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Consumption of the Non-Nutritive Sweetener Stevia for 12 Weeks Does Not Alter the Composition of the Human Gut Microbiota

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Abstract: The use of non-nutritive sweeteners (NNSs) as an alternative to caloric sugars has increased in recent years. Stevia is an NNS that has demonstrated beneficial effects on appetite and energy intake. However, the impact on the gut microbiota is not well understood. Therefore, we investigated how regular consumption of stevia, for up to 12 weeks, impacts the human gut microbiota. Healthy subjects with a normal body mass index participated in our study; the stevia group (n = 14) was asked to consume five drops of stevia twice daily, compared to control participants (n = 13). Faecal samples collected before and after treatment were analysed by 16S rRNA gene sequencing. Stevia did not cause significant changes in the alpha or beta diversity when compared to the control groups. When the relative abundances of taxa were investigated, no clear differences were detected. Conversely, a random forest analysis correctly associated the gut microbiome with the control and stevia groups with an average of 75% accuracy, suggesting that there are intrinsic patterns that could discriminate between control and stevia use. However, large-scale changes in the gut microbiota were not apparent in this study, and, therefore, our data suggest that stevia does not significantly impact the gut microbiota.

Keywords: non-nutritive sweeteners; microbiota; gut; stevia

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1. Introduction

Stevia is a non-nutritive sweetener (NNS) that provides a sweet taste with no calories. Recent evidence supports the beneficial role of stevia and other NNSs on energy intake and body weight [1,2]. More specifically, two randomised controlled trials from our group have shown beneficial effects related to the consumption of stevia-sweetened beverages on appetite and energy intake in healthy adults following acute consumption and also after 3 months of stevia consumption [3,4]. Understanding the mechanisms mediating the effects of NNSs on appetite, food intake and health is of major nutritional and clinical importance, as their wider use could constitute a promising strategy for obesity management.

The gut microbiota is considered one of the key elements contributing to the regulation of host health [5]. Therefore, any changes in the composition or quality of the gut microbiota may have physiological consequences for the host. Host genetics, environmental factors and diet are considered the most important factors affecting microbiota composition [6,7]. There is a well-established relationship between certain microbial populations, insulin resistance and type 2 diabetes mellitus, implying that the gut microbiota affects glucose regulation [8,9]. A potential link has been proposed to exist between sweetener consumption, glucose metabolism and gut microbiota composition. Suez et al. showed that the

ingestion of saccharin by animals and humans induced alterations in metabolic pathways linked to glucose intolerance and dysbiosis in humans [10]. Despite this initial demonstration, more recent studies examining the effects of aspartame, sucralose and saccharin consumption on gut microbiota composition and including larger sample sizes and a longer exposure duration have not shown any significant changes in the gut microbiota [11–13]. However, a further study by Suez et al. showed personalised effects of NNSs on the microbiota [14]. To our knowledge, there are few other human trials that have assessed gut microbiota changes following repeated exposure to stevia, with a dose comparable to general consumption by the public.

Steviol glycosides, the sweet compounds of stevia, do not undergo degradation in the upper gastrointestinal tract, but they enter the colon intact and are degraded into glucose and steviol by the gut bacteria [15]. Steviol is absorbed and reaches the liver, where it conjugates with glucuronic acid to facilitate secretion [16,17]. Therefore, stevia may have a brief point of contact with the gut microbiota. Whether this contact is enough to induce changes in the gut microflora composition remains to be investigated.

Using stored faecal samples from our previously published randomised, controlled and open-label trial that showed an effect on body weight [3], the present study aimed to examine the effects of daily stevia consumption, in real-world quantities, on gut microbiota composition, diversity and community structure in healthy volunteers.

2. Materials and Methods

2.1. Study Design

This study was conducted as detailed in Stamataki et al. [3]. This study is a randomised controlled open-label two-parallel-arm trial conducted in healthy adults with normal body mass index (BMI). Participant assignment was random; using an online tool (www.random.com, accessed on 21 January 2019), an independent person created a random sequence of zeros and ones (zero meant that the participant would receive no treatment and one meant that the participant would receive stevia) that was pre-stratified by gender. Ethical approval was granted by the University of Manchester Research Ethics Committee (2018-4812-7661); all subjects signed an informed consent form before participation in this study and received compensation for their time. The trial was registered at Clinicaltrials.gov under the registration number NCT03993418.

In the current study, participants were randomly allocated to either daily consumption of stevia—in liquid form, administered as 5 drops of a commercially available product (SweetLeaf Stevia Sweet Drops Clear, Sweetleaf®, Gilbert, AZ, USA) in habitual beverages twice a day, ideally before lunch and before dinner (total 10 drops; 5 drops of stevia correspond to the sweetness of one teaspoon of table sugar)—or to the control group, where no changes in usual diet were required.

2.2. Participants

The full participant criteria are detailed in Stamataki et al. [3]. In brief, healthy adults with a normal BMI (18.5–24.9 kg/m^2), aged 18–40 years old, who were non-habitual consumers of NNSs ($\leq$1 can of diet beverages per week or $\leq$1 sachet of NNSs per week) and non-restrained eaters (restraint eating score in the Dutch Eating Behaviour Questionnaire (DEBQ) $\leq$ 3) were recruited. Other inclusion criteria were fasting blood glucose $\leq$6 mmol/L, stable weight for the last 12 months ($\pm$5 kg), willingness to comply with the study protocol and no self-reported food allergy or intolerance to foods supplied during our study. Exclusion criteria were being on a diet or having ceased a diet in <4 weeks, following any special diets for weight maintenance, being vegetarian or vegan, having alcohol consumption of more than 14 units a week, more than 10 h of vigorous physical activities per week and/or planning to increase or decrease physical activity levels in the future, having ceased smoking in the last 6 months and female participants who were or may have been pregnant or were lactating.

