

Artificial Sweetener Consumption Increasing Type II Diabetes Risk Revealed by Gut Microbiome

Peizhi Li

Shanghai Starriver Bilingual School, Shanghai, China

Abstract. Artificial sweeteners, low-caloric sugar substitutes, are widely applied in modern food industry and regularly consumed by people. However, recent studies have shown the increasing disease risk by consuming artificial sweeteners, especially metabolic diseases. In this study, we investigated the effect of three artificial sweeteners, namely, saccharin, acesulfame-potassium, and stevia on Type II diabetes risk by gut microbiome. The study utilized the 16S rRNA gut microbiome data from rat fecal samples to analyze the gut microbiome abundance, composition, and difference between four groups, i.e., acesulfame-potassium, saccharin, stevia, and control group. The most significant gut microbiota changes were identified and used to determine whether the altered bacteria taxa have correlation with glucose intolerance and Type II diabetes. In the end, it is found that the *Faecalibacillus* genus and *Prevotellax* genus have significant changes and are closely related with higher risk of Type II diabetes, suggesting acesulfame-potassium and saccharin consumption may increase diabetic risk via altering gut microbiome, while no bacteria taxa change in stevia group is found to be related with glucose intolerance or Type II diabetes. This gut microbiome-based study revealed the key disrupted gut microbiota by artificial sweeteners that were associated with Type II diabetes.

Keywords: Artificial sweeteners, Microbiome, Saccharin, Acesulfame-potassium, Stevia, Type II diabetes.

1. INTRODUCTION

Artificial sweeteners (AS), represented by polyhydric sugar alcohols including erythritol, xylitol, sorbitol, and several other naturally extracted sweetening compounds like stevia, are metabolized differently by human organs, and thus, provide people sweetness with less unnecessary calories and nutrition [1]. The increasing consumption of artificial sweeteners had raised concerns over whether artificial sweetener intake increases the risk of chronic disease for humans. For instance, through recent immunohistochemical analysis and reassessment, aspartame, one of the widely used artificial sweeteners in the food industry, had demonstrated causal relation with inflammation, tumors, and, ultimately, cancer [2]. Type II diabetes mellitus (T2DM) is a chronic disease that is associated with predominant insulin resistance and a relative deficiency in insulin secretion [3]. It is recognized that higher sugar intake associates with a higher risk of the incidence of T2DM [4].

Currently, a few studies suggested that artificial sweetener consumption correlates with an increasing rate of T2DM, and thus, suggesting artificial sweeteners are not a healthy sugar alternative but a potential risk factor [5]. Nonetheless, the molecular mechanism behind artificial sweetener consumption and diabetes incidence remained unclear, demanding further research. According to research, gut microbiota has close relation with the pathophysiology of most chronic diseases and is found to be associated with T2DM via affecting glucose metabolism [6]. Research had demonstrated that the consumption of artificial sweeteners such as Aspartame, Saccharin, and Sucralose could alter the animal microbiota, and change the subjects' glucose tolerance, adding to the risk of T2DM [7]. Hence, investigating specific artificial sweeteners' impact on gut microbiota could build the connection between artificial sweeteners consumption and a higher risk of T2DM.

For this research, we focus on three commonly used artificial sweeteners, stevia, saccharin, and acesulfame-potassium (Ace-K). Scientists had discovered the negative correlation between saccharin consumption and glycemic control as well as insulin regulatory ability, implying that Saccharin intake increases T2DM risk [8]. A similar result was found for Ace-K as the experiment indicated that long-term consumption of Ace-K within the acceptable daily intake could still increase blood glucose,

glycated hemoglobin, total cholesterol, triglyceride, and other factors associated with T2DM [9]. Although there was a study pointing out the antioxidant function of stevia which can potentially alleviate diabetes [10], we were still taking stevia into account as other research demonstrated that Stevia consumption would impact the gut microbiome, which is a key influencing factor to both artificial sweetener metabolization and T2DM condition [11].

In this study, we used gut microbiome data to analyze artificial sweetener consumption's impact on microbiome diversity and composition, followed by the analysis of glucose tolerance. The data used for analysis were collected from the fecal samples of rats fed with stevia, Ace-K, saccharin, and water, and the changes in the gut microbiome were recorded. Following the results, we focused on the phyla of the bacteria that changed most significantly and investigated the relationship between the specific phyla of bacteria and glucose tolerance based on previous studies. The alpha diversity and beta diversity analysis indicated a noticeable change in bacteria phyla and lower classification levels such as *Faecalibacillus*, *Prevotella*, *Bacteroidaceae*, *Phocaeicola*, and *Frisingicoccus* in the fecal sample of the rat. The bacteria *Faecalibacillus* and *Prevotella* are significantly correlated with higher risk of T2D as other studies have shown, implying the possible relationship between certain types of artificial sweetener consumption and higher T2DM incidence.

2. RESEARCH METHOD

2.1. Dataset

The gut microbiome dataset was acquired on European Nucleotide Archive (ENA) database under the accession ID PRJNA632038. The data were collected on *Rattus norvegicus* (Norway rat) fed with different sugar substitutes including saccharin (15 mg/kg), acesulfame potassium (Ace-K) (15 mg/kg), and stevia (4 mg/kg) via solution.

