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Effects of Steviol Glycosides and Steviol on the Relative Abundance and Diversity of the Human Gut Microbiome in the Fecal Homogenates from Healthy Adults and Children

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Abstract: Steviol glycosides extracted from *stevia rebaudiana* plant are increasingly used as sweeteners. The minor steviol glycosides in the stevia extract, such as rebaudioside D and rebaudioside M, have superior sensory quality compared to the major components (e.g. rebaudioside A and stevioside). Due to the limited availability of rebaudiosides M and D in stevia leaf, they can be commercially produced by using enzymatic bioconversion and fermentation processes. The effect of steviol glycosides produced by different processes and their common metabolite steviol on human colonic bacteria is not adequately studied or published. There are limited research findings of the effect of artificial sweeteners, such as sucralose and Ace-K, on microbiomes. The key findings on artificial sweeteners are often extended, albeit incorrectly, to present the effect of all sweeteners including stevia, a natural sweetener on human microbiome. This study was designed to examine the potential influence of steviol glycosides and metabolite on the diversity and relative abundance of colonic bacteria genera in human fecal homogenates collected from adult male, female and young children. During the *in vitro* biotransformation of steviol glycosides to steviol as a final common metabolite in human fecal homogenate, the metabolic fate of steviol glycosides and the effect of stevia metabolites on the bacterial population were investigated by employing the next-generation genomic sequencing and informatics to record the bacterial diversity and relative abundance in the fecal microbiota. From *in vitro* anaerobic incubation samples, 20 major bacteria genera were observed, accounting for a range of 75% to 85% of the freshly collected colonic populations in male, female and children. Based on hierarchical clustering analysis, beta diversity differences were noted in bacterial genera between adult male, female and children fecal homogenate incubation samples as anticipated to be influenced by dietary variables and age as previously recognized by other researchers. The relative abundance of colonic bacterial populations in the incubation samples were different from the corresponding raw stool samples due to the influence of enrichment medium during fecal homogenate metabolic incubation. However, the principal coordinate analysis of bacteria genus data indicated a general lack of differences in alpha and beta diversity between samples incubated for 72 hours in the presence and absence of steviol glycosides and steviol metabolite amongst fecal homogenates collected from adult male, female and children. The relative abundance data (%) of 30 bacteria genus showed some effect of experimental incubation time, but there was no apparent difference between the fecal homogenate samples incubated in the presence and absence of steviol glycosides and steviol. The adult male, female and children bacteria genera data from this study have contributed to a conclusion that steviol glycosides used at typical low concentrations as a non-nutritive sweetener did not result in observable alternations of the bacterial population.

Keywords: steviol glycoside metabolism; effect on colonic bacteria; microbiome; adult and children

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1. Introduction

Steviol glycosides isolated from *Stevia rebaudiana* are a group of over 60 identified naturally occurring glycosides containing a common steviol aglycone [1,2]. Previous *in vitro* and *ex vivo* toxicology studies [3,4] have demonstrated that steviol glycosides are not absorbed from the gastrointestinal tract but undergo deglycosylation by bacterial hydrolysis to steviol as a final stable metabolite, which can either be excreted in stool or absorbed and metabolized to steviol glucuronide, which is excreted primarily via the urine in human. The rapid hydrolysis of steviol glycosides is the result of bacterial enzymatic activity in the lower bowel. The gut microbiome, which is amongst the most extensively studied of the human microbiomes, is highly diverse and contains thousands of different bacterial species [5,6]. The diverse community of bacteria is composed of a small number of abundant species plus a large number of species found at low abundances [7]. One of the abundant bacterial family is the *Bacteroidaceae*, predominantly *Bacteroides*, that have previously been identified as being primarily responsible for the enteric biotransformation of steviol glycosides [4,8].

Steviol glycosides derived from stevia leaf extract have been increasingly used commercially as a natural low caloric sweetener along with artificial non-nutritive sweeteners in food and beverages. A review of published literature on the commonly used low caloric non-nutritive sweeteners including aspartame, saccharin acesulfame K, and steviol glycosides and their influence on the diversity and abundance of the human gut microbiome revealed that limited amount of human data and the interpretation of animal experimental data did not adequately support a conclusive attribution between sweeteners and their influence on the human gut microbiome [9]. Another literature review [10] on the effects of sweeteners towards metabolic syndrome in the obese population characterized by insulin resistance and cardiovascular risks concluded that sweeteners could interact with the gut microbiota and intestinal sweet-taste receptors, thus considerations in daily intake, sweeteners composition and human metabolism are useful in considerations in their use in a sugar-free product.

Despite of the historical safe use of steviol glycosides in small quantities commonly found in dietary exposure, detail information on the influence of steviol glycosides and the common metabolite steviol on the beta-diversity and relative abundance of the human gut microbiome is limited. Hence, this study employed a well-established *in vitro* metabolic incubation protocol [11–13] involving the use of pooled human fecal homogenates to investigate a possible influence of steviol glycosides and the common final deglycosylation metabolite, steviol, on the gut microbiome diversity and relative abundance based on fecal materials collected from healthy adults and children donors. In this investigation, the use of *in vitro* pooled human fecal homogenates was designed to provide representative gut microbiome materials to control the complexity and interplay between individual variations in the gut microbiome due to diet and other confounding variables amongst the subjects.

The highly diverse human gut microbiome contains thousands of enteric bacterial species. Technological advances in high-throughput sequencing of genome have accelerated the analysis of the human gut microbiome since the majority of the enteric bacterial species cannot be readily cultured using standard laboratory culture techniques [14–16]. The most common sequencing approach to analyze the microbiome is amplicon analysis of the 16S ribosomal RNA (rRNA) gene, which was extensively used to compile most of the data collated by the Human Microbiome Project [17]. The annotation of the 16S method is based on the presumed association of the 16S rRNA gene with a taxa defined as an operational taxonomic unit (OTU), which are generally analyzed at the phyla or genera level with limited precision at the species level. The use of different sequencing methods to analyze the human fecal microbiome has been reviewed [16]. The authors concluded that whole genome shotgun sequencing (WGS) is more effective and accurate in defining a taxa at the species level. The WGS method uses sequencing with random primers to sequence overlapping regions of a genome. However, the WGS method generally requires sequencing a genome with high coverage which involves extensive data analysis and specialized database capability.

