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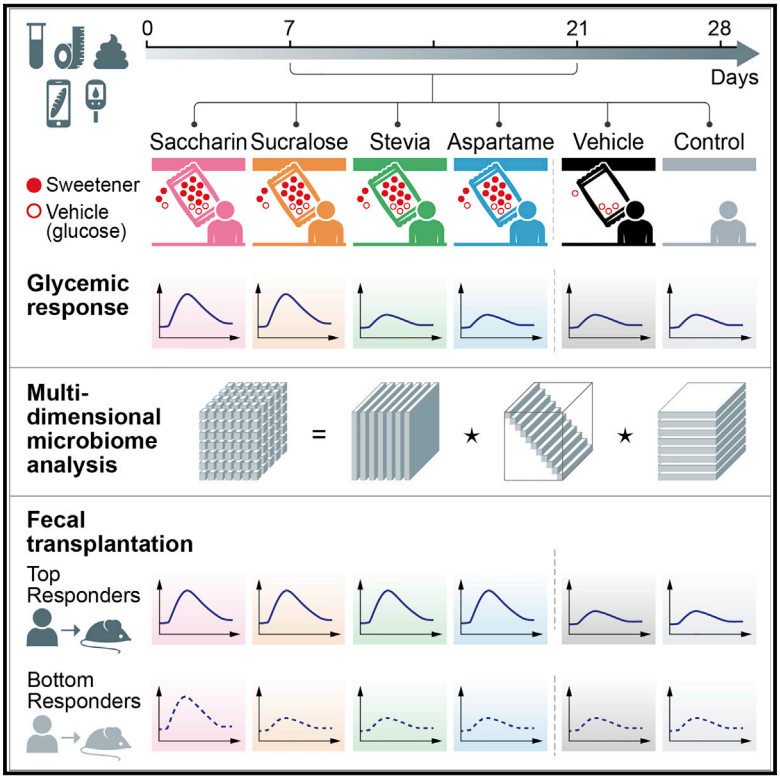
Personalized microbiome-driven effects of non-nutritive sweeteners on human glucose tolerance

Graphical abstract



Highlights

- Randomized-controlled trial on the effects of non-nutritive sweeteners in humans
- Sucralose and saccharin supplementation impairs glycemic response in healthy adults
- Personalized effects of non-nutritive sweeteners on microbiome and metabolome
- Impacts on the microbiome are causally linked to elevated glycemic response

Authors

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In brief

A study of the effects of non-nutritive sweeteners on human metabolism as well as their microbiomes reveals how these can induce individual-specific, microbiome-dependent changes to glycemic responses, warranting follow-up clinical studies to understand long-term impact.

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Personalized microbiome-driven effects of non-nutritive sweeteners on human glucose tolerance

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SUMMARY

Non-nutritive sweeteners (NNS) are commonly integrated into human diet and presumed to be inert; however, animal studies suggest that they may impact the microbiome and downstream glycemic responses. We causally assessed NNS impacts in humans and their microbiomes in a randomized-controlled trial encompassing 120 healthy adults, administered saccharin, sucralose, aspartame, and stevia sachets for 2 weeks in doses lower than the acceptable daily intake, compared with controls receiving sachet-contained vehicle glucose or no supplement. As groups, each administered NNS distinctly altered stool and oral microbiome and plasma metabolome, whereas saccharin and sucralose significantly impaired glycemic responses. Importantly, gnotobiotic mice conventionalized with microbiomes from multiple top and bottom responders of each of the four NNS-supplemented groups featured glycemic responses largely reflecting those noted in respective human donors, which were preempted by distinct microbial signals, as exemplified by sucralose. Collectively, human NNS consumption may induce person-specific, microbiome-dependent glycemic alterations, necessitating future assessment of clinical implications.