Acesulfame-potassium (Ace-K), 5,6-dimethyl-1,2,3-oxathiazin-4(3H)-one 2,2-dioxide [12], exhibit the physical property as a white, crystalline material which is highly stable despite store for long periods in normal conditions. Saccharin, chemically defined as o-sulfabenzamide (2,3-dihydro-3-oxobenzisulfonazole), is one of the earliest artificial sweeteners used in the food industry. It is a highly soluble weak organic acid that is very stable in solid state and decomposes into toxic fumes of nitrogen oxides and sulfur oxides when heated to 380 °C, the temperate that would not be reached in the typical food process. Stevia, also referred to as steviol glycoside or stevioside, is a naturally present chemical compound in the plant *Stevia rebaudiana*, with a structure of a glucosyl and a sophorosyl residue attached to the aglycone steviol, that contains a cyclopentanone hydrophenanthrene skeleton [13]. The control group was fed with equivalent volume/body weight water. Each group of mice included females (n = 10) and males (n = 10), and the mice were fed via rodent sipper tubes with vinyl caps. The fecal samples of the mice were collected and underwent 16S amplicon sequencing for exploring the gut metagenome.

2.2. Raw data processing of gut microbiome data

The gut microbiome data were analyzed in this study to investigate the impact of artificial sweetener consumption on rat gut microbiome diversity and richness. The raw metagenomic data was processed using R 4.3.0 with a few libraries, including dada2, phyloseq, DESeq2, ggplot2, structSSI, vegan, and ggnetwork. First, the data were read, filtered, and trimmed for inspecting and ensuring the quality of the data. Then, the identical sequencing reads were combined to avoid redundant computation via dereplication in the dada2 package. Afterward, the sequence table was constructed and the chimeras were removed. The microbiome taxonomy was assigned, and the processed data was used to construct the phylogenetic tree using the DECIPHER package and the phangorn package. Following the amplicon sequencing data were synthesized and organized with the phyloseq package into phyloseq-class data object.

2.3. Downstream data analysis

The downstream data analysis included alpha-diversity, beta-diversity analysis, visualizations of the gut microbiota components in different subjects, and statistical analysis between these groups. Alpha-diversity describes the diversity of a single sample through two metrics: richness, the number of species in an environment, and diversity, the sample richness, and sample evenness. In the study, the alpha diversity of the rat gut microbiome was analyzed with phyloseq and vegan R package. Beta-diversity demonstrates the diversity among different samples through several metrics including (abundance, phylogenetic information, etc). Using phyloseq, vegan, and ggplot2 R package, we computed the beta diversity of the fecal samples to guide further research. The bar plot is created with the phyloseq package and ggplot2 to show the abundance of bacteria taxa in all the reads. The ordination plot was plotted with the plot_ordination function using the phyloseq, ggplot2, and plyr packages. The plot was based on phylum level bacteria taxa identified in the microbiome dataset. The net plot and the heatmap were also graphed to demonstrate the distribution and changes in bacteria taxa in the gut microbiome samples. Following, we compared each test group (Ace-K, Saccharin, Stevia) with the control group with the linear discriminant analysis Effect Size (LEfSe). The LEfSe result was graphed in a bar chart showing the abundance of specific bacteria taxa of each pair.

2.4. Function Analysis

The function analysis was conducted using the Tax4Fun2 R package to make functional prediction based on the genome data. The OTU table and the fastq. files containing gene sequence data were imported first, and the reference data in the Tax4Fun2 package were combined with the inputted data for an further analysis. Through searching, profiling, and merging abundance data with functional data, we acquired the final prediction about the bacteria functions.

3. RESULTS AND DISCUSSIONS

3.1. Study design

Figure 1 shows the study design of evaluating the glucose tolerance by different artificial sweeteners. The study used metagenomics data collected from fecal samples acquired from rats fed with saccharin, stevia, Ace-K, and water, and the dataset underwent metagenomics analysis by R. With downstream data process, we analyzed the alpha and beta diversity through plotting richness, followed by identification of significant microbiome changes. The changes were then further investigated and connected to the influence on glucose tolerance, which was utilized to bridge the consumption of artificial sweeteners and the risk of T2DM.

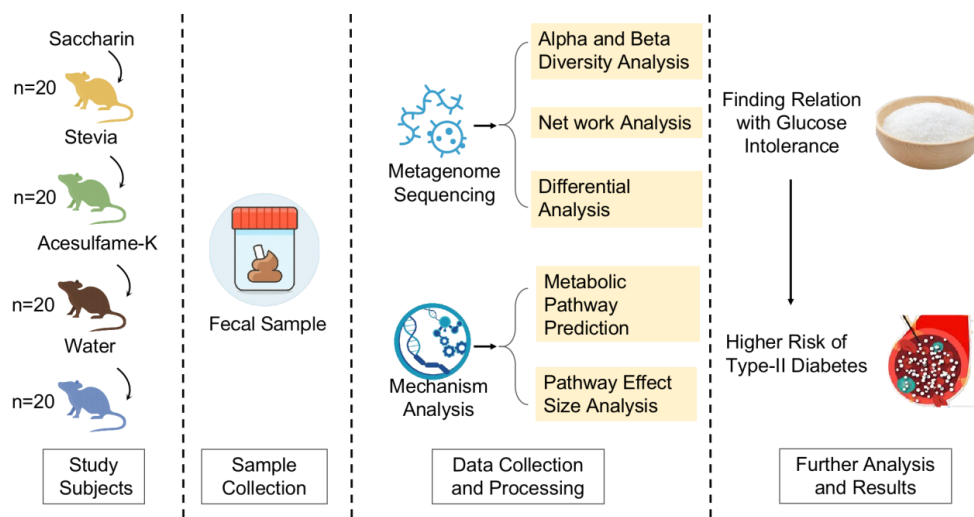


Figure 1. Workflow of gut microbiome-T2DM association analysis by different artificial sweeteners.