Within the children population, the gut bacterial composition changes readily during the first few years of life after birth, evolving with adaptation throughout the lifetime of the individual [18–

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21]. The gut microbiome is acquired at birth from the mother and is influenced by the method of delivery [18]. The development of the gut microbiota from birth to approximately 2.5 years of age showed that bacteria of the *Firmicutes* phylum generally dominated the early stages of the gut microbiota, whereas the introduction of solid foods promoted the establishment of a gut microbiome akin to adults, dominated by *Firmicutes* and *Bacteroidetes* in addition to low abundance of *Proteobacteria* and other enteric Gram-negative bacteria [18,22,23]. In adults, the composition of the gut microbiome is dominated by members of the *Bacteroidetes* and *Firmicutes*, and to a lesser extent, *Proteobacteria, Actinobacteria, Fusobacteria, Spirochaetes, Verrucomicrobia, and Lentisphaerae* [21,22,24–27].

It has been described that by the time solid foods are introduced to infants, the gut microbiome has reached a significant level of diversity, including a high proportion of *Bacteroidetes* expressing metabolic enzymes including beta-glycosidases and beta-glucuronidases which are responsible for deglycosylation of steviol glycosides to steviol as a final metabolite [22,24,28]. As a result, the gut microbiome in children within a few months of birth would be expected to develop a capacity to hydrolyze steviol glycosides. Those steviol glycosides that are not completely hydrolyzed would simply transit through the intestines and excreted since steviol glycosides have been previously shown (3, 4, 7) to remain non-absorbed at normal consumption levels. Accordingly, in very young children with a lower capacity to metabolize steviol glycosides to steviol compared to adults, a lower systemic exposure of steviol would be expected relative to adults.

An increasing number of published articles have discussed the linkages of the human gut microbiome in a wide range of human diseases and conditions including inflammatory bowel disease [29], irritable bowel syndrome [30], obesity [31], type 2 diabetes [32], multiple sclerosis [33], Parkinson's disease [34], autism [35], and colorectal cancer [36]. These studies revealed the association of a distinctive pattern in the diversity and relative abundance of the human gut microbiome in disease subjects compared with control healthy subjects and emphasized the need to investigate potential alterations in the gut microbiome in relations to the etiology of many human diseases.

2. Materials and Methods

2.1. Healthy Adult and Children Human Fecal Donors

A summary of the experimental scheme is summarized in Table 1. A total of six male adult donors aged between 22 to 58, six female adult donors aged between 23 to 60, and six children donors aged between 24 to 36 months were enrolled with reference to study inclusion and exclusion criteria established for this study. Donors provided a self-declaration with absence of dietary disease/allergy, consumption of sweeteners, prebiotic/fiber and probiotic supplements within 2 weeks, recent history of gastrointestinal illness, history of Type 1 or Type 2 diabetes and cardiovascular disease, history of gastrointestinal or bariatric surgery, alcohol abuse, use of laxatives within 2 weeks, antibiotic therapy or chemotherapy within 3 months, inflammatory disorders, and not enrolled in weight-loss diet.

Fecal material collection and handling involved the use of a customized stool collection kit provided to each donor. Upon voiding, an estimated 10 g to 20 g wet weight of fecal material from each donor was immediately collected in a pre-labelled 100 mL screw-capped polypropylene specimen container pre-filled with 50 mL 0.01 M isotonic anaerobic phosphate buffer (pH 7.0) containing Brain-Heart Infusion broth (BHI). The initial fecal-to-buffer ratio of the collected sample was determined based on the tare-weight of the screw-capped fecal sample container with the initial volume of buffer used. Based on the initial dilution determined for each fecal material, a subsequent dilution with 0.01 M isotonic anaerobic phosphate buffer (pH 7.0) is performed to provide a 12.5x diluted fecal homogenate. All fecal materials were stored at room temperature before being used in homogenate preparation within 48 hours.

2.2. In Vitro Incubation of Steviol Glycosides in Pooled Human Fecal Homogenates

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A commercial steviol glycoside mixture extracted from stevia leaf (Zeta-M, Lot No A95-27A) was provided by PureCircle™ for this study. Metabolic incubation in the presence and absence of steviol glycosides (Lot A95) was performed over a period of 72 hours and post-incubation samples were stabilized for DNA metagenomic analysis to provide species diversity and relative abundance data.

Under anaerobic conditions, each of the 6 adult male, 6 adult female and 6 children fecal materials collected in 0.01 M isotonic phosphate buffer (pH 7.0) was homogenized by shaking and mixing with a disposable stirrer. Fecal materials from 2 subjects of the same gender or group were pooled (n =2 pooled) to provide 3 lots of pooled fecal homogenates of adults male, adults female and children. So, total 9 fecal homogenates were used for incubation. The sample preparation of the pooled fecal homogenate had been previously detailed [11–13].

Steviol glycosides were *ex vivo* spiked and incubated at a concentration of 200 µg/mL in each of 3 lots of human fecal homogenate, each pooled from 2 subjects. Incubation was carried out over a time intervals of 0, 12, 24, 48 and 72 hours. Into each pre-labelled sample vial, 10 µL of a Test Article (A95) or the positive control (Reb A) or the negative control (no steviol glycosides) stock solution was accurately dispensed followed by the addition of the 0.5 mL pre-incubated fecal homogenate to each vial performed inside an anaerobic nitrogen hood purged with high purity nitrogen to ensure <1% oxygen level. Samples were capped, vortex mixed and incubated under an anaerobic environment at 37°C±5°C in a calibrated environmental chamber. Independent triplicate samples were prepared for each time point with each of three lots of pooled adult male, female and paediatric fecal homogenate. An aliquot of each incubation sample with or without Test article (A95) was stabilized for DNA metagenomic analysis to generate species diversity and relative abundance data.