INTRODUCTION

Over the past 4 decades, the global prevalence of overweight, obesity, and hyperglycemia has markedly increased in both children and adults, constituting a considerable health threat due to the association of these conditions with type 2 diabetes and cardiovascular disease (NCD Risk Factor Collaboration, 2017; Sarwar et al., 2010), coupled with substantial economic ramifications (Imes and Burke, 2014). As sugar consumption is strongly associated with weight gain (Hu, 2013), one of the most common dietary strategies in combating obesity and hyperglycemia involves dietary sugar replacement with non-nutritive sweeteners (NNS), such as saccharin, sucralose, aspartame, acesulfame-K, and stevia, that do not contain calories and are thereby presumed to be inert and not elicit a postprandial glycemic response. This strategy is immensely popular. In a survey conducted between 2009 and 2011, 25.1% of children and 41.4% of adults in the United States reported consuming NNS, a marked increase compared with 1999 (Sylvetsky et al., 2017a). Over 50% of children reported consumption of NNS in a multi-national study (Katzmarzyk et al., 2016), whereas countries enforcing labeling of sugar-containing products observe a

high concomitant consumption of NNS-containing products (Martínez et al., 2020).

Nonetheless, the efficacy of this strategy remains uncertain. Although some randomized-controlled trials (RCTs) report improvement in metabolic markers in subjects supplemented with NNS (Blackburn et al., 1997; Ebbeling et al., 2020; Katan et al., 2016; Masic et al., 2017; Miller and Perez, 2014; Tate et al., 2012), other RCTs report neither a detrimental nor a beneficial effect (Ahmad et al., 2020a; Thomson et al., 2019) and do not support the intended benefits of this approach (Azad et al., 2017; Lohner et al., 2020; Toews et al., 2019). Furthermore, some cohort studies (Azad et al., 2017, 2020; Romo-Romo et al., 2016; Swithers, 2013) and RCTs (Bueno-Hernández et al., 2020; Dalenberg et al., 2020; Lertrit et al., 2018; Méndez-García et al., 2022; Romo-Romo et al., 2018) counterintuitively suggest that NNS may even contribute to the obesity and diabetes pandemic in some contexts. As many of the studies associating NNS with negative impacts on human health are observational, it is often difficult to interpret their findings due to reverse causality (i.e., whether NNS cause weight gain and hyperglycemia, or alternatively whether individuals with these conditions consume NNS). The heterogeneity in outcomes and methodology between RCTs further complicates interpretation. In the absence of strong evidence for causality and a clear mechanism demonstrating how "metabolically inert" substances can affect human metabolism, consumption of NNS is still widely endorsed by clinicians and dietitians for adults (Gardner et al., 2012), although a more cautious approach has been lately recommended for children (Johnson et al., 2018).

To partially circumvent the limitations of human studies, feeding trials in animals are commonly used to causally link NNS intake with an effect on cardiometabolic diseases. However, although animal studies are less heterogeneous than human RCTs, some reported an adverse impact of NNS on metabolic health (Abou-Donia et al., 2008; Bian et al., 2017a, 2017b; Collison et al., 2012; Feijó et al., 2013; Gul et al., 2017; Leibowitz et al., 2018; Mitsutomi et al., 2014; Nettleton et al., 2020; Olivier-Van Stichelen et al., 2019; Otero-Losada et al., 2014; Palmnäs et al., 2014; von Poser Toigo et al., 2015; Shi et al., 2019; Suez et al., 2014; Swithers et al., 2008; Uebanso et al., 2017), whereas others reported a beneficial effect or no effect (Bailey et al., 1997; Parlee et al., 2014; Risdon et al., 2020; Serrano et al., 2021; Tovar et al., 2017).