3.2. Gut microbiome profiling analysis

The alpha diversity (**Figure 2**) demonstrates a similar pattern for most of the bacteria taxa among all four experimental groups. Therefore, the gut microbiome analysis does not generate a significant difference between the alpha diversity of rats fed with stevia, saccharin, Ace-K, and water, implying that the general abundance of distinct bacteria phylum in all four study groups is not significantly different. Similar conclusion was acquired through beta diversity analysis (**Figure 3**) results. Hence, we focus on the differentially expressed gut microbiota in subsequent analysis.

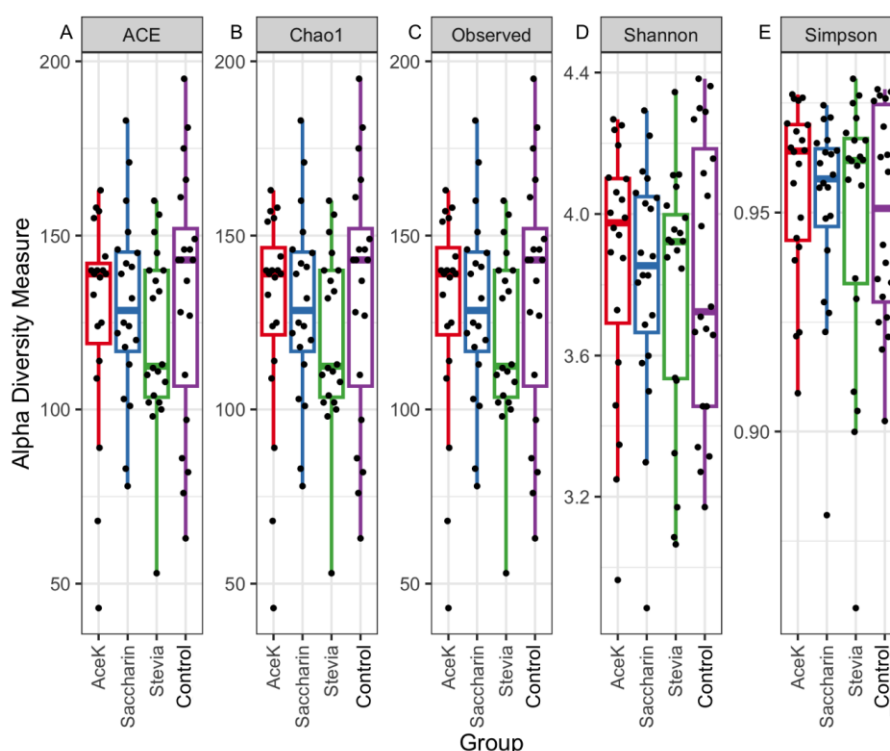


Figure 2. Alpha diversity plot of fecal sample of rat administrated with stevia, saccharin, Ace-K, and water. For each plot, the x-axis indicates the type of low-calorie artificial sweetener used and the y-axis measures the alpha diversity richness value in the number of taxa present. Each separated figure demonstrates the richness of rare taxa under a specific index (A: ACE, B: Chao1, C: Observed, D: Shannon, E: Simpson).

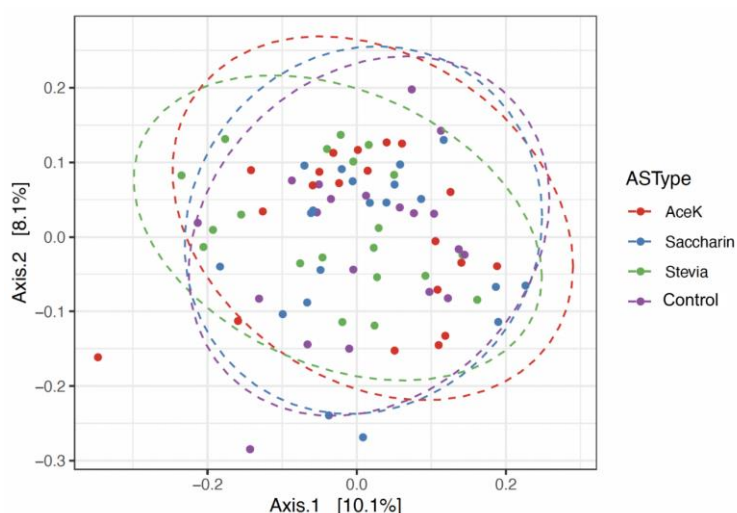


Figure 3. Beta diversity plot of fecal sample of rat administrated with stevia, saccharin, Ace-K, and water (Control). For each plot, the x-axis and y-axis are two measurements for diversity. The dash-line circles indicate the distribution range of bacteria taxa of each group.

