcaceae* (p=0.02 HF, 0.016 stevia), *Ruminioccocus*_1 (p=0.014 HF, 0.022 stevia), and *Erysipelatoclostridium* (p=0.014 HF, 0.051 stevia).

Decreases in several genera were also observed just among stevia and HF groups, including a decrease in *Aneraeroplasma* (p=0.0039 HF, 0.0078 stevia) and a decrease in *Mollicutes_RF9_ge* (p=0.039 HF, 0.0078 stevia). A decrease in OTU was also observed in *Oscillibacter* (p=0.015) in HF. While the OTU count of *Oscillibacter* in stevia did not increase significantly over the course of treatment, there was an increase as measured by difference in mean while the difference in mean of saccharin, in contrast, increased its difference in mean OTU count of *Oscillibacter* over treatment. The only genera whose OTU count changed during treatment in the stevia group and not in the HF group was in the *Lachnospiraceae* family, genus *Coproccus*_1 (p=0.016).

These distinct changes specific to stevia and to stevia and HF alone suggest there are distinct microbiome profiles for each sweetener beyond changes due simply to a HF diet, and that stevia may not be altering OTU count as strongly as saccharin which produced much more distinct changes in OTU abundance due to treatment.

Alpha diversity

To further explore the effect of stevia on the microbiome, alpha-diversity metrics were evaluated first using the Chao index of species richness.

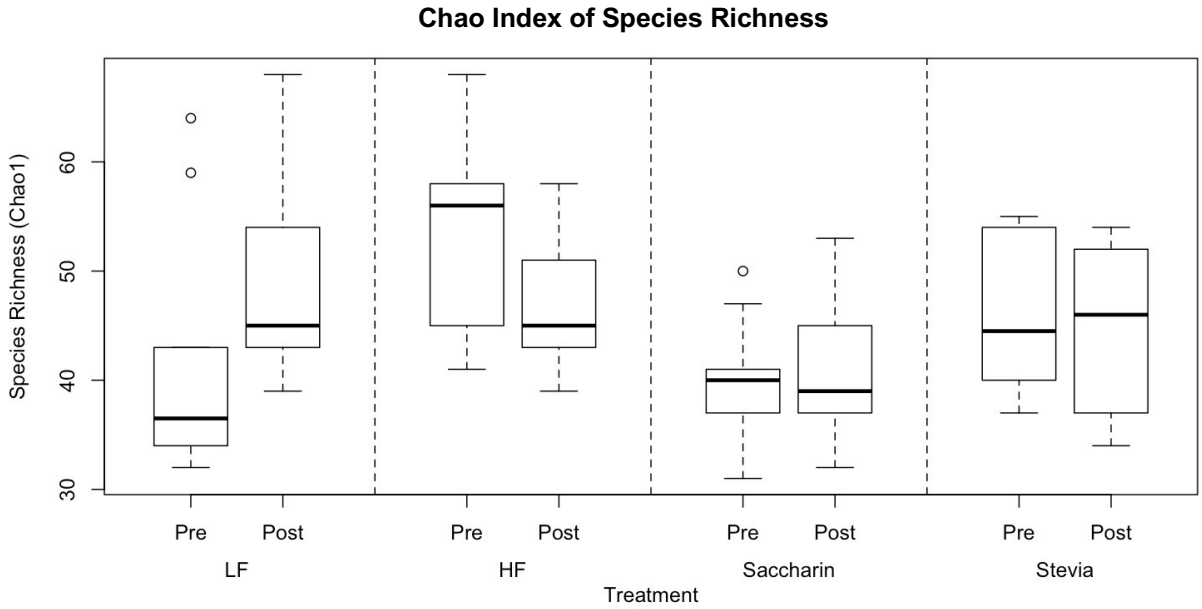

Figure 7*. Chao index of treatment groups before (pre) and after treatment. No significant differences within or between groups.*

No significant differences were observed within groups due to treatment, but there was a significant difference in groups initially (p=0.0061). Over the course of treatment, this difference in richness dissipated as there was no significant difference between groups at the end of treatment.