This noted and often confusing heterogeneity between trials could potentially be resolved through consideration of the gut microbiome. The human gastrointestinal tract harbors trillions of microorganisms that play critical roles in multiple aspects of human physiology and pathologies, including cardiometabolic health (Fan and Pedersen, 2021). Importantly, the assemblage of microorganisms varies between individuals (and between animals in different research vivaria), leading to personalized responses to diets (Berry et al., 2020; Korem et al., 2017; Kovatcheva-Datchary et al., 2015; Spencer et al., 2011; Zeevi et al., 2015) and therapeutics (Ananthakrishnan et al., 2017; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; Zmora et al., 2018). Indeed, NNS have been shown to affect microbial growth in culture (Harpaz et al., 2018; Omran et al., 2013; Rettig et al., 2014; Wang et al., 2018) and modulate the microbiome of animals (Abou-Donia et al., 2008; Anderson and Kirkland, 1980; Bian et al., 2017a, 2017b, 2017c; Cheng et al., 2021; Chi et al., 2018; Dai et al., 2021; Daly et al., 2014; Guo et al., 2021; Hanawa et al., 2021; Harrington et al., 2022; Li et al., 2021; Lyte et al., 2016; Martínez-Carrillo et al., 2019; Nettleton et al., 2019, 2020; Olivier-Van Stichelen et al., 2019; Palmnäs et al., 2014; Rodriguez-Palacios et al., 2018; Sánchez-Tapia et al., 2020; Sünderhauf et al., 2020; Uebanso et al., 2017; Wang et al., 2018; Zheng et al., 2022). Furthermore, an NNS-modulated microbiome is sufficient to promote glucose intolerance in germ-free (GF) mice (Nettleton et al., 2020; Suez et al., 2014), providing a possible causal link between NNS, microbiome, and metabolic health of the host. Although a small pilot study in humans suggested that the microbiome may constitute a potential determinant of a negative effect of saccharin on glycemic response in some individuals (Suez et al., 2014), there is a dearth of evidence on the effects of NNS on the human microbiome, and the few available studies are inconclusive, with some suggesting an effect (Frankenfeld et al., 2015; Laforest-Lapointe et al., 2021; Méndez-García et al., 2022), as opposed to others (Ahmad et al., 2020b; Serrano et al., 2021; Thomson et al., 2019). Notably, most of these studies were limited in their ability to screen and exclude individuals already incorporating NNS into their diets, and their microbiome was profiled using 16S rRNA gene sequencing or qPCR of target microbes, which may lack sufficient resolution to determine functional and species-level effects of NNS on the microbiome.

Here, we present the result of a multi-arm RCT assessing NNS effects on human metabolic health and the microbiome. We demonstrate that both sucralose and saccharin supplementation impairs glycemic responses in strictly non-NNS-consuming healthy volunteers, an effect that is not observed in non-supplemented control groups. Four different NNS functionally alter the microbiome. Importantly, by performing extensive fecal transplantation of human microbiomes into GF mice, we demonstrate a causal and individualized link between NNS-altered microbiomes and glucose intolerance developing in non-NNS-consuming recipient mice.

RESULTS

A randomized controlled interventional clinical trial

The trial featured four NNS intervention arms: aspartame, saccharin, sucralose, and stevia. All NNS were given as commercially available sachets containing glucose as a bulking agent (2 sachets/3 times a day), corresponding to 8%, 20%, 34%, and 75% of the acceptable daily intake (ADI) of each of the supplemented NNS, respectively (US Food and Drug Administration, 2018) (Figures 1A–1C, STAR Methods). To control for the potential confounding effect of the glucose vehicle, routinely incorporated into the sachet mixtures, we supplemented participants in a fifth arm with an equivalent amount of glucose (5 g day^{-1}). A sixth group did not receive any supplement (no supplement control, NSC). The study consisted of three phases: 7 days of baseline measurements of metabolic, metabolomic, and microbial parameters were followed by 14 days of exposure to the various nutritional interventions, after which supplementation was ceased and participants were followed up for 7 additional