The ordination analysis and the net plot indicate the relation and mutual influence between different types of bacteria, which was further considered and analyzed. From the ordination plot in **Figure 4**, we could conclude that most changes happen in *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* bacteria phyla and fewer changes in the rest of the phyla. We then paid attention to the changes in unique classes of bacteria in the net plot (**Figure 5**). The distance label showed the relatively close relations between *Bacteroidia*, *Bacilli*, and *Erysipelotrichia*. Many bacteria from the *Clostridia* class demonstrated both close relation and distant relation with the other three classes of bacteria, requiring more order-level research.

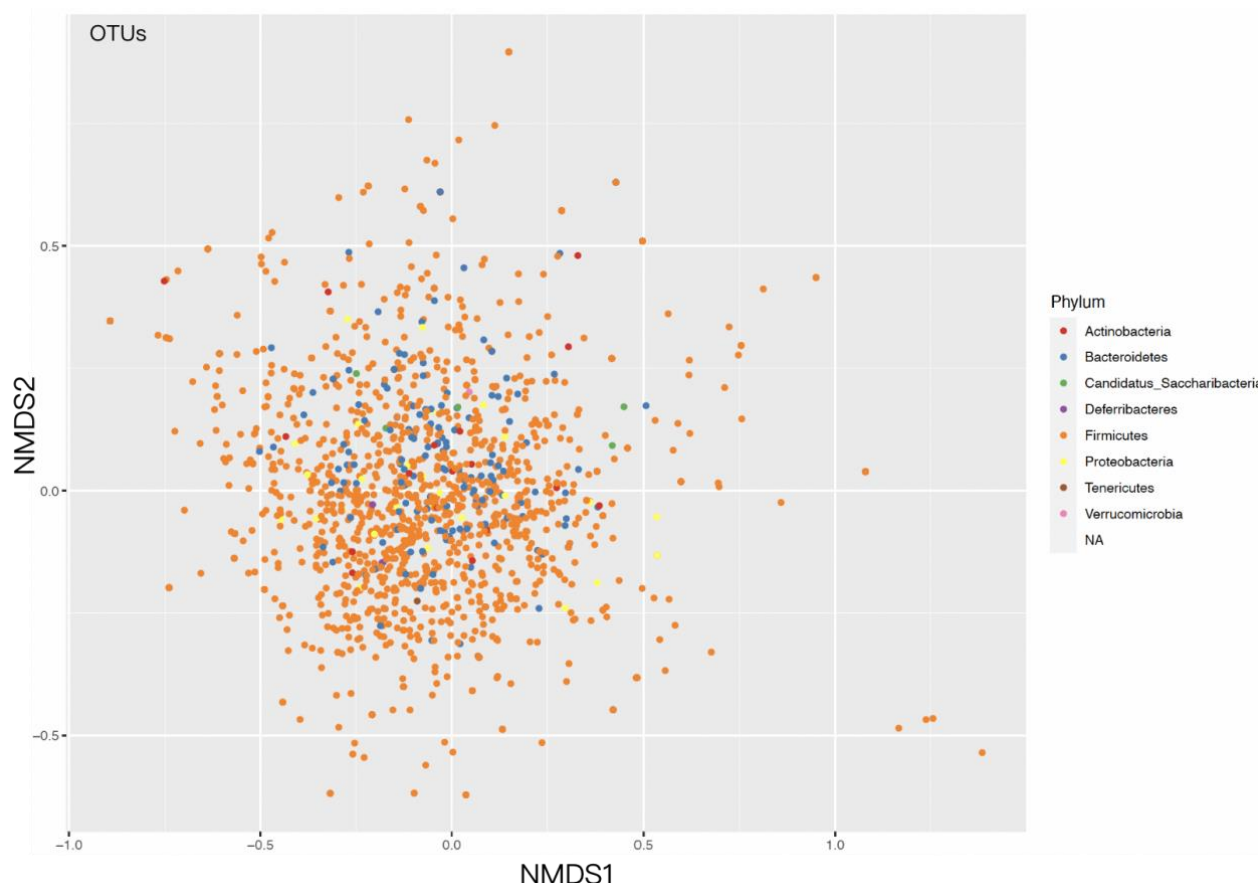


Figure 4. The ordination analysis of bacteria taxa by phylum for the fecal sample of rats administrated with stevia, saccharin, Ace-K, and water. It is a multivariate analysis based on Bray-Curtis distance and NMDS ordination. Both axes are in the NMDS scaling value to indicate distribution and relations.