However, the Chao index is an imperfect model of bacterial populations and ignores species evenness. This necessitates use of the Shannon index which is a more comprehensive metric as it takes into consideration both richness and evenness of taxa present in a sample and is more sensitive to rare OTU [Figure 8]. Shannon's index is a measure of "information content"

and has a minimum of 0 when there is only one species present and a maximum, equal to ln(richness) when all species are present in the same proportion.

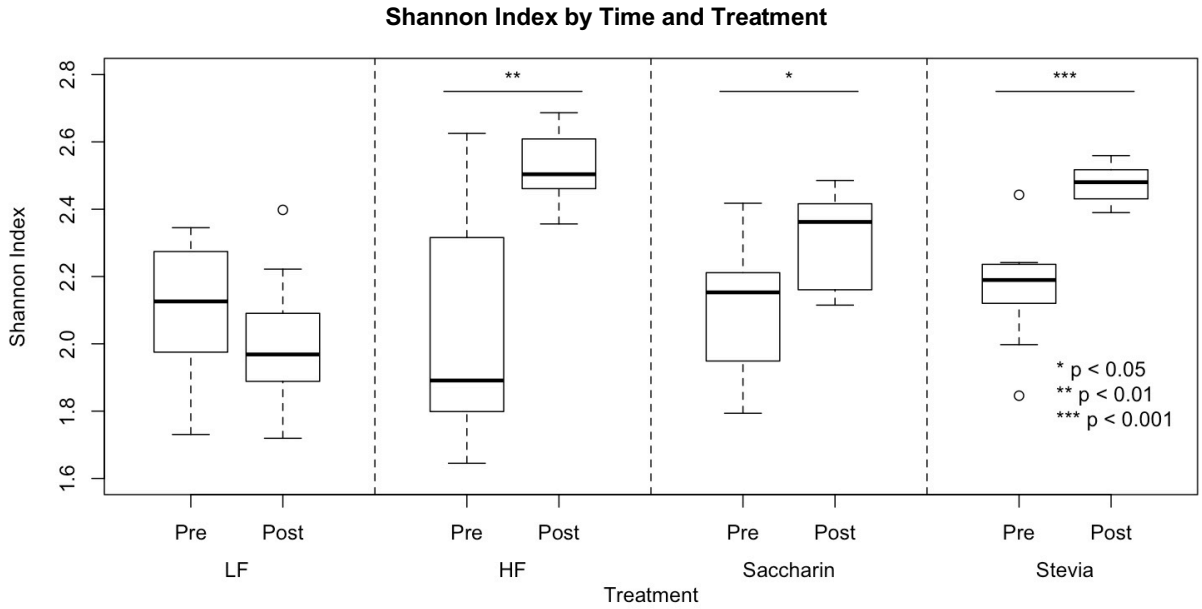

Figure 8. *Shannon index, a measure of richness (number of species present in a sample) and evenness (distribution of these samples based on relative abundance) in a sample which is used to evaluate alpha-diversity. A higher Shannon value indicates a greater degree of diversity. Each group has two time points: pre and post treatment. HF p=0.001, saccharin p=0.016, stevia p=0.0001).*