Participants who were eligible to participate completed an online screening questionnaire, and if inclusion criteria were satisfied, they were invited to a screening session on site following an overnight fast. Fasting blood glucose, weight and height were measured, and all details of our study were explained to participants. Eligible participants were invited to the full study and consented prior to participation.

A sample size calculation was conducted for the primary outcome, which was glucose response to an oral glucose tolerance test (OGTT), as described in our previous publication [3]. A total of 28 participants completed our study, and a detailed flowchart is provided (Figure S1). Faecal samples were collected from 14 participants in the stevia group and 13 participants in the control group.

2.3. Study Protocol

Participants randomised to the stevia group were required to start consuming stevia (in liquid form) with their habitual drinks twice a day, while the control group received no intervention. During the intervention period, participants were required to avoid all other NNSs in beverages or foods, and they received training regarding the hidden sources of any NNS by a dietician. In addition, participants were asked to refrain from consuming any probiotic supplements. The stevia product administered in this study was selected based on its purity, as it contained only stevia leaf extract and water. Due to the nature of this study, blinding could not be performed. Participants were aware of the trial arm to which they had been randomised (the stevia arm or the control arm). Therefore, we did not eliminate cognitive factors that may have occurred when subjects consumed stevia.

Participants attended 3 study visits: one at baseline, one at 6 weeks and the last one at 12 weeks. For visit week 0 and visit week 12, participants fasted overnight; for visit week 6, they had to abstain from foods and drinks for 2 h prior to the scheduled time visit. Dietary intake was assessed via three 24 h diet recalls at week 0, week 6 and week 12 (results can be found in our previous publication [3]). All study procedures were conducted at the Neuroscience and Psychiatry Unit at the University of Manchester. A summary of the study schedule for assessment is presented (Table S1).

A detailed description of the outcomes presented in the original study, such as the glucose and insulin response to the oral glucose tolerance test, weight, energy intake, blood pressure and appetite questionnaires, can be found in Stamataki et al. [3].

2.4. Faecal Sample Collection

Participants were required to collect a faecal sample at home one day prior to the study visit. Faecal sample collection kits were provided to the participants. They were advised to collect samples from 3 random sites of the stool. After collection, participants were asked to keep the sample immediately in cold storage (−4 °C) for up to 24 h and bring it with them in the morning. Once samples were collected at our lab, they were stored at −80 °C until further analysis. Although our study was open-label, the processing and analysis of faecal samples were conducted by a blinded researcher.

2.5. Isolation of Genomic DNA and PCR

A total of 200 mg of stool was collected from each participant, and microbial DNA was extracted using a Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Manchester, UK), in line with the manufacturer's instructions. First-round polymerase chain reaction (PCR) was performed to amplify the V4 hypervariable region (V4_515fb.F: 5′-TCGTCGGCAG CGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3′ and 4_806rB.R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3′) using KAPA HiFi Hotstart ReadyMix (Roche, Burgess Hill, UK). The PCR products were submitted for sequencing as detailed below.

2.6. 16S rRNA Sequencing

A total of 54 samples, including additional experimental controls, were submitted for 16S rRNA sequencing. The Illumina 16S metagenomic Sequencing Library Preparation protocol was followed. All samples underwent amplicon PCR clean-up using AMPure beads (Beckman Coulter, Indianapolis, IN, USA). Illumina sequencing adapters and dual-index barcodes were added to each library using an Index PCR Illumina XT Index Kit v2 (Illumina Inc., San Diego, CA, USA) followed by PCR clean-up with AMPure beads (Beckman Coulter). Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (Thermo Fisher Scientific, Waltham, MA, USA). Library fragment-length distributions were analysed using the Agilent TapeStation 4200 and the Agilent D1000 ScreenTape Assay (Agilent Technologies, Santa Clara, CA, USA). Libraries were then pooled in equimolar amounts. Library pool quantification was performed using the KAPA Library Quantification Kit for Illumina (Roche). The library pool was sequenced on an Illumina MiSeq using a MiSeq v2 500 cycle Kit (Illumina; MS-102-2003, San Diego, CA, USA) to generate 250 bp paired-end reads.

2.7. Microbiota Data Analysis

Data were analysed using QIIME 2.0 [18], with DADA2 [19] as the de-noising step. The mean number of reads in the experimental samples after quality filtering was 179,850 (range 93,060–426,047). There were 3 reads in a negative control of purified water after de-noising, which was deemed comparatively negligible. Microbiota data were processed using the Phyloseq R package [20], version 1.3.6. To avoid bias caused by sequencing depth during diversity analysis, data were rarefied to 61,353 reads (the lowest read depth). Beta diversity was calculated using weighted UniFrac [21] and plotted using principle coordinates analysis (PCoA) with the 'plot_ordination' function in the Phyloseq package. Jaccard Index values were also calculated amongst all samples and plotted as non-metric multidimensional scaling (NMDS) graphs using the 'plot_ordination' function, checking to ensure convergence in all cases. A scree plot was generated using the dimcheckMDS function in the 'goeveg' R package [22], version 0.5.1, to ensure that stress values were below the 0.2 acceptability threshold [23].

The relative abundance of each taxon was calculated at each taxonomic level. Taxa that made up less than 0.01% of the total abundance for phyla, class and order; less than 0.02% for family; and 0.011% for genera were merged into one group. Differential abundance between taxa was calculated using the DESEQ2 R package [24], version 1.32.