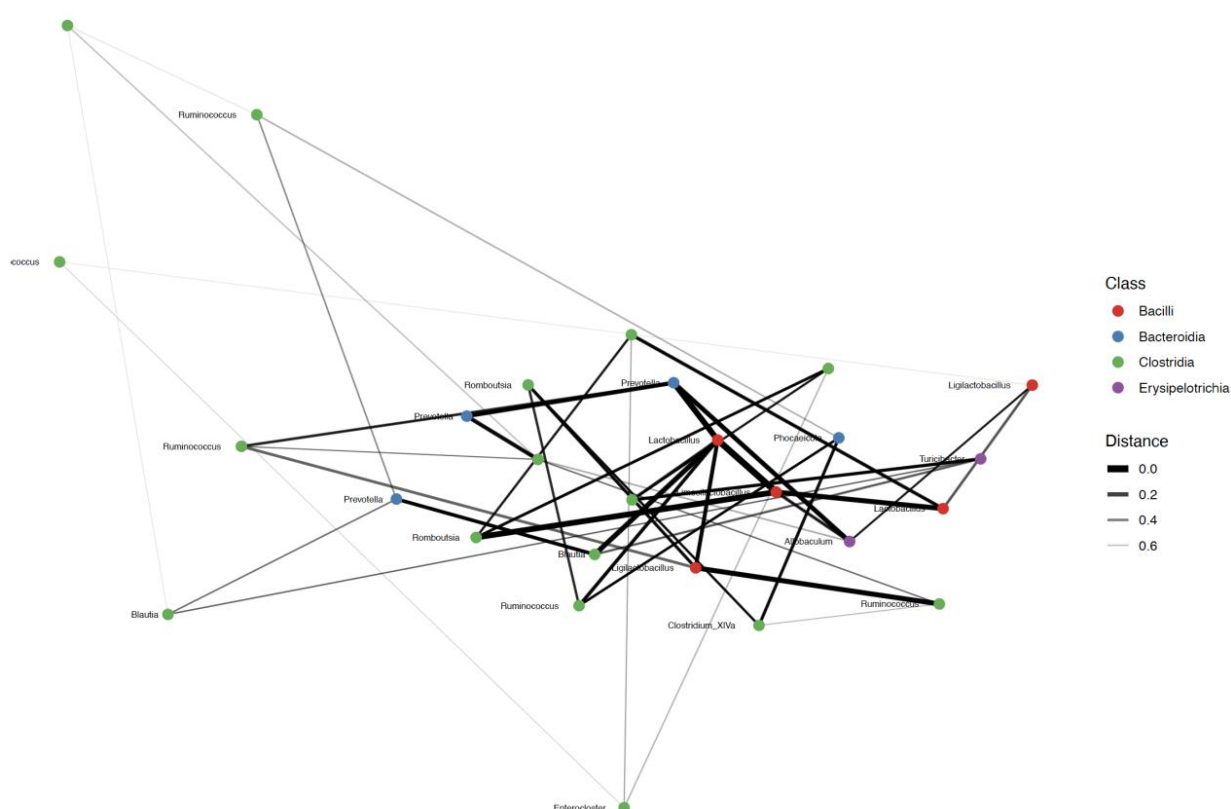


Figure 5. The network of the bacteria distribution in the fecal samples of rats administrated with stevia, saccharin, Ace-K, and water by class level. Different classes of bacteria are labeled in different colors (Red: *Bacilli*, Blue: *Bacteroidia*, Green: *Clostridia*, Purple: *Erysipelotrichia*). The thickness of the line between each point indicates the strength of the relation between two genera of bacteria.

3.3. Significant modulating gut microbiota between different AS groups

Through LDA analysis (**Figure 6**), the change in the microbiome was compared and demonstrated. Among the difference, the bacteria taxa related to glucose intolerance and T2DM were found to have a significant change in abundance in experimental groups that consume Ace-K and saccharin. In the comparison between the control group and the saccharin group (**Figures 6A** and **6B**), the *Faecalibacillus* bacteria genus had the greatest LDA score indicating a significant difference between the control group's microbiome and the saccharin group's microbiome. This genus of bacteria had been correlated with T2DM in previous research [14]. Therefore, saccharin consumption is likely to increase the risk of T2DM via changing the gut microbiome and influencing the glycemic status. Besides, in the comparison between the control group and the Ace-K group (**Figures 6C** and **6D**), there is a significant change in the abundance of *Prevotella*, which was also discovered to be abundant in patients with T2DM compared to ones not having T2DM [15]. Thus, Ace-K consumption could lead to higher T2DM risk via changing gut microbiome abundance and affecting the microbiome metabolism. Aside from these two artificial sweeteners, the stevia and the control group's comparison (**Figure 6E** and **6F**) only demonstrated a significant increase in LDA score for *Fournierella* and *Adlercreutzia* in the control group and an increase in LDA score for *Bacteroidacea*, *Phocaeicola*, and *Frisingicoccus* in the stevia group, meaning these were the most important bacteria genera to look at. However, these phyla are not indicated to be correlated with T2DM or glucose intolerance strongly, meaning stevia is still unlikely to increase T2DM risk with changing gut microbiome.