2.3. Human Gut Microbiome Shotgun Metagenomic Sequencing

A proprietary BoosterShot™ assay (CoreBiome™, Minneapolis, USA) particularly suited for human fecal samples was used to provide shotgun metagenomic sequence data. BoosterShot™ is a proprietary shotgun sequencing approach based on converting random DNA sequences into direct gene and species-level markers to generate accurate taxonomic and functional profiles of the microbiome with species-level resolution. Pooled human fecal homogenates in the presence and absence of steviol glycosides at each of the incubation time points were transferred into a commercial OMNIgene™ GUT fecal collection tube to provide contamination-free storage and stabilization for ambient temperature storage for up to 8 weeks to allow ease of handling prior to frozen storage until BoosterShot™ NGS assay.

2.4. Statistical Analysis of relative abundance of microorganism

Statistical Analysis was conducted on bacterial abundance data of 30 microorganisms using General Linear Model (GLM) function of ANOVA menu in Minitab 19 software of Minitab LLC (https://www.minitab.com). The factors for the GLM analysis were three Groups (Male, Female, Paediatric), five incubation times (0, 12, 24, 48, and 72 h) and two treatments (with and without stevia). The abundance (in percentage) of microorganism (genus level) was used as response. Two-way interactions for each factor were included in the analysis and ANOVA result was used to eliminate insignificant factors. Model was retested until residuals showed normality and constant variance. Transformation of response was conducted when appropriate using Box-Cox transformation. A 95% confidence interval (p-value 0.05) was used to determine statistical significance. The analysis was performed for each microorganism separately.

3. Results

3.1. Metabolism of Steviol Glycosides by Human Gut Microbiome

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Metabolic incubation in the presence and absence of steviol glycosides was performed over a period of 72 hours and post-incubation samples were assayed for remaining steviol glycosides and steviol metabolite as previously described using a published LC-MS method [11,1213]. Metabolic hydrolysis data had been reported elsewhere [2]. The mixture of steviol glycosides containing predominantly rebaudioside D and rebaudioside M were rapidly and nearly completely (>95%) deglycosylated during 12-16 hours of incubation in the pooled fecal homogenates collected from adult male, adult female and children. The formation of steviol as a final metabolite expressed as molar % equivalent over the incubation time intervals is summarized in Figure 2.

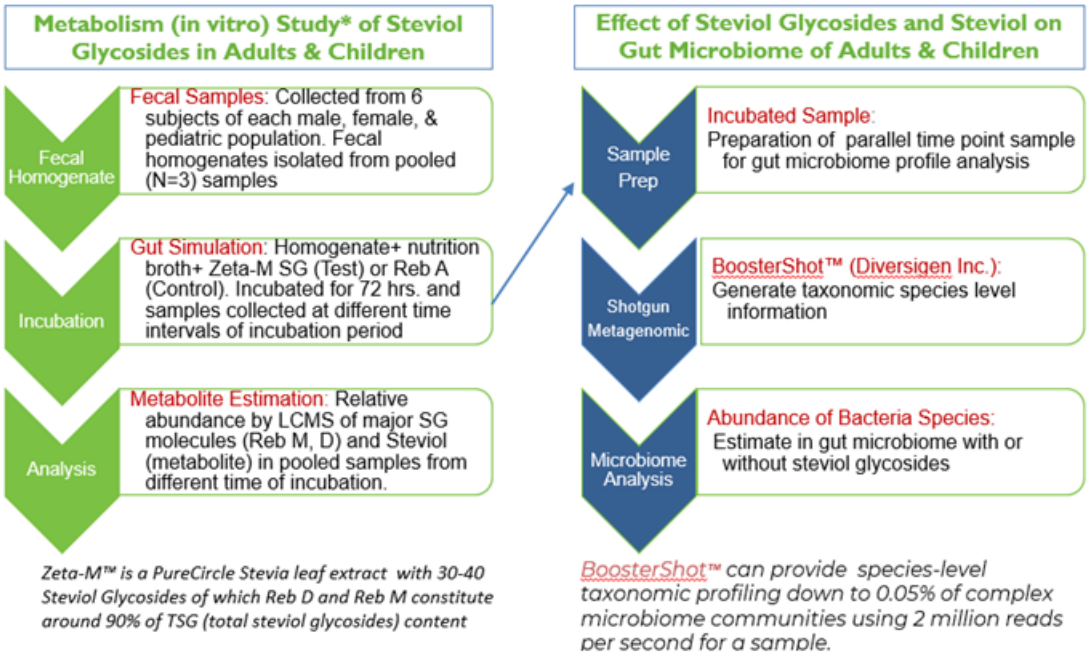
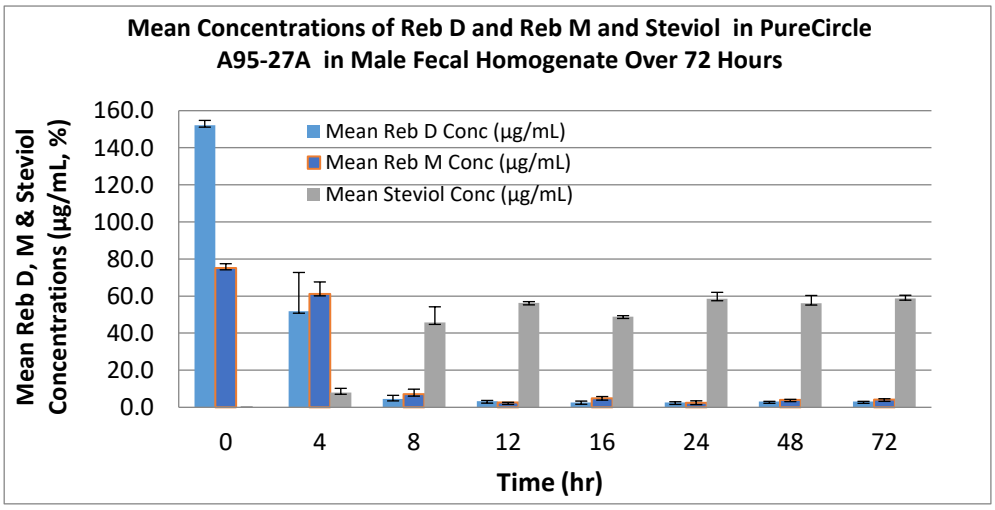


Figure 1. Experimental scheme.