Random forest analysis was performed with the 'randomForest' R package, version 4.6, using relative abundances based on identified genera. 'Out-of-Bag Error' (OOB) values were obtained and used to estimate the predictive accuracy of the model in finding associations between the microbiota and the different treatment groups. To validate these associations, taxon abundance and samples were randomised to create a 'negative control' model in order to see whether these associations were simply due to chance. Each forest was controlled for all other treatments (i.e., a random forest-predicting treatment also included time as an explanatory variable). Taxa most important for making distinctions were identified based on their 'MeanDecreaseAccuracy' values.

2.8. Statistical Analysis

Statistical differences between groups in beta diversity were calculated with PERMANOVA, using the adonis function in the 'vegan' [25] R package, version 2.5.7. Differences in alpha diversity between groups were calculated using pairwise Wilcoxon Rank Sum tests using the 'pairwise.wilcox.test' function in R. Statistical analysis on differential abundance was performed with the calculation of geometric means before estimation of size factors in the same software using the DESeq2 package and the DESeq function. Significantly different taxa were defined as those with $p < 0.01$.

3. Results

3.1. No Changes in Beta and Alpha Diversity between Treatments

To investigate large-scale patterns in the data, beta diversity was calculated using weighted UniFrac and plotted as a PCoA (Figure 1). It can be seen that separation along the *x*-axis explained 90.9% of the variation within the data, which visually appears to be separated by the stevia treatment. However, PERMANOVA suggested that there were no significant differences in beta diversity between any of the comparisons, combining all treatments ($p = 0.947$), comparing time (i.e., 0 weeks vs. 12 weeks ($p = 0.121$)) and comparing treatment (i.e., control vs. stevia ($p = 0.978$)). A similar analysis using the Jaccard Index also showed no differences between groups, comparing all treatments combined ($p = 0.981$), time ($p = 0.9964$) and treatment ($p = 0.981$) (Figure S2). The stress value was 0.17 (Figure S2), within the 0.20 acceptability threshold.

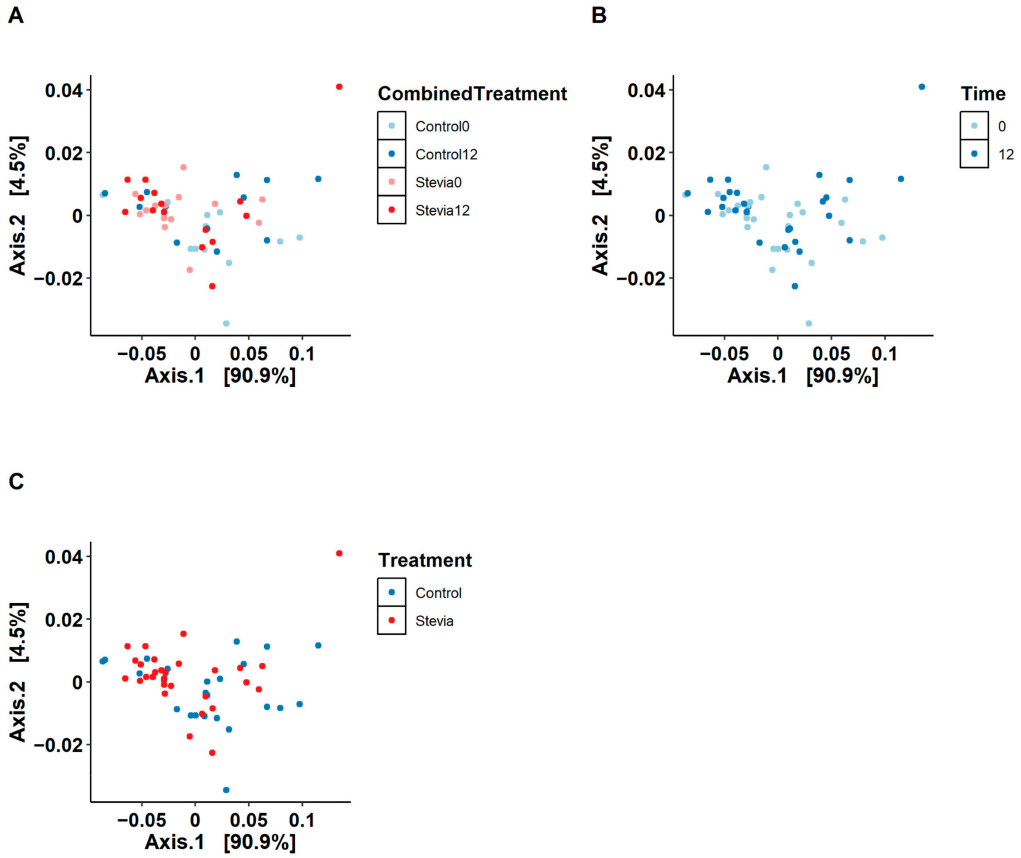

Figure 1. Principle Coordinates Analysis (PCoA) of gut microbiota data from control vs. stevia groups. Healthy participants were asked to consume five drops of the sweetener stevia twice daily, compared to control participants. Stool samples were collected from these participants at baseline (0 weeks) and 12 weeks after the intervention, and 16S rRNA sequencing was performed to analyse the gut microbiome. PCoA was plotted using the weighted UniFrac method, comparing all experimental groups (**A**), time only (**B**) and control vs. stevia irrespective of time (**C**).