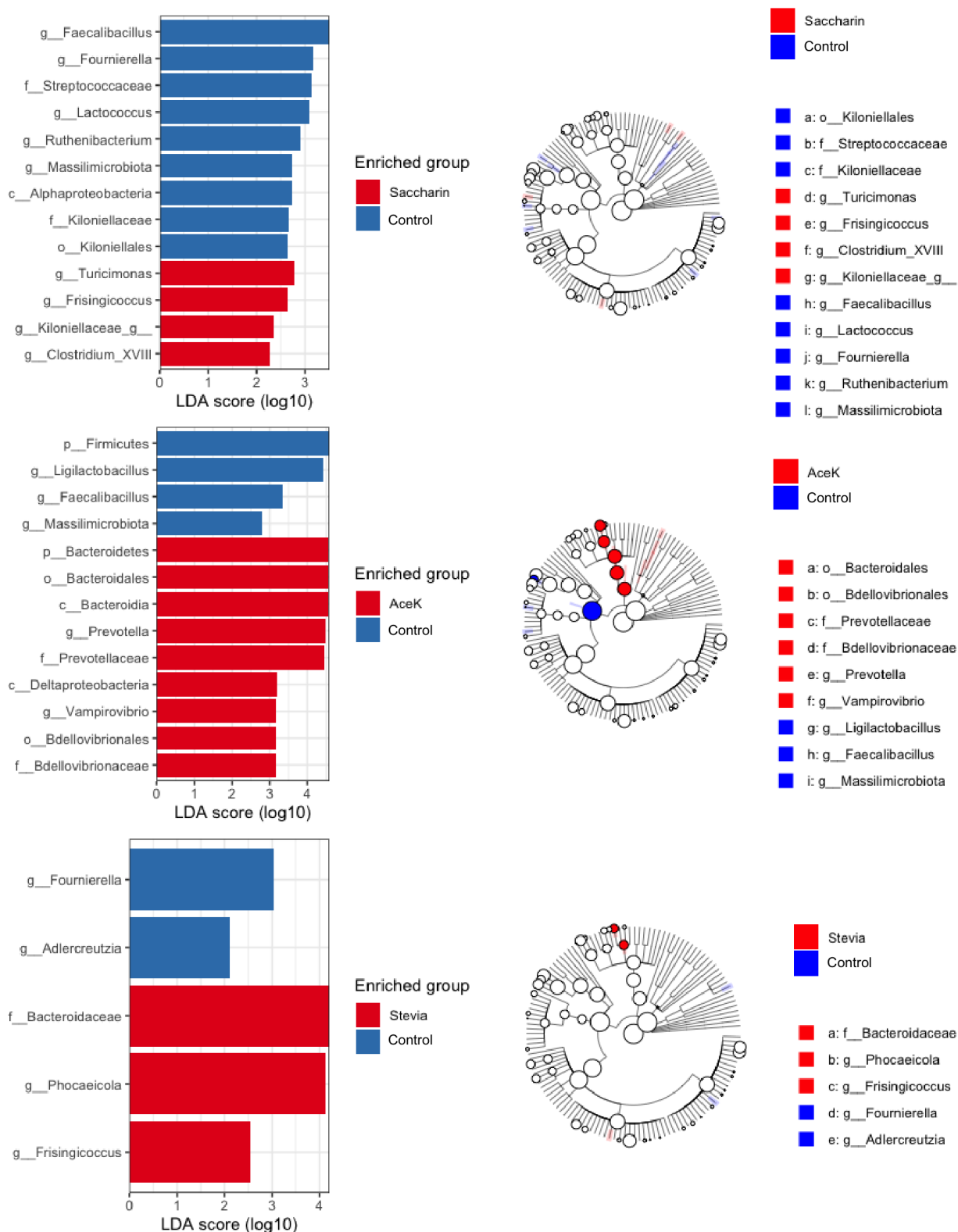


Figure 6. The LDA score bar plot and cladogram of each AS group compared with control group. The results are shown in log scale LDA score, presenting the most important genera of bacteria in the comparisons (A: saccharin and control; C: Ace-K and control; E: stevia and control). The result from the LDA analysis are plotted into the cladogram with important genera highlighted using either red or blue color that correspond with the legend (B: saccharin and control; D: Ace-K and control; F: stevia and control). The cladogram demonstrates the relation between the bacteria taxa; the relative size of the circles indicates the abundance of bacteria taxa.

Since saccharin's discovery in 1878, it is widely applied in food and beverage manufacture as a non-caloric substitute [16]. Due to its 200 times sweetness compared with table sugar, Ace-K is also commonly used in minute amounts in food and beverages, especially soft drinks, for flavoring and sweetening [17]. Despite sweeteners are commonly accepted by the food industry and are widely present in people's daily lives, based on the analysis results, the consumption of the artificial sweeteners saccharin and Ace-K should be lowered or strictly controlled within the suggested consumption amount per day for avoiding higher risk of T2DM and abnormal glycemic control. Stevia, on the other hand, could be considered safe to consume for now, but the consumption should still be controlled within suggested consumption amount due to its possible effect on altering gut microbiome. Further research on other types of artificial sweetener could focus on the sweetener consumption's impact on subject gut microbiome as well, as changing gut microbiome is shown as a possible pathway for artificial sweeteners to increase health disease risk.

There is a couple of limitations in the study. Since the study is conducted using rat gut microbiota sample data and no clinical experimental data is used, the result may not be simply generalized to human subjects. Besides, the total sample size is 80, which individual data may influence the result more, suggesting that a larger sample size is needed in order to improve the reliability of the result. Further research could be better conducted with larger sample and human participants.