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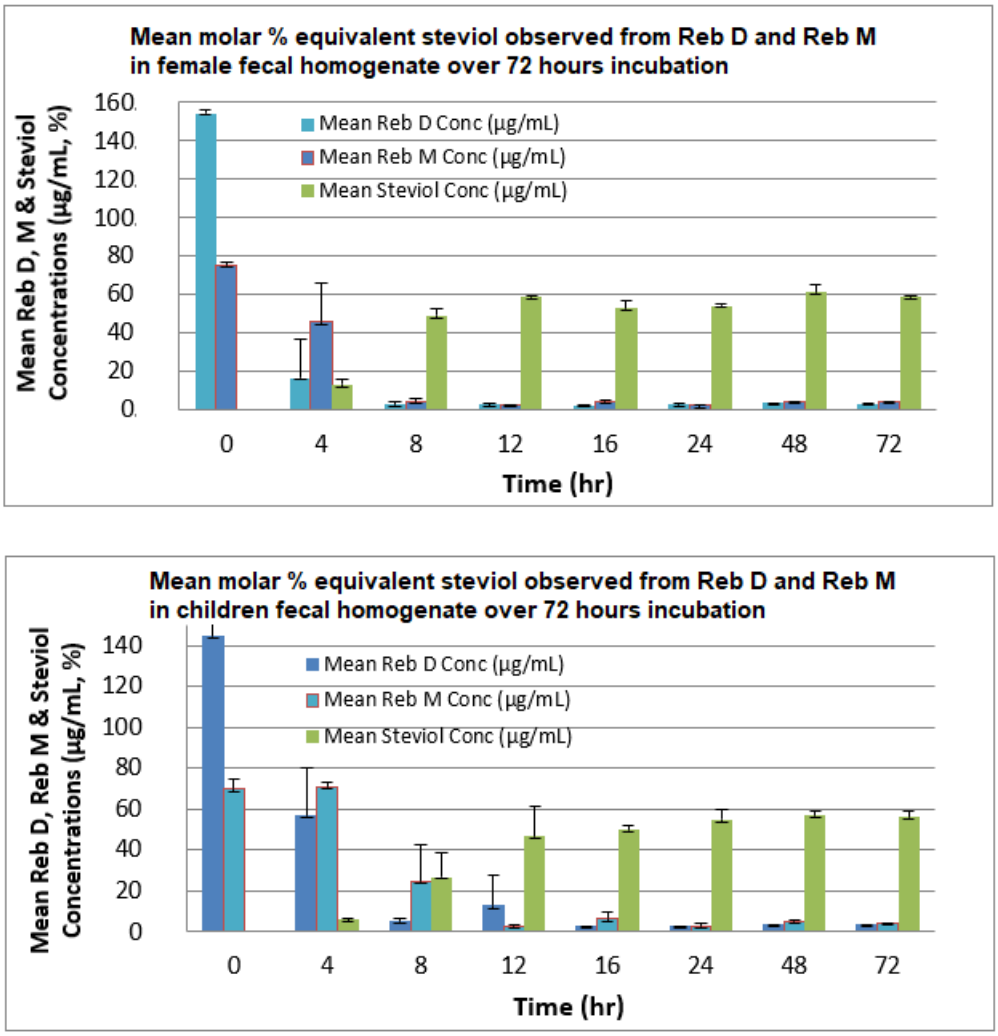

Figure 2. Pooled human fecal homogenate collected from adult male (top), adult female (middle) and children (bottom) donors showing rapid and near completely (>95%) deglycosylation of Rebaudioside D and Rebaudioside M and formation of molar equivalent % steviol metabolite observed during metabolic incubation over a 72-hour period.

3.2. Steviol Glycoside Influence on Human Gut Microbiome Diversity and Relative Abundance

From the metagenomic data on the pooled human fecal homogenate incubation samples, 20 major bacteria genera were observed and contributed to the overall relative abundance ranging from 75% to 85% of the gut microbiome observed in adult male, adult female and children.

To investigate the potential influence of steviol glycosides and steviol on the beta diversity of the human gut microbiome, the % relative abundance of the most abundant bacterial genera observed in adult male, adult female and children pooled fecal homogenates over the metabolic incubation period are presented in Figure 3. Differences in beta diversity in the gut microbiome between adult male, adult female and children were observed in this study, which is likely an influence of dietary [26] and age [18] differences between the study groups as previously discussed in the literature. In this study, no marked differences in the alpha and beta diversity of the human gut microbiome were observed when comparing the presence and absence of steviol glycosides within adult male, adult female and children fecal microbiome samples over a metabolic incubation period of 72 hours (Figure 4).