Alpha diversity was calculated to give a measure of sample richness (number of different taxa) and evenness (spread of taxa abundance) between groups (Figure 2). All groups had a median of around 300 observed OTUs, with no significant difference between groups ($p = 0.966$ when comparing all treatments). An analysis of evenness showed that all groups were comparable with relatively even community composition (median values > 0.75). There were no significant differences when comparing all treatments ($p = 0.346$), and there were similar levels of intra-group variability (Figure 2B). An analysis of the Shannon Index, which takes into account both evenness and richness, showed no

significant difference between groups when comparing all treatments ($p = 0.652$) (Figure 2C). All groups had a large amount of variability.

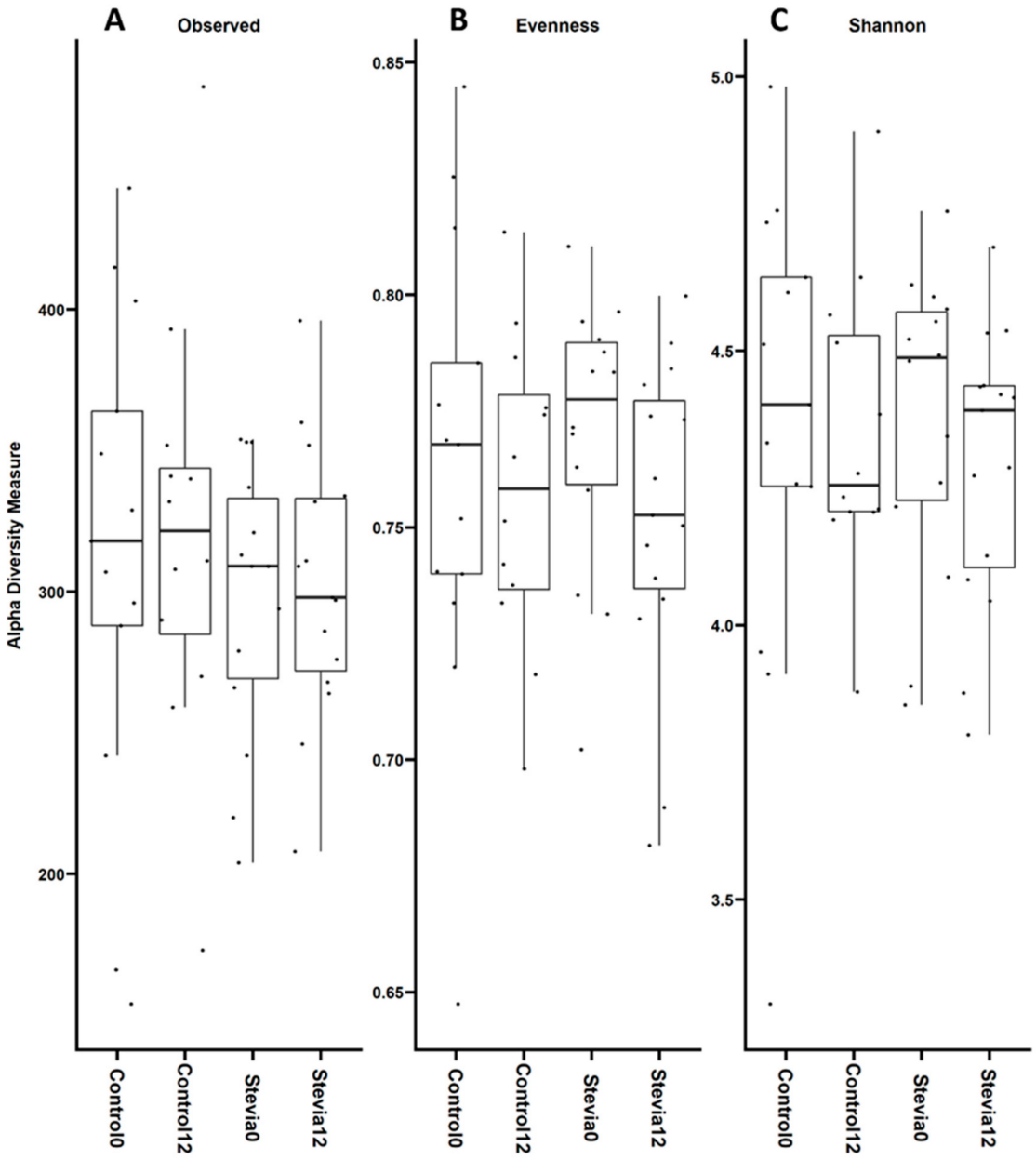

Figure 2. Alpha diversity analysis of gut microbiota data from control vs. stevia groups. Healthy participants were asked to consume five drops of the sweetener stevia twice daily, compared to control participants. Stool samples were collected from these participants at baseline (0 weeks) and 12 weeks after the intervention, and 16S rRNA sequencing was performed to analyse the gut microbiome. Alpha diversity (in terms of observed taxa (**A**), evenness (**B**) and Shannon Index (**C**)) was plotted comparing all groups. Individual data points are also shown on each graph.

3.2. Taxa Abundance Is Similar between Control and Stevia Groups

The taxonomic composition of the data comprised 15 phyla, 27 classes, 42 orders, 68 families and 130 genera (the numbers refer to unique taxa only). When taxa abundance was investigated at the phylum level (Figure 3A), the dominant phyla were Firmicutes, Bacteroidetes and Actinobacteria. At baseline (0 weeks), the control and stevia groups had similar proportions of phyla, although the proportion of Actinobacteria appeared to be lower in the stevia group at both timepoints. Additionally, virtually no Proteobacteria in the

stevia group could be visualised. At the genus level (Figure 3B), relative abundances were similar between the control and stevia groups. However, at 12 weeks, *Clostridium* and *Dorea*, which were not present at baseline, could be identified in the stevia group. *Clostridium* and *Megamonas* were no longer present in the control group at 12 weeks, whereas *Dorea* could also be identified at 12 weeks in this group. Relative abundances at the class, order and family levels were also plotted (Figure S3), although no major differences were visualised.