3.4. Key functional pathways show association between modulated microbiota and glucose metabolism

The analysis result for functional pathways was plotted in **Figure 7**, showing the microbiota related functional pathways with significance difference between each AS and the control group. In all three comparisons, phosphotransferase system (PTS) and glycolysis/gluconeogenesis stood out to be the top two correlated functional pathways. In both pathways, the scores for AS groups were lower compared with the scores for the control group, and the results were likely to be caused by difference in microbiota abundance between the groups. Since both PTS and glycolysis were pathways that were highly related to glucose metabolism, the analysis indicated a possible decrease in glucose metabolic function in groups consumed AS. Subsequently, the groups could have higher risk of glucose intolerance which led to higher T2DM risk.

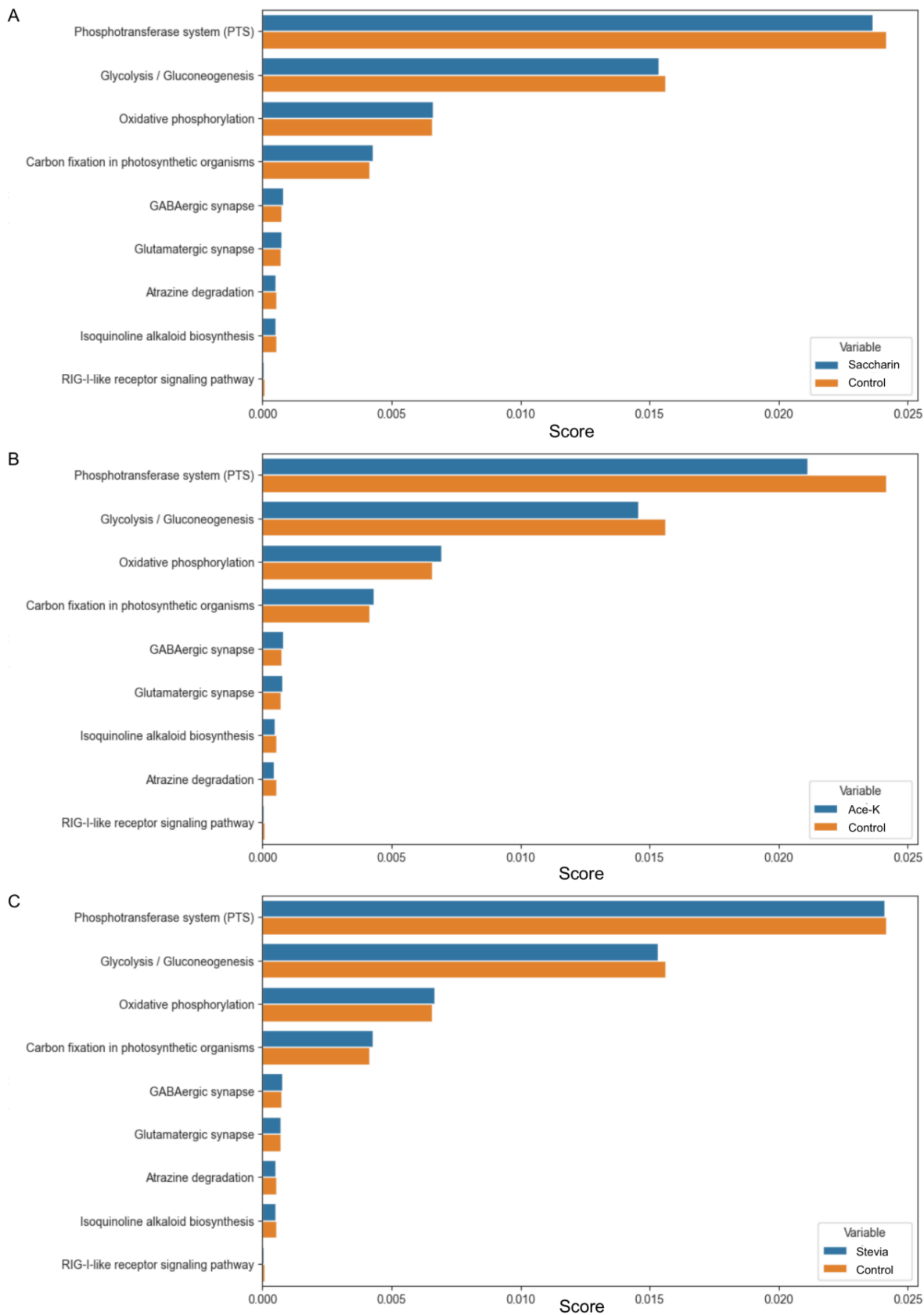


Figure 7. Tax4Fun 2 pathway prediction analysis result for each AS group compared with the control group (A: saccharin and control; B: Ace-K and control; C: stevia and control).