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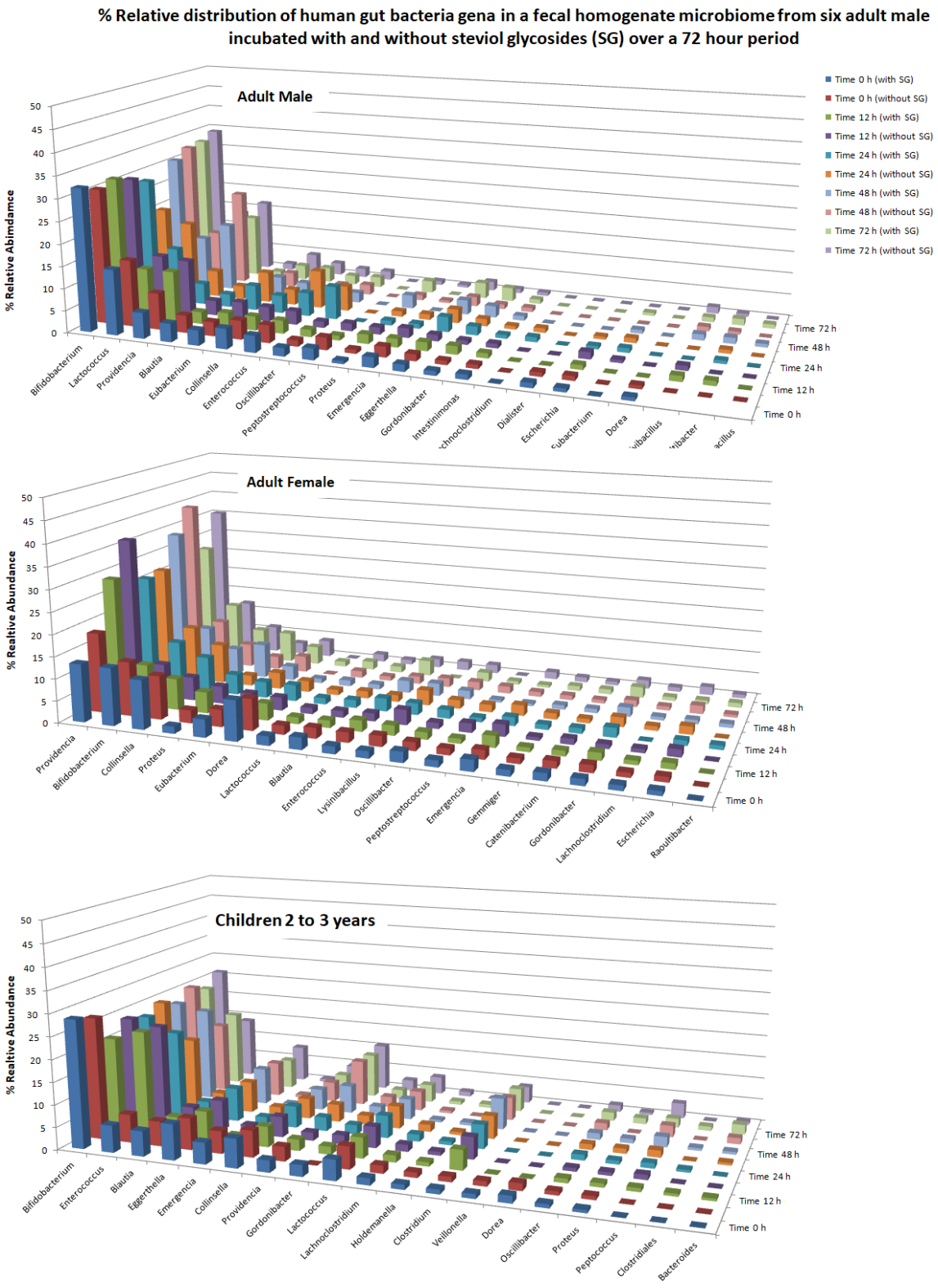

Figure 3. Percent relative abundance of major human gut bacteria genera observed in pooled fecal homogenate from six males, six females, and six children incubated with and without steviol glycosides over a 72-hour period.

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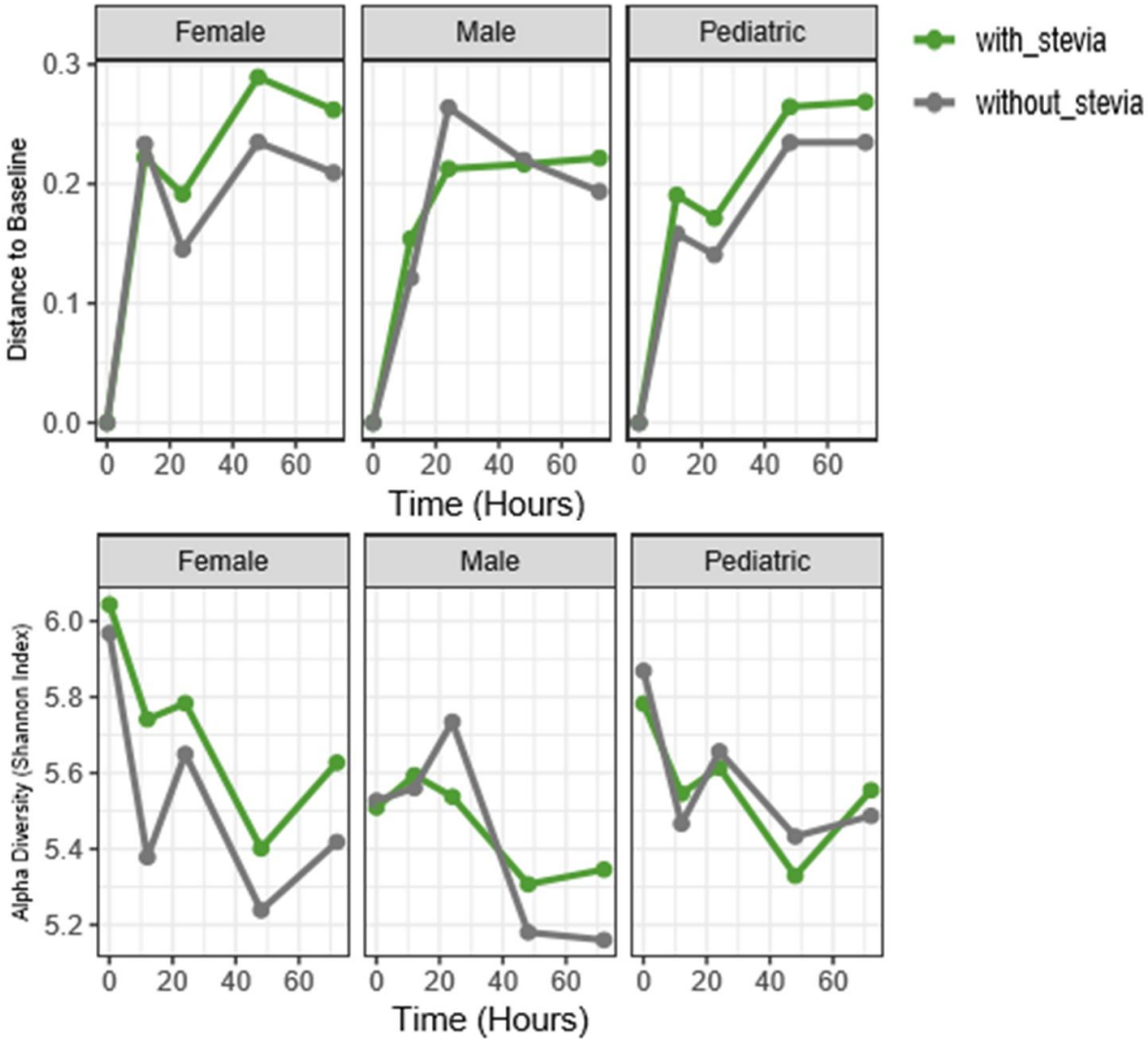

Figure 4. No apparent differences in the alpha and beta diversities observed in the pooled fecal homogenate microbiomes in male, female and children materials during metabolic incubation in the presence and absence of steviol glycosides over a 72-hour period.