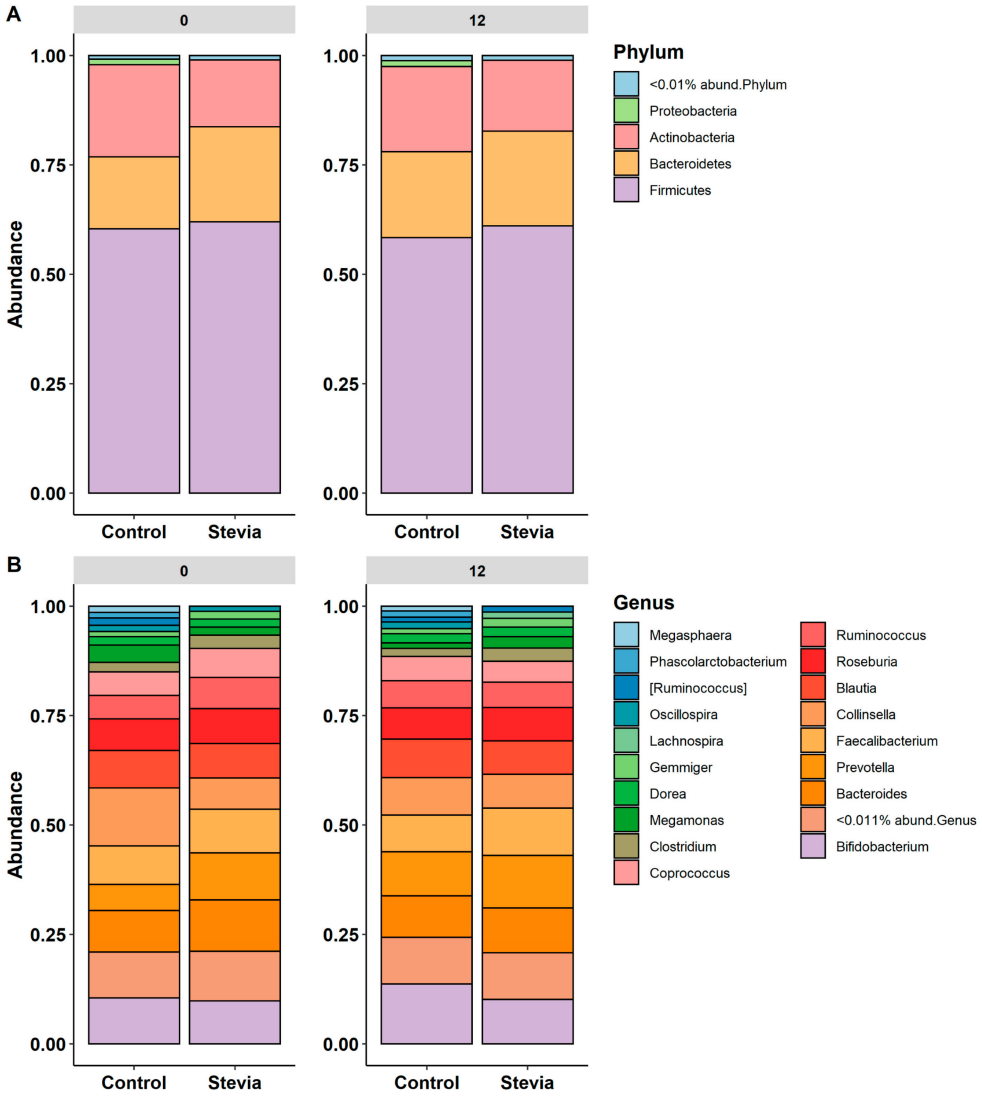

Figure 3. Comparison of taxa abundance between control and stevia groups. Healthy participants were asked to consume five drops of the sweetener stevia twice daily, compared to control participants. Stool samples were collected from these participants at baseline (0 weeks) and 12 weeks after the intervention, and 16S rRNA sequencing was performed to analyse the gut microbiome. Taxa abundance was plotted for phyla (**A**) and genera (**B**), with taxa having less than 0.01% or 0.011% abundance, respectively, combined into a single fraction.

Differential abundance was calculated to identify which taxa, if any, were statistically significantly different between groups. When the control groups were compared at baseline and 12 weeks, 15 genera were significantly different between the groups (Figure 4A). Variation also existed at baseline between the control and stevia groups (Figure 4B), with 19 significantly different genera. When stevia levels at baseline and at 12 weeks were compared (Figure 4C), there were only nine significantly different genera between the two timepoints. Notably, stevia treatment led to a significant decrease in *Akkermansia* and

an increase in *Faecalibacterium*. When the control and stevia groups were compared at 12 weeks (Figure 4D), many of the significantly different taxa were already significantly different at baseline. *Butyricoccus* was the only genus identified as significantly different at 12 weeks that was not already different at baseline. However, some species were significantly different at baseline, but not after 12 weeks of stevia consumption. These bacteria included *Collinsella* and *Aldercreutzia*. Additionally, there were two *Coprococcus* species identified as significantly different at baseline (one higher and one lower when comparing stevia vs. control). However, after 12 weeks, both species were significantly elevated in the stevia group.