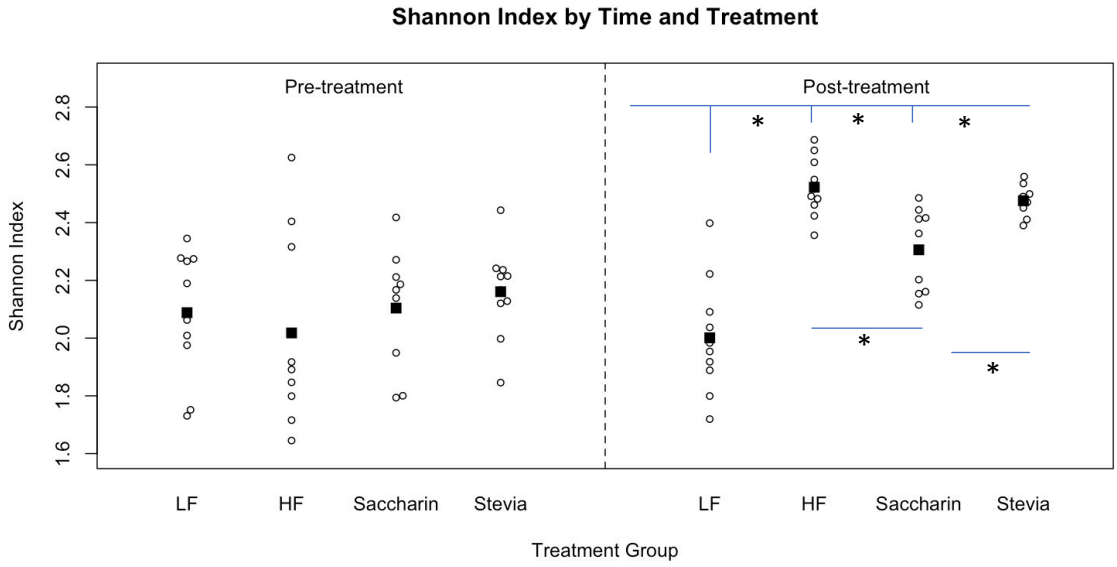

Figure 9*. Beeswarm plot of Shannon index. HF, saccharin, and stevia significantly different than LF (p<0.00). HF and stevia significantly different than saccharin (p=0.002 HF, 0.008 stevia). * indicates p-value <0.05.*

No initial differences were observed between treatment groups, however HF, saccharin, and stevia showed a significant difference in their Shannon indices over the course of treatment ($p=0.001$ HF, 0.016 saccharin, and 0.0001 stevia) [Figure 8]. At the end of treatment, all groups on HF diet were significantly different than LF ($p<0.001$) and there was no difference between HF and stevia [Figure 9]. However, saccharin was significantly different from both HF and stevia ($p=0.002$ HF, 0.008 stevia). Therefore, saccharin exerts an effect on diversity not observed in stevia or HF as there was no difference between stevia and HF at the end of treatment. This data suggests saccharin has a significant effect on alpha diversity not observed in stevia or HF.

Beta diversity

Beta diversity was analyzed by two metrics: Bray-Curtis distances and weighted UniFrac distances. PERMANOVA analysis indicated a significant impact of treatment on both group and time, with the interaction of group and time highly significant ($p=0.004$). Initially, animals are clustered together, showing there is little diversity between groups prior to treatment [Figures 10, 12]. However, group has a significant effect on diversity following treatment in both males and females ($p=0.021$, 0.007). While much of this difference is largely due to the distinct clustering of groups on a HF diet and is thus likely driven by diet [Figures 11, 13], PERMANOVA analysis of just HF, saccharin, and stevia groups following treatment indicated a highly significant interaction of group ($p=0.0009$). Subsequent Hotelling's multivariate t-tests comparing these groups at the end of treatment did not show significance except for a trend toward significance in males between HF and saccharin ($p=0.054$). Therefore, a weighted

UniFrac was performed to further evaluate the differences in beta-diversity present between all groups on a HF diet.