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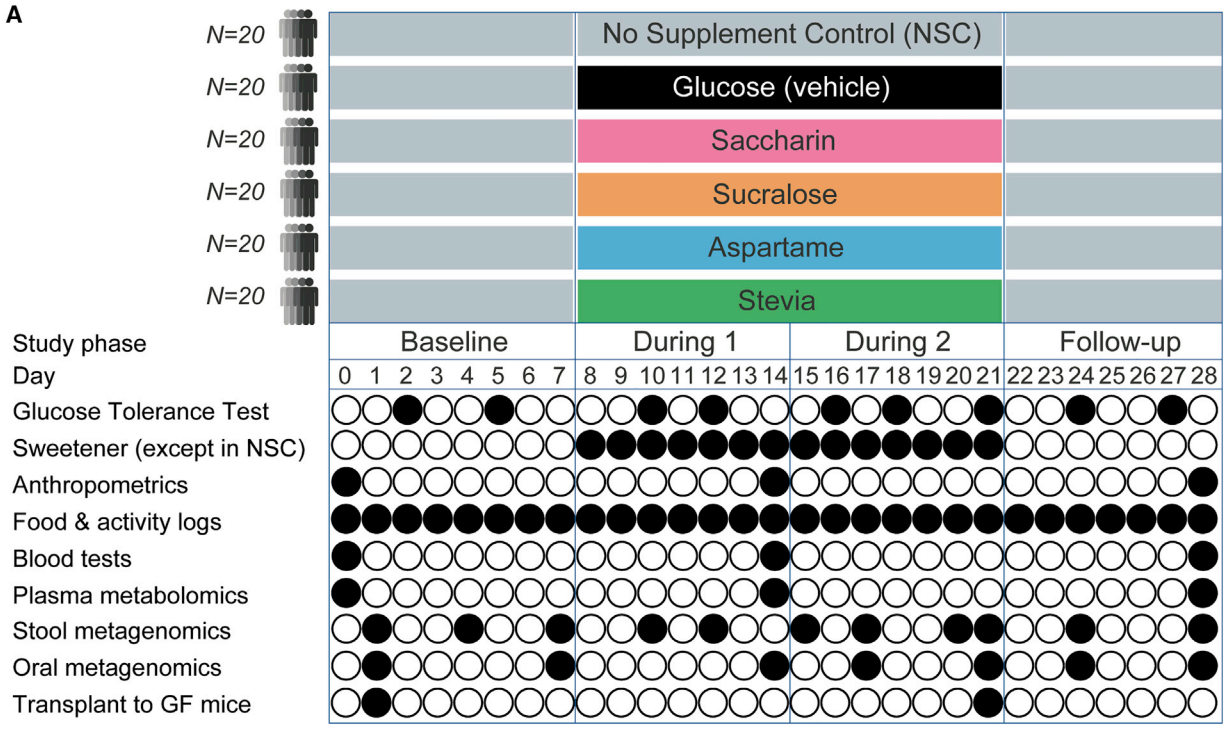
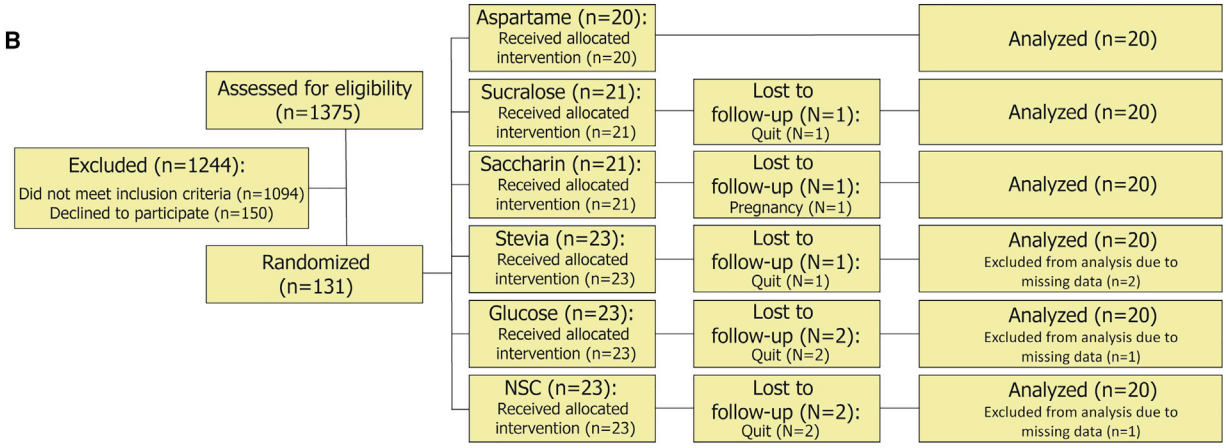
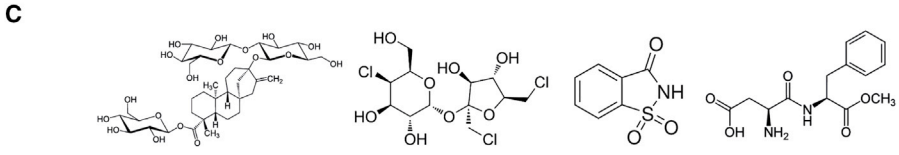
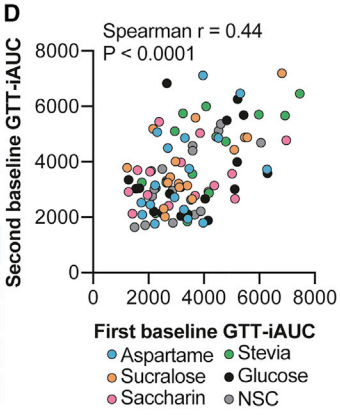