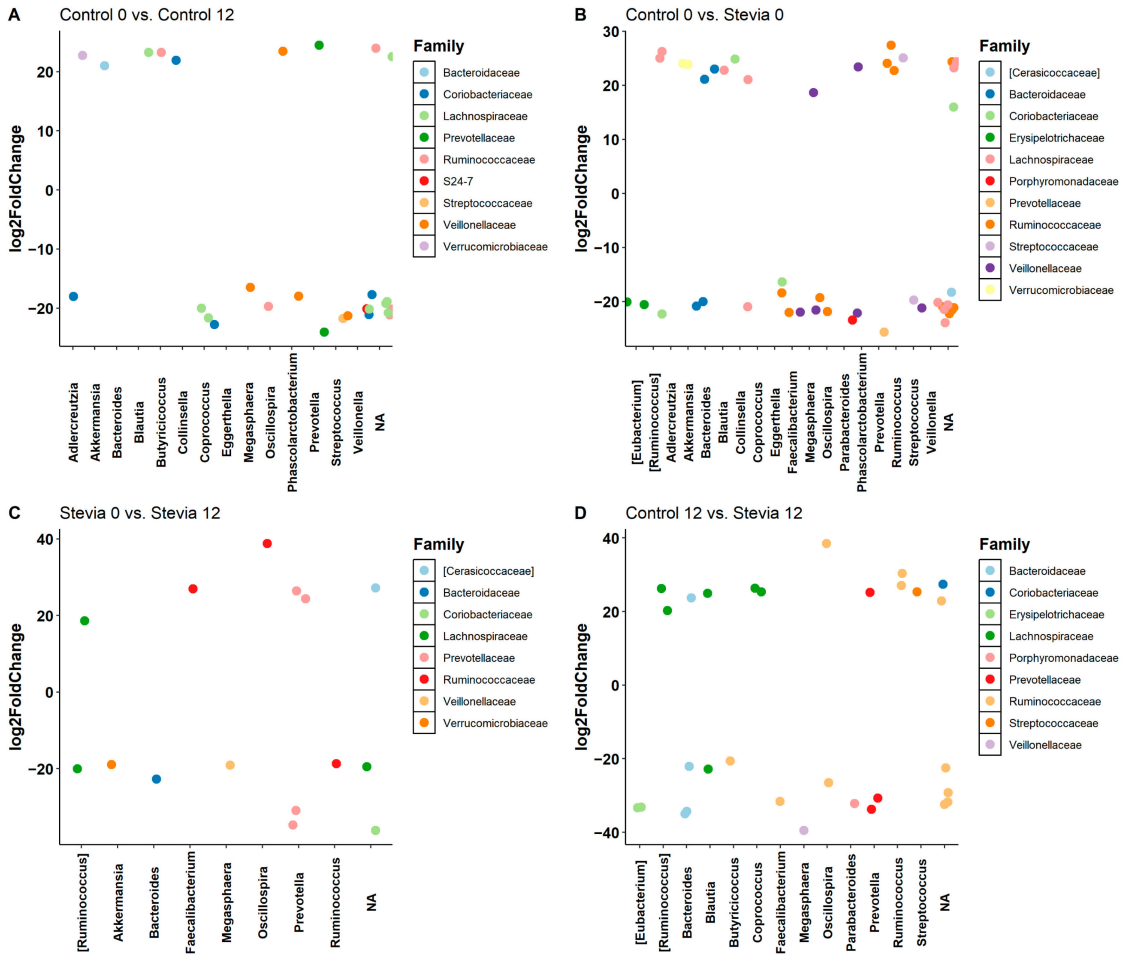

Figure 4. Differentially expressed bacteria between groups. Healthy participants were asked to consume five drops of the sweetener stevia twice daily, compared to control participants. Stool samples were collected from these participants at baseline (0 weeks) and 12 weeks after the intervention, and 16S rRNA sequencing was performed to analyse the gut microbiome. The differential abundance of taxa between groups (Control 0 weeks vs Control 12 weeks (**A**), Control 0 weeks vs Stevia 0 weeks (**B**), Stevia 0 weeks vs Stevia 12 weeks (**C**) and Control 12 weeks vs Stevia 12 weeks (**D**)) was calculated using the DESeq2 R package. Taxa that could not be resolved at the genus level were referred to as NA.

3.3. Random Forest Finds Associations between Microbiota and Stevia Group

A random forest was employed to investigate any associations between the gut microbiota and the different groups in this study (Figure 5). Significant associations could be found between the gut microbiota when comparing control vs. stevia (Figure 5A) ($p < 0.05$). Indeed, the model was able to associate the microbiota with each treatment correctly with approximately 75% accuracy. The negative control model had only ~50% accuracy. The taxa

most strongly associated with this comparison were *Dehalobacterium*, *Methanobrevibacter*, *Oscillospira* and *Oxalobacter*. No significant associations could be found when comparing time (Supplementary Figure S4A) or when the treatment groups were combined (Supplementary Figure S4B). The taxa that were identified as most important for time are shown in Figure S5A. Notably, two genera were shared between the treatment-only comparison and the comparison in which the treatment groups were combined (*Dehalobacterium* and *Oxalobacter* (Figure S5B)).