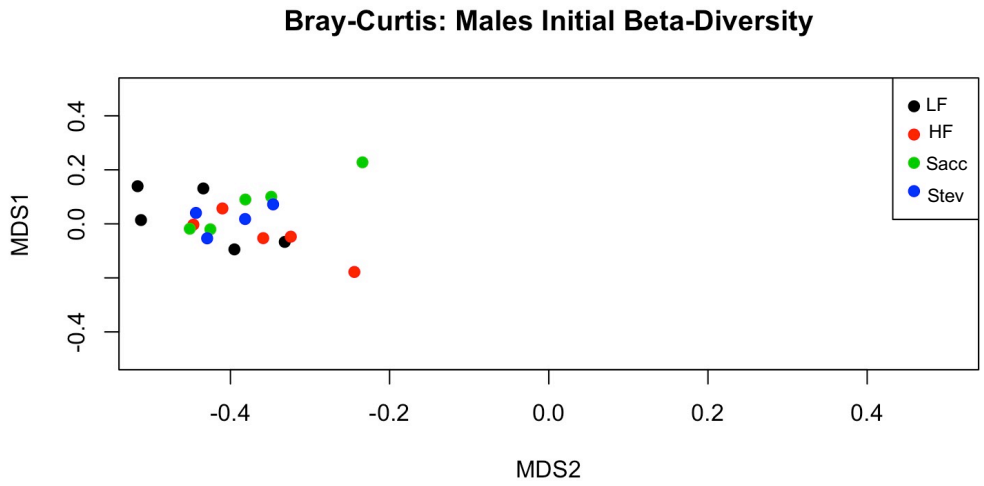

Figure 10. *Bray-Curtis plot of males at the beginning of treatment. No difference between treatment groups.*

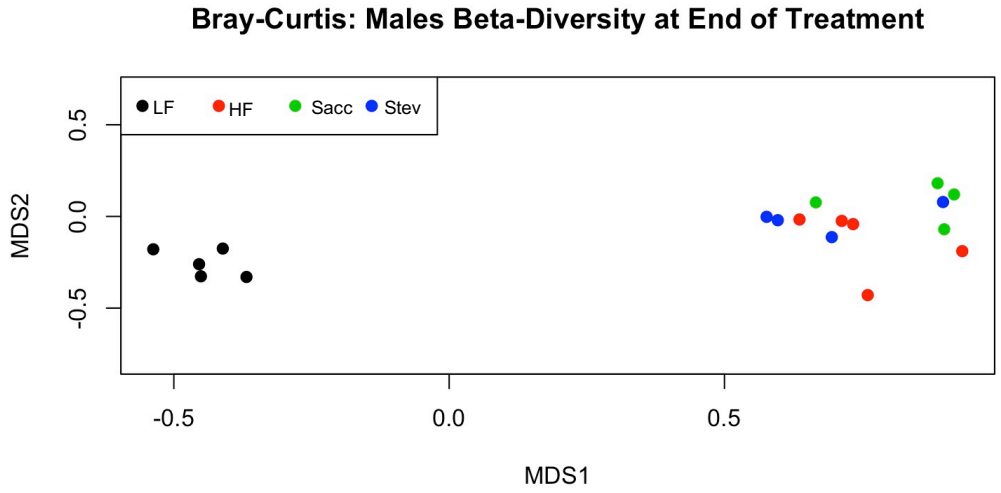

Figure 11. *Bray-Curtis of males at end of sweetener treatment. Groups have clustered based on treatment, with most of the variation coming from the interaction of group and time (p=0.004). The HF and Saccharin groups display a trend toward a difference in beta diversity (p=0.054)).*