Principal coordinate analysis (PCoA) of the bacterial compositions observed in the raw stool samples and the corresponding pooled fecal homogenate samples of adult males, adult females and children is presented in Figure 5. The differences observed amongst different groups are likely due to the differences in diet and age. The raw stool materials without dilution by incubation medium were observed to be qualitatively different from the incubation samples at a 1:50 dilution in the BHI broth, which likely have resulted from enrichment during the metabolic incubation period. Nevertheless, additional hierarchical clustering analysis based on PCoA plots of the incubation samples in the presence and absence of steviol glycosides indicated the lack of steviol glycoside influence on the pooled fecal homogenate microbiome composition in adult male, adult female and children (Figures 6 and 7) as indicated by their relative high p-values, low R-squared values and the overlapping coordinates between treatments in the presence and absence of steviol glycosides.

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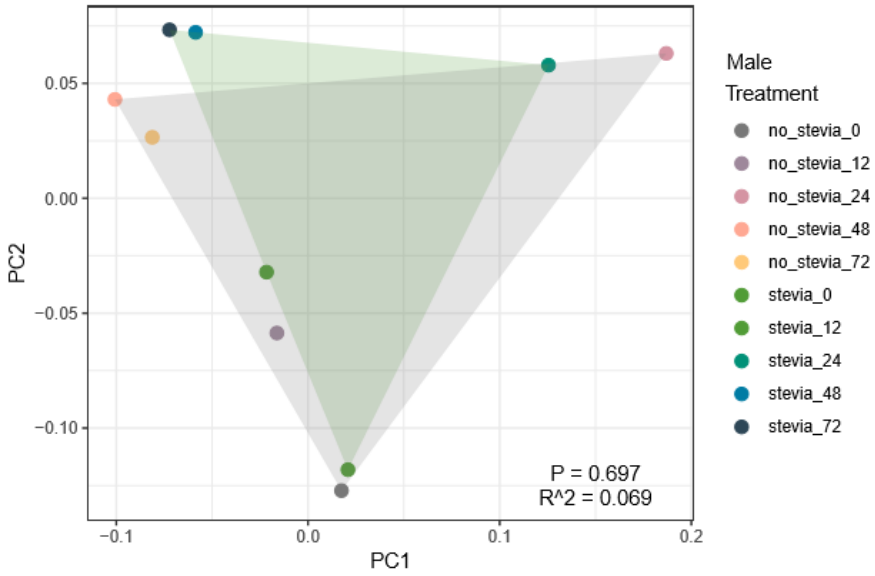
Figure 5. Principal coordinate analysis (PCoA) of bacterial composition between male, female and children pooled fecal homogenates.



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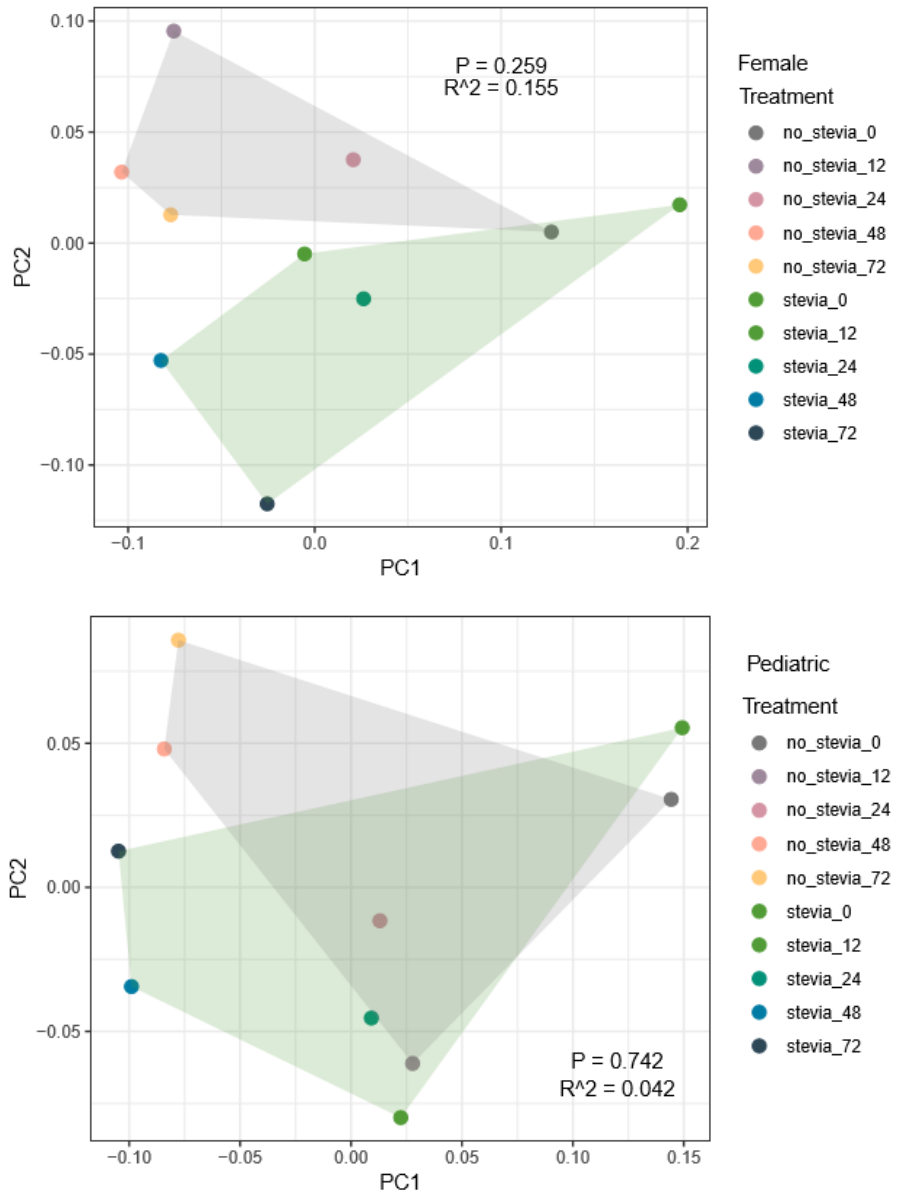

Figure 6. Principal coordinate analysis (PCoA) of bacterial composition in male (top), female (middle) and children (bottom) pooled fecal homogenates in the presence and absence of steviol glycosides over the 72-hour incubation time intervals.