	Stevia	Sucralose	Saccharin	Aspartame
#Sachets/day	6	6	6	6
NNS mg/day	180	102	180	240
ADI (mg/kg/day)	4	5	15	50
%ADI (per 60kg)	75%	34%	20%	8%



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days. To determine the effect of NNS on glycemic control, participants wore a continuous glucose monitor (CGM) throughout the 29 days of the trial, and glucose tolerance tests were performed on pre-determined days. Anthropometrics and blood tests were conducted on days 0, 14, and 28. Microbiome samples from the stool and the oral cavity were collected at pre-determined time points. Participants logged all food intake and physical activity in real time using a dedicated smartphone application. The study design is summarized in Figure 1A.

Between 2018 and 2020, we screened 1,375 individuals for eligibility (see exclusion criteria in STAR Methods). A unique feature of the trial consisted of inclusion only of participants defined as complete NNS abstainers according to a detailed food frequency questionnaire based on NNS-containing products on the Israeli market (STAR Methods). Indeed, using the stringent screening protocol, the vast majority of ineligible candidates were found to consume NNS, in many cases unknowingly, in line with a similar finding in a US cohort (Sylvetsky et al., 2017b). A total of 120 participants, 20 in each group, successfully completed the trial and had enough glucose measurements for analysis (for the consolidated standards of reporting trials [CONSORT] participants flow diagram, see Figure 1B and STAR Methods). Table S1 shows the baseline characteristics of the cohort and each group. Participants in all groups were in good metabolic health, featuring normal body mass index (BMI), waist-hip ratio, hemoglobin A1c (HbA1c), C-reactive protein (CRP), total and high-density lipoprotein (HDL) cholesterol, blood pressure, heart rate, serum alanine transaminase (ALT), and aspartate transaminase (AST). 65% of participants were women, and the median age was 29.95 (interquartile range [IQR] 26.93–35.23). None of the following covariates was significant in any of the groups: age, sex, BMI, smoking, and habitual diet (Table S2).

Saccharin and sucralose impair glucose tolerance in healthy adults

As a standardized measure of glucose tolerance, all participants performed oral glucose tolerance tests (GTT) with 50 g of glucose consumed in the morning after an overnight fasting on pre-determined days, twice during weeks 1, 2, and 4, and 3 times on week 3 (a total of 9 GTTs). GTTs were performed by participants at home, and the CGM recorded interstitial glucose every 15 min, after which the incremental area under the glucose curve (iAUC) was calculated. Performing GTTs using continuous glucose monitoring at home (rather than inviting the participants to perform the GTT at the testing center) enabled to longitudinally assess the effect of acute NNS consumption and reduce noise (Bailey et al., 2014). Although considerable person-to-person heterogeneity was observed in the GTT-iAUC (range 1,225–7,458 mg dL^{-1} min^{-1}), baseline GTTs performed by the same individual were similar and significantly correlated with each other (Spearman $r = 0.44$, $p < 0.0001$, Figure 1D), in line with previous findings (Zeevi et al., 2015).