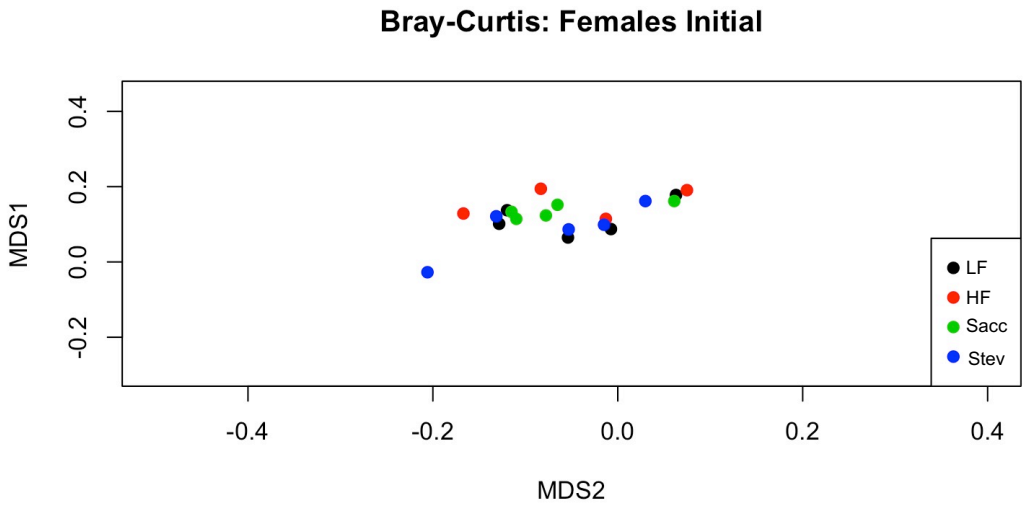

Figure 12. *Bray-Curtis plot of females at the beginning of treatment. No difference between treatment groups.*

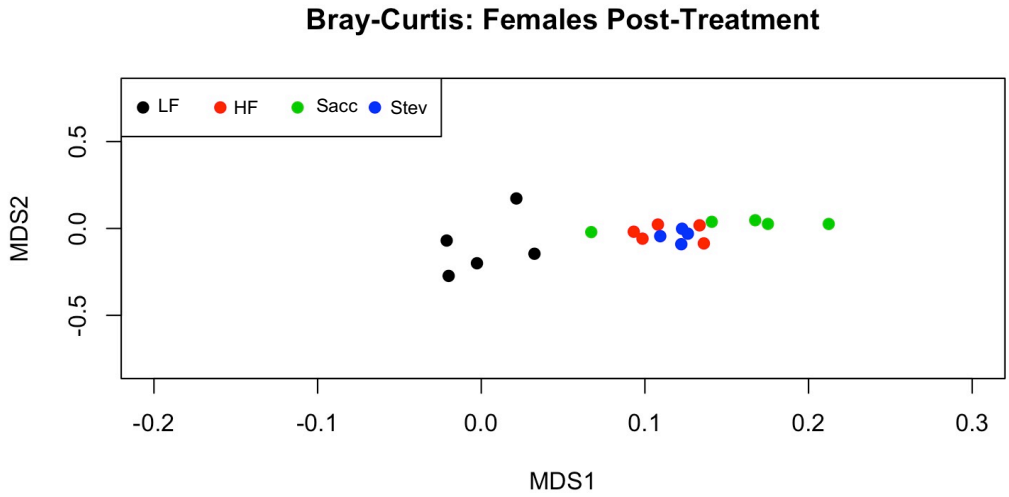

Figure 13. *Bray-Curtis plot of females at end of treatment. LF is significantly different than HF, saccharin, and stevia (p=0.007). No difference between treatment group on HF diets.*

UniFrac is another scaling system which evaluates the phylogenetic distance between microbial communities using both the presence and abundance of OTU and the relative abundance to calculate distances for the principal coordinate analysis. As phylogeny and

abundance are both taken into consideration, a weighted UniFrac evaluates the relationship of taxa to each other and for this reason is more widely used in the microbiome community. Like in the Bray-Curtis, initially all treatment groups were clustered together, indicating there was no difference in beta-diversity which was corroborated by statistical testing [Figures 14, 16]. Following treatment, there was a significant effect of treatment on clustering with the LF group and HF, saccharin, and stevia clustering separately in both males and females (p=0.029, 0.006) [Figures 15, 17].