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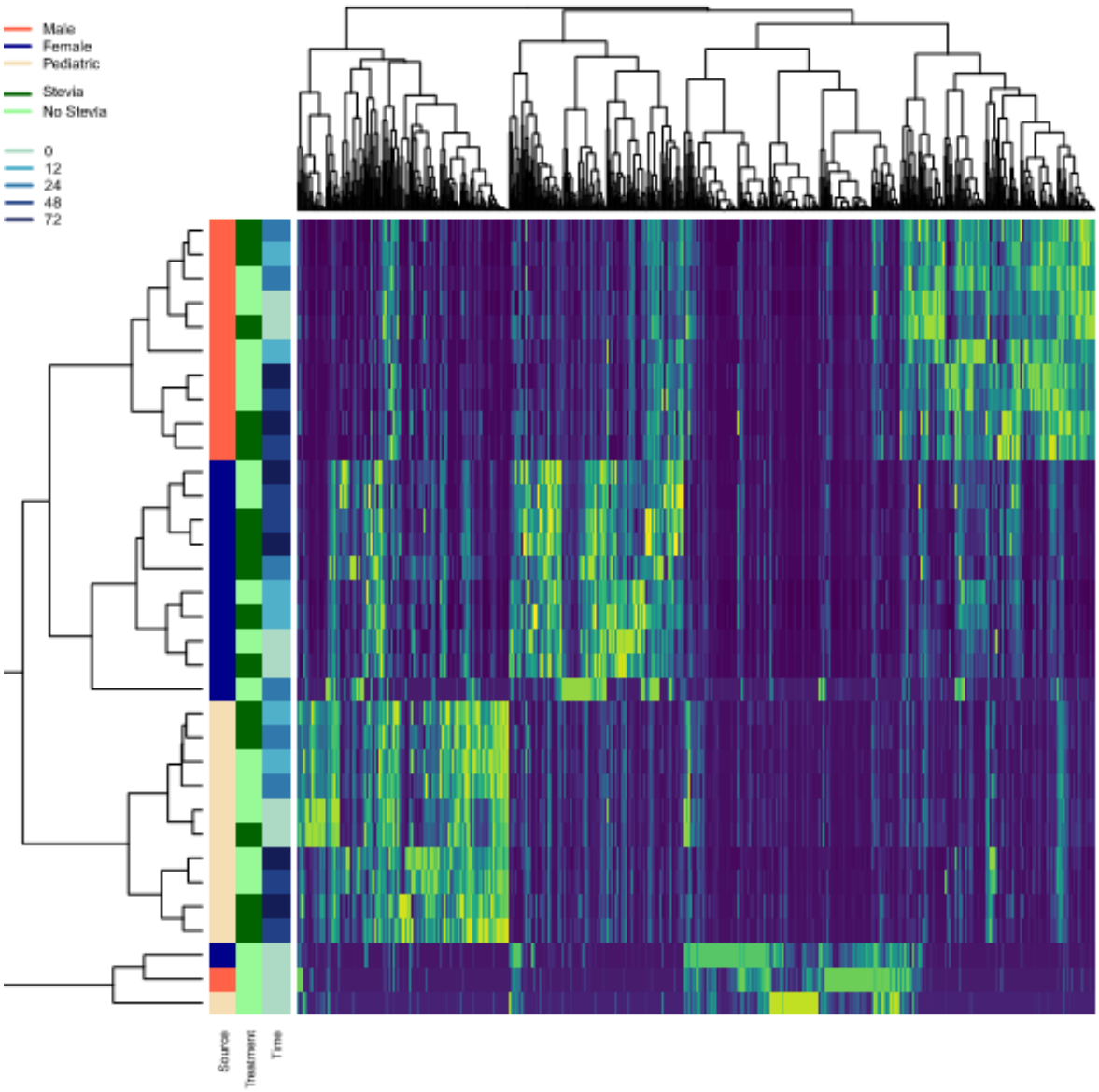

Figure 7. Hierarchical clustering analysis based on PCoA plots of the incubation samples in the presence and absence of steviol glycosides indicated the lack of steviol glycoside influence on the pooled fecal homogenate microbiome composition in adult male, adult female and children.

To evaluate whether steviol glycosides influenced the bacteria genera relative abundance data in a statistically significant manner, ANOVA data from each of the bacterial genera relative abundance revealed significant differences at 95% confidence interval (P-value <0.05) over the incubation times and between adult male, adult female and children study groups. These differences observed across the bacteria genera agrees with the expected changes in bacteria relative abundances during metabolic incubation, and between pooled adult male, adult female and children fecal homogenates. However, differences in the relative abundance of each bacteria genus were generally not significantly different between the presence and absence of steviol glycosides indicating that steviol glycosides at the relatively low concentration evaluated in this study to reflect a low dietary consumption level did not impact on bacteria relative abundance. ANOVA data is in agreement with PCoA analysis supporting a conclusion that steviol glycosides did not influence the bacteria genera relative abundance.

4. Discussion

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A study on the short-term and long-term macronutrients influences on the relative abundance of the human gut microbiome have been published [37]. For instance, short-term consumption of a predominant animal-based diet increased the relative abundance of bile-tolerant *Alistipes, Bilophila,* and *Bacteroides*, while decreased *Firmicutes* such as *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii* associated with metabolism of plant polysaccharides. The study revealed that microbes transiently colonize the human gut microbiome and can rapidly respond to changes in dietary components. The significance of diet in shaping the human gut microbiome has been reviewed [38]. There is a consensus that dietary alterations can induce large, temporary and predictable shifts in microbial populations within 24 hours and the authors suggested that dietary approach can be utilized to manipulate bacteria genera populations responsible for host immune response and metabolic parameters with broad potential applications for human health.