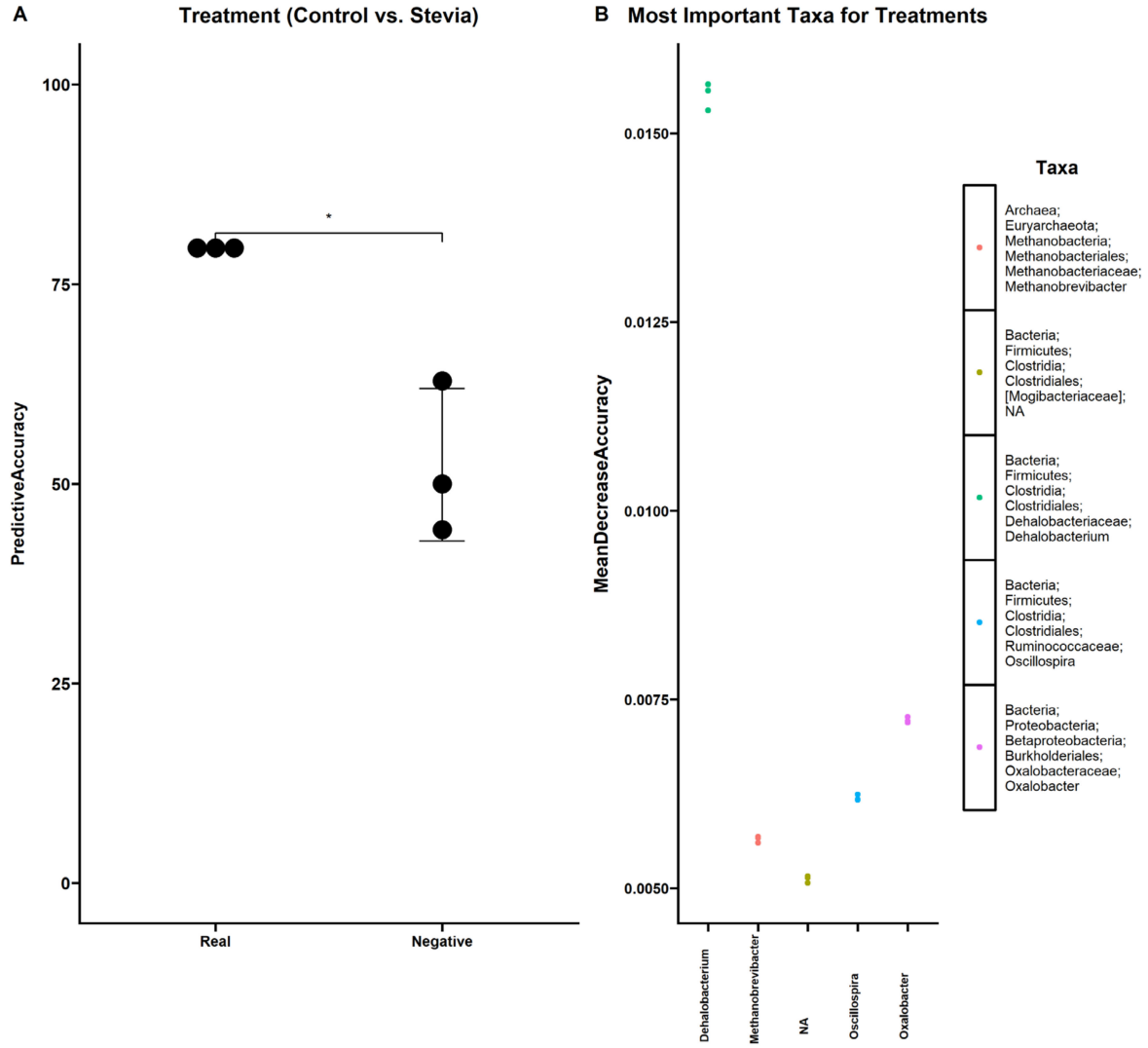

Figure 5. Associations between the gut microbiota and stevia group. Healthy participants were asked to consume five drops of the sweetener stevia twice daily, compared to control participants. Stool samples were collected from these participants at baseline (0 weeks) and 12 weeks after the intervention, and 16S rRNA sequencing was performed to analyse the gut microbiome. Random forest was used to find associations between the relative abundance of identified genera and the accuracy of the model (**A**) and the genera most strongly associated with these groups (**B**). Individual data points are shown (±SEM). Taxa that could not be resolved at the genus level were referred to as NA. * $p < 0.05$.

4. Discussion

We investigated how the use of stevia could impact the human gut microbiota in a randomised controlled open-label trial. Various studies have investigated the impact of stevia in different contexts, such as mice [26] or using in vitro models [27], but to our knowledge, few clinical studies have specifically investigated the impact of stevia on the

gut microbiota. Some clinical studies investigated the use of alternative NNSs, such as saccharin [10,13], sucralose [11] and aspartame [11,28]. Suez et al. [10] conducted a key previous investigation into the impact of NNSs on the microbiota and reported that saccharin consumption led to glucose intolerance induced by microbial gut dysbiosis. They followed this study with a comprehensive assessment of various other NSSs, including stevia [14]. Although this study was conducted over a shorter period of time (2 weeks) compared to the current study (12 weeks), Suez et al. [14] found that stevia had no impact on glucose tolerance, supporting our previous findings [3]. In terms of the gut microbiota, they reported that stevia had no significant impact on the microbiota composition but did significantly impact the microbiota function. Upon investigating the factors underlying this change in function, they reported that stevia had a prominent impact on fatty acid biosynthesis, with other NNSs impacting different functions. Different sweeteners have different chemical compositions (summarised by Turner et al. [29]), and, therefore, their functional impacts on the host and their metabolism by the microbiota are likely to be different.