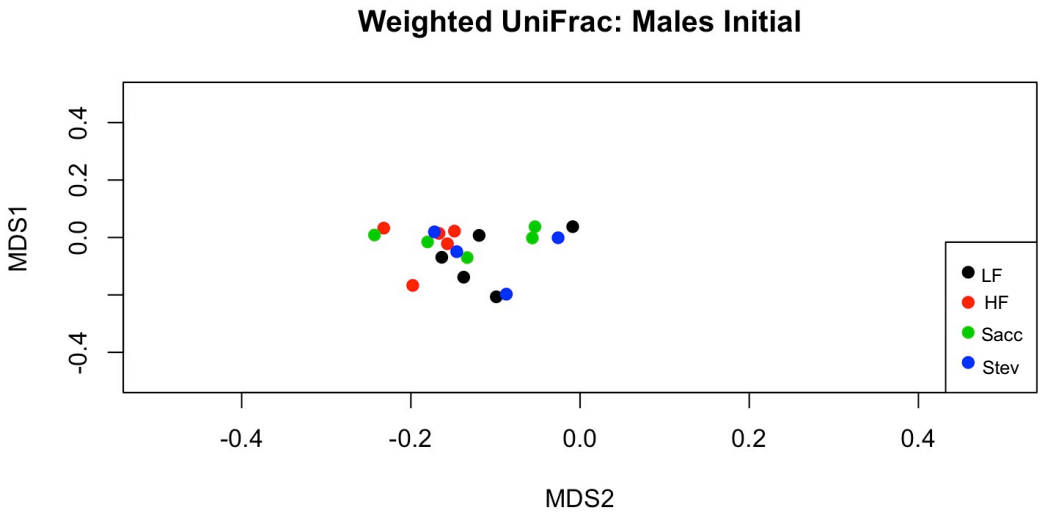

Figure 14. *Weighted UniFrac of males prior to treatment. No difference between groups.*

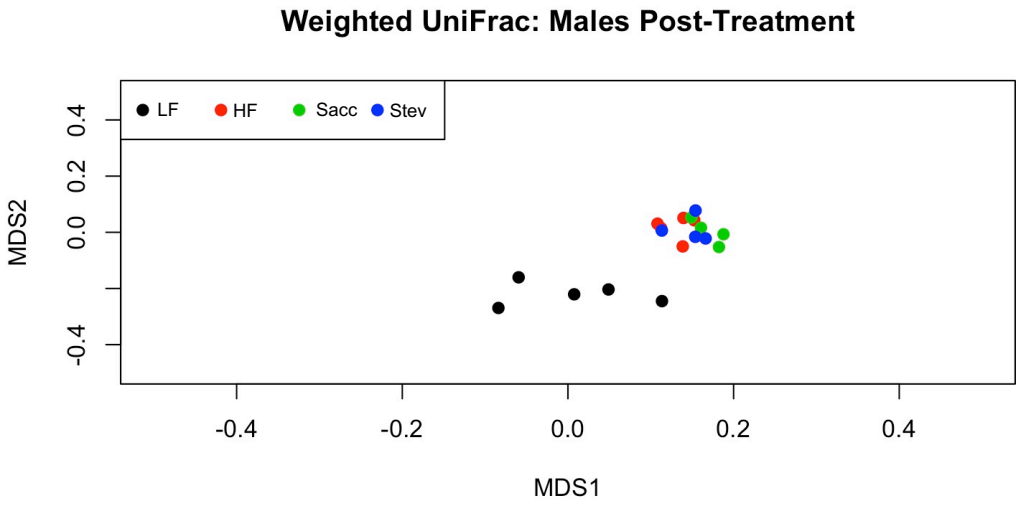

Figure 15. *Weighted UniFrac of males after treatment. LF is significantly different than HF, saccharin, and stevia (p=0.029).*