Specifically, dietary intake of low-caloric non-nutritive sweeteners and their potential impact on the gut microbiome have been reviewed and studied in preclinical models [39]. The authors concluded that the use of sweeteners in animal models have provided evidence of putative unfavorable effects on the gut microbiome and metabolic endpoints. In contrast, a recent review of published data from double-blind randomized controlled clinical trials involving non-nutritive sweeteners, including aspartame, acesulfame potassium, sucralose, saccharin and stevia did not reveal a meaningful attribution of non-nutritive sweeteners with alternations on the relative abundance and diversity of the human gut microbiome and metabolic health endpoints [40,42–44]. Furthermore, asides from the evaluation of relative distribution and diversity of the gut microbiome, the concept of "Keystone Species" of gut bacteria which can exert key influences responsible for individuality of the gut microbiome and health status have been proposed [45]. For instance, *Bacteroides fragilis* and *Bacteroides stercosis* have a disproportionate influence on the structure of the gut microbiome even though these species are only found in moderate abundance. Importantly, the presence of both species is associated with an increased risk of colon cancer [46]. These studies have highlighted the complex multivariate roles of the human gut microbiome in human health. The interplay between dietary influences and the bacteria keystone species associated with the status of a specific human health condition must be taken into consideration during interpretation of clinical data when evaluating the influence of steviol glycosides on the gut microbiome and its potential influence on human health.

In vitro human fecal homogenate culture conditions have also been previously evaluated to determine the impact of culture media conditions on microbial growth [47]. The potential health effects of four bacterial culture media including the use of a gut microbiota medium, a bacterial growth medium, a brain-heart infusion broth, and a fastidious anaerobic broth were investigated based on the formation of short-chain fatty acids under each in vitro culture conditions. The results from this study indicated that all four *in vitro* culture media exhibited different effects on the metabolism and composition of the human fecal microbiota. The limited number of published studies on human fecal homogenate culture conditions provided an initial understanding that the relative abundance and diversity of the fecal microbiome are susceptible to alterations by *in vitro* culture media conditions, hence, relevant culture conditions and appropriate study designs with experimental controls are required to allow valid interpretation of results. Clearly, more research is warranted.

An *in vitro* and *in vivo* study on the effects of synthetic sweeteners acesulfame potassium (AceK), saccharin, and sucralose, including the sweetener stevia, have also been reported [48]. The authors concluded that the synthetic and non-nutritive sweeteners all exerted strong bacteriostatic effects on the gut bacterial phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (E. coli). For sucralose, the authors reported an IC50 at 58.4 mM, equivalent to 23 mg/mL, which is 100 to 1000-fold of the levels found in commercial soda drink products labeled from 19 to 380 ug/mL. Similarly, the authors studied saccharin, AceK, and Reb A at 2.5% (25,000 ug/mL), which is in far excess of the typical 100 ug/mL concentrations of these sweeteners labelled in commercial soda drink products. Therefore, it is necessary to interpret the results from this study with caution with respect to the excessively high

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sweeteners concentrations studied and the relevant use of mouse gut microbiome data for inference to human gut microbiome.

In the current *in vitro* metabolic incubation investigation conducted under relevant experimental parameters, the results clearly showed that both Reb D and Reb M along with other minor glycosides in a stevia leaf extract were rapidly deglycosylated by the gut microbiome to steviol as a final major metabolite within 12 hours in a comparable manner in healthy adults (male and female) and in young children.

Beta diversity data from this study indicated that *Bifidobacterium* spp. is a predominant bacteria found in the fecal homogenate incubation samples from adult male, adult female and children. In addition, *Providencia* was also a predominant bacterium in both adult male and female, while *Enterococcus spp*. was observed in the children between 2 to 3 years of age. Amongst these predominant bacteria genera in the fecal homogenate incubation samples, *Bifidobacterium* spp. has been recognized as an important human gut microbiome genus in mediating beta-glucosidase activity [49], and the common predominant presence of *Bifidobacterium spp* in adult male, adult female and children fecal samples from the current investigation is in agreement with the observed functional efficiency of the human gut microbiome in the metabolic clearance of steviol glycosides.

5. Conclusion

The effects of steviol glycosides and their final common metabolite, steviol, on the human fecal homogenates indicated that the 20 most abundant bacteria genera were commonly observed in the pooled fecal homogenates collected from male, female and children. The observed abundant bacteria genera contributed to the overall abundance ranging from 75% to 85% of the diverse colonic population found in the representative fecal homogenate preparation. During the 72-hour metabolic incubation period, the overall % microbial abundance in the study preparations was observed to remain comparable between male, female and children. In addition, the potential influence of steviol glycosides and steviol on beta diversity of the microbial population was further evaluated and the % relative abundance of 22 major bacteria genera observed between male, female and children samples remained comparable over the entire metabolic incubation period.

Beta diversity data from this study indicated that *Bifidobacterium* spp. is a common predominant species found in representative fecal homogenates from male, female and children. Amongst the predominant bacteria genera, *Bifidobacterium* spp. has been recognized as an important human gut microbiome genus in mediating beta-glucosidase activity, and the common predominant presence of *Bifidobacterium spp* in male, female and children fecal samples are in the agreement with the observed functional efficiency of the human gut microbiome in deglycosylation of steviol glycosides. ANOVA (P-value <0.05) data from each of the most abundant 30 bacterial genera revealed non-significant differences in the relative abundance between steviol glycoside and placebo control, indicating that steviol glycosides at the relevantly low concentration evaluated in this study to reflect a low dietary consumption level did not reveal an observable impact on bacteria relative abundance. Hierarchical clustering analysis and PCoA plots of the incubation samples in the presence and absence of steviol glycosides and steviol indicated the lack of influence on the gut microbiome composition amongst the representative male, female and children pooled fecal homogenates.

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Conflict of Interest: The authors declare that no known completing financial interests or personal relationships that could have influenced the work and results reported in this paper. BRI Biopharmaceutical Research Inc. (a Frontage Laboratory company) is a pharmaceutical research laboratory commissioned to conduct this study for Purecircle™.

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