We also investigated other metrics of community composition, specifically beta and alpha diversity, where we found no significant differences between the treatment groups (Figures 1 and 2). These findings are in line with previous studies that investigated the impact of stevia on the gut microbiota, although these studies were conducted using an in vitro model [30] or in rats [31]. Although we do not see significant differences in diversity, our PCoA plots showed strong separation along the *x*-axis (Figure 1A–C), which can also be seen visually in how the stevia and control participants separate on the graph. While these data may imply baseline differences in the community composition between the control and stevia participants, it was not a significant difference. Notably, all groups had comparable alpha diversity, suggesting that the community composition in each group was relatively even over time and equally diverse.

Changes in the gut microbiota, at any taxonomic level, are often associated with functional impacts on the host [32,33]. In our study, at the phylum level, the taxonomic profile closely resembled a typical gut profile (Figure 3A), dominated by Firmicutes and Bacteroidetes, but Actinobacteria were also prevalent, with a smaller fraction of Proteobacteria and other phyla. The ratio of Firmicutes to Bacteroidetes has often been explored in the context of obesity [34,35], and, indeed, the use of NNSs is frequently associated with energy intake and weight [1,36]. As discussed, our previous study investigated the impact of stevia on glucose tolerance, but body weight and energy intake were also explored [3]. We found that although there were no differences in glucose tolerance, there were significant differences in energy intake after 12 weeks of stevia consumption [3]. Based on our current observations, the impact on energy intake is unlikely to be mediated by the Firmicutes-to-Bacteroidetes ratio between the control and stevia participants. The microbiota is strongly associated with the production of short-chained fatty acids (SCFAs) that may mediate relevant functional effects on the host [37,38]. One aspect that could be investigated in our future work is the production of SCFAs in response to stevia treatment, alongside microbial profiling.

In terms of specific differences in the gut microbiota, differential abundance analysis revealed that only one taxon identified was significantly different between the control and stevia groups after 12 weeks: Butyricoccus. This was downregulated after the consumption of stevia. One study investigated the impact of various sweeteners in rats and found that honey downregulated *Butyricoccus pullicaecorum* [39], a butyrate-producing species [40]. Blautia was significantly upregulated at baseline and significantly downregulated after 12 weeks of stevia consumption. In contrast, an in vitro study revealed that stevia treatment of human faecal samples led to no significant differences in the expression of *Blautia coccoides* [27]. Blautia is associated with the production of butyric and acetic acids [41]. Thus, it could be that stevia leads to a reduction in butyrate-producing species. Indeed, after 12 weeks compared to baseline, the stevia group had a significant reduction in *Megasphaera* bacteria, with some species associated with butyrate production [42]. However, this was not significantly different when compared to the control group.

Our random forest identified taxa that were strongly associated with either the control or stevia participants (Figure 5) but were not identified by differential abundance analysis. Notably, the most important microorganism identified was *Dehalobacterium*, a bacterium previously associated with a high BMI [43]. A study in mice revealed that sucralose treatment had a notable impact on the *Dehalobacteriaceae* family [44]. Curiously, these bacteria were also found in significant abundance in the intestinal mucus of a colitic mouse model [45]. Previous studies have highlighted the difference between stool and mucus-resident bacteria [30,46]; therefore, there could be changes in the mucus bacteria that we do not see in this study.

One of the strengths of our study is that, presently, this is one of a limited number of studies to investigate the effects of stevia consumption on the human gut microbiota, where the dose of stevia reflects a normal dose to simulate regular consumption by the general population. The stevia used was also a pure stevia product (stevia in water), so any effects demonstrated can be linked to stevia. However, some limitations should be reported. Our study was powered by the primary outcome, which was postprandial glucose response to an oral glucose tolerance test [3]. Gut microbiota analysis was an exploratory secondary outcome, as reported in the study registration. Additionally, as discussed, more functional analysis with respect to metabolite analysis could further strengthen this study. Participants were selected to be healthy and with a normal body mass index, as it is crucial to first establish the impact of low-calorie sweeteners on the gut microbiota in healthy individuals before investigating their effects on other population groups (such as people with obesity, overweight, or type 2 diabetes mellitus), which would nevertheless be an appropriate subsequent research question.

5. Conclusions

Overall, although there may be several individual taxa that are associated with stevia use, we found no significant differences in overall community composition after 12 weeks of stevia consumption at real-life doses. Therefore, our data suggest that regular, long-term consumption of stevia does not significantly impact the human gut microbiota.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16020296/s1, A flow chart summarising the clinical study is provided (Figure S1: Participant flow chat). We have also included supplementary data pertinent to ordination (Figure S2: NMDS of gut microbiota data plotted using Jaccard Index), relative abundance (Figure S3: Comparison of relative taxa abundance between control and stevia groups) and our random forest models (Figure S4: Associations between gut microbiota and stevia group; Figure S5: Important taxa identified for random forest model). We have also included a table summarising assessment criteria and procedures (Table S1: Summary of the study procedures and assessment).

Author Contributions: G.S. prepared samples for sequencing, performed the microbiota analysis, made figures and co-wrote the manuscript. A.J.M. supervised the microbiota aspect of the project and critically reviewed and edited the manuscript. J.T.M. designed and supervised the clinical aspect of the project and critically reviewed and edited the manuscript. N.S.S. designed and facilitated the clinical study, made figures and tables and co-wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the University of Manchester (project number: 2018-4812-7661, date of approval: 11 December 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Sequence data analysed during the current study will be made available. Code to reproduce the main text figures produced in R will be available on FigShare (https://doi.org/10.48420/24970158, accessed on 11 January 2024).

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Conflicts of Interest: The authors declare no conflict of interest.

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