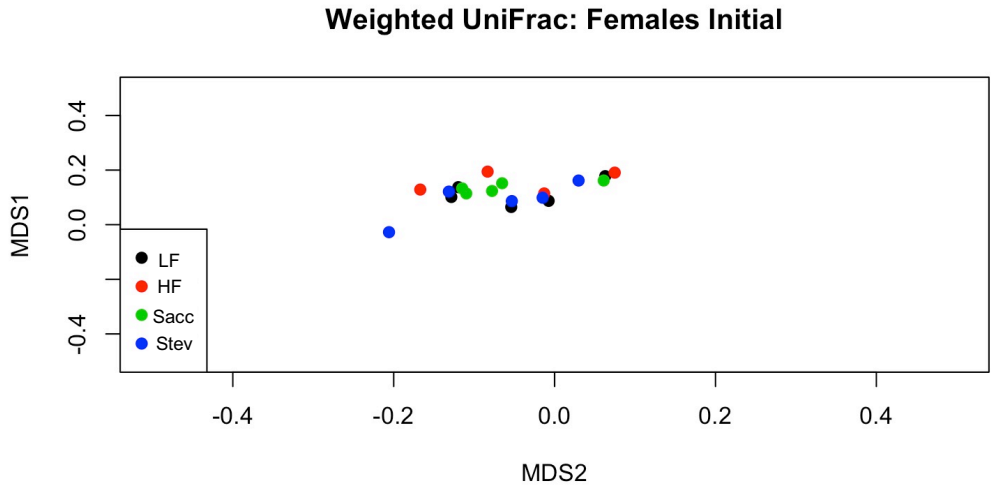

Figure 16. *Weighted UniFrac of females prior to treatment. No difference between groups.*

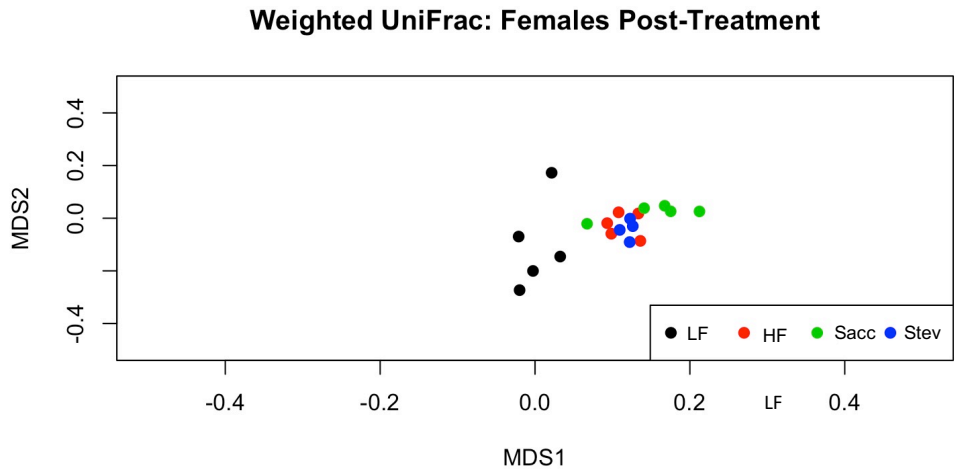

Figure 17. *Weighted UniFrac of females after treatment. LF is significantly different than HF, saccharin, and stevia (p=0.006). No difference between groups on HF diet.*

However, this difference seems to be largely driven by diet as there was no significant difference between groups on HF diet at the end of treatment. Therefore, it seems while alpha-diversity and OTU abundance are more affected by sweetener addition, the primary driver of beta-diversity is the HF diet. This may be due to relative proportions of sweetener vs. HF food in the animals' diets as they consumed a much higher percentage of food than liquid and so it would understandably exert a stronger role on the composition and structure of the microbiome.