3.5. Microbiome revealed association between AS consumption and T2DM

With the data of significantly different bacteria taxa between each AS group and the control group identified by LEfSe analysis, we conducted subsequent association research using the MicrobiomeAnalyst 2.0 online analysis platform. Significant correlation between the identified bacteria taxa and diseases as well as biomarkers were noticed. In the host-intrinsic taxon sets analysis for saccharin group, the bacteria taxa were significantly ($p < 0.05$) associated with T2DM and several other diseases (The related diseases with $p < 0.05$ were listed in **Table 1**). For Ace-K, the bacteria taxa were significantly ($p < 0.01$) correlated with T2DM and other diseases (116 related diseases have $p < 0.05$ and 42 of them have $p < 0.001$; diabetes and related diseases' results were listed in **Table 2**). At the same time, in the diet association analysis, the result demonstrated a significant ($p < 0.01$) correlation with high fructose and glucose consumption, further supporting the association between Ace-K consumption and higher T2DM risk. In the analysis for stevia, the bacteria taxa were not significantly correlated with T2DM directly nor T2DM related factors such as diet in glucose or metabolites associated with glucose intolerance.

Table 1. Microbiome diseases association analysis result for saccharin

Group	Disease	Related Taxa	P-value	Effect
Saccharin and Control	Acute-On-Chronic Liver Failure	Streptococcaceae	0.0135	Increase
Saccharin and Control	Anorexic	Streptococcaceae	0.0135	Increase
Saccharin and Control	Blastocystis	Streptococcaceae	0.0135	Decrease
Saccharin and Control	Severe pain and Chronic morphine treatment	Streptococcaceae	0.0135	Increase
Saccharin and Control	Colorectal Neoplasms	Lactococcus, Streptococcaceae	0.0164	Increase
Saccharin and Control	Acute viral gastroenteritis with complication	Streptococcaceae	0.0189	Increase
Saccharin and Control	Parkinson Disease	Alphaproteobacteria	0.0189	Increase
Saccharin and Control	Colorectal Carcinoma	Streptococcaceae	0.0216	Increase
Saccharin and Control	Sleep Deprivation	Streptococcaceae	0.0242	Increase
Saccharin and Control	Colorectal Adenomatous Polyposis, Autosomal Recessive	Lactococcus	0.0269	Increase
Saccharin and Control	Coronary Artery Disease and Type-2 Diabetes Mellithem	Streptococcaceae	0.0269	Increase
Saccharin and Control	Liver Cirrhosis	Streptococcaceae	0.0348	Increase
Saccharin and Control	Inflammatory Bowel Diseases	Streptococcaceae, Lactococcus	0.0363	Increase
Saccharin and Control	Obesity and Metabolic Diseases	Alphaproteobacteria	0.0401	Decrease
Saccharin and Control	chromogranin A	Alphaproteobacteria	0.0427	Increase

Table 2. Microbiome diseases association analysis result for Ace-K

Group	Disease	Related Taxa	P-value	Effect
Ace-K and Control	Constipation	Bacteroidetes, Prevotellaceae, Prevotella, Firmicutes	9.01E-7	Decrease
Ace-K and Control	Obesity	Bacteroidetes, Prevotella, Firmicutes, Deltaproteobacteria, Bacteroidales	3.4E-6	Decrease
Ace-K and Control	Diabetes Mellitus	Firmicutes, Bacteroidetes, Prevotella, Bacteroidales	6.39E-5	Increase
Ace-K and Control	Diabetes Mellitus, Type 2	Prevotella, Firmicutes	4.08E-4	Increase
Ace-K and Control	Diabetes Mellitus	Bacteroidetes, Prevotella, Firmicutes	4.62E-4	Decrease
Ace-K and Control	Intestinal Diseases and Fatty Liver	Bacteroidetes, Firmicutes	4.62E-4	Decrease
Ace-K and Control	Obesity	Firmicutes, Prevotella, Bacteroidetes	0.00172	Increase

4. LIMITATION

The abundance and depth of the 16s rRNA data used for this study may not be enough for more accurate and profound analysis. It is noticed that there were still multiple unlabeled or not clearly labeled sequences for bacteria taxa in the current data set. Therefore, this study's result is based on limited amount of identified bacteria taxa, and the conclusion would be correlational.

5. CONCLUSION

This study examined the consumption of saccharin, Ace-K, and stevia, three commonly used artificial sweeteners, and the impact on rat gut microbiota to investigate the possible mechanism of artificial sweetener consumption increasing T2DM risk. We observed significant microbiome change in bacteria genera *Faecalibacillus* and *Prevotellax*, which were found to be correlated with T2DM incidence when comparing the saccharin sample group and the Ace-K sample group with the control group. For the determined microbiome changes in the stevia group, no correlation between the changes and T2DM was observed. Thus, the study result suggested that consuming saccharin and Ace-K would likely increase the risk for T2DM by altering the gut microbiome and affecting normal glycemic control. Meanwhile, the observation did not demonstrate stevia could affect T2DM risk by influencing gut microbiome, suggesting that stevia could be considered safe in terms of T2DM risk.

We had further conducted functional pathway analysis and microbiota diseases association analysis to investigate AS consumption's impact on T2DM risk. The functional pathway analysis revealed the decreased score in the PTS and glycolysis pathways in AS group compared with control group, showing the correlation between all types of AS consumption and higher risk of glucose intolerance as well as T2DM. The disease association analysis demonstrated significant correlation between saccharin consumption and Ace-K consumption with T2DM and obesity as well as diet of increased glucose and fructose. Both results reinforced the conclusion that saccharin and Ace-K consumption were highly correlated with higher T2DM risk while indicating stevia as a potential risk factor for T2DM.

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