Discussion

This study presents the first characterization of the effects of stevia on the gut microbiome, suggesting stevia consequentially affects microbiota composition by altering alpha-diversity, but perhaps less consequentially than saccharin. The differences in alpha-diversity largely map those of the HF group, suggesting the changes induced by stevia may not be as severe as those induced by saccharin. This is supported by the changes in OTU count following treatment which are largely homologous between HF and stevia, with saccharin not displaying the same differences in OTU as HF and stevia. At a family level, HF and stevia showed abundant differences in *Ruminoccaceae* and *Clostridiales* which have both been associated with a HF diet [80]. In humans, the *Clostridiales* order is known to be associated with T2D [81], however the *Clostridiales* order is very diverse and reference genomes are thought to not be specific enough to account for the diversity of this order. Thus, it is possible differences in this order may not have been classified in this study and changes due to treatment may have been lost. *Ruminoccaceae* have been associated with a HF diet [82], and are thought to be implicated in intestinal cell health, possibly acting to protect epithelial cells from the stress of a HF diet by triggering a protein through their production of the short-chain fatty acid butyrate [83, 84].

The only genera whose OTU count changed during treatment in the stevia group and not in the HF group was in the *Lachnospiraceae* family, genus *Coproccus*. While little is known about the specific functional effect of genus *Coproccus*, there is some indication it may be correlated with increased lipid levels [85] and the family *Lachnospiraceae* are associated with a HF diet [81]. Increases in species *Lactobacillus* and *Streptococcus mutans,* as observed in the saccharin group, are also associated with T2D [81]. Though the lowest taxon rank identified in

this study was at the genus level, we did demonstrate significant differences in the saccharin group alone on *Lactobacillus* and *Streptococcus*, with significant increases in both genera suggesting there are likely significant differences at a species level as well.

Therefore, the changes observed in OTU count in both HF and stevia are likely due to the effect of the HF diet rather than an independent effect of stevia while saccharin may be exerting a more metabolically consequential effect on the microbiome. This positions stevia as a potentially beneficial sweetener alternative as it appears to not exert as detrimental effects on the microbiome as saccharin. However, stevia was not able to reduce glucose intolerance or weight gain, both of which contribute to numerous metabolic syndromes. Suez et al. did not report measures of alpha diversity so it is difficult to compare our results and explore the effect of alpha vs. beta-diversity of saccharin vs. stevia.

While these results are promising, further research should be conducted before translating the findings of this research to humans to better understand the functional consequences of stevia on the microbiome and how it may be acting on a metagenomics level as compared to HF and LF. It is interesting that there was no difference in glucose tolerance observed between the three groups on HF diet, as Suez et al. previously demonstrated a significant difference in AUC between HF and saccharin [1]. Furthermore, we did not observe a difference in beta-diversity between HF, saccharin, and stevia following treatment [1]. This discrepancy may reflect insufficient dosing of animals due to limitations of our study design as we changed sweetener dosages weekly and calculated concentrations based off group body weight and liquid consumption averages rather than every three days and by each individual animal's weight and liquid intake per the Suez protocol.

However, our findings are also reflective of the current findings in the field. There is mixed evidence on the glycemic effects of artificial sweeteners, including stevia. In a review authored by Suez himself, he acknowledges that rodent and human models have produced mixed results, with saccharin shown to lead to increased weight gain by some studies while others report saccharin can relieve weight gain and exert anti-hyperglycemic effects in both diet [86] and genetically induced models of obesity [87]. These differences are attributed to differences in methodology as well as differences in facility environment which may have contributed to differences in microbiome composition and function, thus leading to the differences observed in results [88]. While it seems clear the microbiome is an important regulator of glucose metabolism, NAS have been shown to have varying results on glucose tolerance. Therefore, given the wide consumption of NAS products, further testing of both products is necessary to ensure consumer safety and health as well as to validate the relative role of the microbiome in such result discrepancy. The results of our study support stevia's inclusion in further NAS research as it may present a neutral alternative to other NAS products by not affecting species diversity and richness as severely as saccharin, positioning stevia as a beneficial alternative to traditional artificial sweetener products for those managing or predisposed to metabolic syndrome and T2D. However, future research should employ a functional genomics and metabolomics approach to understand the functional and systemic consequences stevia may be exerting on glucose metabolism.

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