The effects of the NNS and controls on glucose tolerance are summarized in Figure 2. To consider each individual GTT, we used two linear-mixed-effects models (LMMs) to assess the impact of NNS consumption over time, either with the seven GTTs performed during baseline and exposure weeks (model A) or all GTTs including follow-up (model B; Figure 2A). Both saccharin and sucralose significantly elevated glycemic response during exposure (model A) compared with glucose vehicle ($p = 0.042$ and 0.004, respectively) and NSC ($p = 0.018$ and 0.001; Figure 2A). The significant effect on GTT-iAUC was largely limited to the exposure period (model B saccharin versus glucose $p = 0.18$ and versus NSC $p = 0.21$; model B sucralose versus glucose $p = 0.051$ and versus NSC $p = 0.059$). Aspartame and stevia did not show a significant effect in both models. Importantly, no significant effect on glucose tolerance was observed in the glucose vehicle or NSC groups (Figure 2A; Table S2). To compare the magnitude of the effect between groups, we normalized the iAUC-GTT of each individual with their average baseline iAUC-GTT. During the 1st week of exposure, the normalized glycemic response was significantly higher in the sucralose group compared with the glucose vehicle (Figure 2B, two-way ANOVA & Dunnett $p = 0.037$) and the NSC groups (Figure 2B, $p = 0.047$) and in the saccharin group compared with the glucose vehicle group (Figure 2B, $p = 0.023$). The significantly elevated glycemic response persisted during the 2nd week of exposure compared with either glucose vehicle (Figure 2C, saccharin $p = 0.047$, sucralose $p = 0.017$) or NSC (Figure 2C, saccharin $p = 0.003$, sucralose $p = 0.002$) but declined toward baseline during follow-up (Figure 2D). Importantly, despite considerable inter-individual heterogeneity of glycemic responses, there were no significant differences in GTT-iAUCs between the groups at baseline (one-way repeated-measures ANOVA $F = 0.67$, $p = 0.64$). As an additional output, we compared each individual with their own baseline (repeated-measures two-way ANOVA & Dunnett's). Saccharin significantly elevated glycemic response, starting from the 1st week of exposure (Figure 2E, $p = 0.0073$, iAUC mean difference 783.5, 95% confidence interval [CI] 204.3–1,363) and persisting to the 2nd week of exposure ($p = 0.0094$, mean 811.2, CI 190.6–1,432). These differences abated upon cessation of saccharin intake ($p = 0.39$). Sucralose supplementation likewise resulted in significantly elevated glycemic response during week 1 (Figure 2F, $p = 0.018$, mean 855.8, CI 134.8–1,577) and week 2 ($p = 0.0092$, mean 976.3, CI 231.3–1,721), which did not persist in the follow-up week ($p = 0.34$). In contrast, neither aspartame (Figure 2G) nor stevia (Figure 2H) had a significant effect on glucose tolerance during the 1st ($p = 0.9$ and 0.47, respectively)

Figure 1. Randomized-controlled trial to determine the effects of NNS on glycemic control and the microbiome

(A) Study design in humans. Data and sample collection days are denoted by black circles.

(B) CONSORT flow diagram. Participants flow during the process of recruitment, randomization, follow-up, and data analysis in the study.

(C) Doses and chemical structures of the NNS used in this study. ADI, acceptable daily intake; 60kg, human body weight.

(D) Baseline glucose tolerance tests show high inter-personal and low intra-personal variability, related to Figure 2. Two oral glucose tolerance tests were performed with 50 g of glucose during a baseline week prior to any dietary intervention. Spearman correlation was calculated between the incremental area under the glucose curve for each individual. Colors correspond to the groups.

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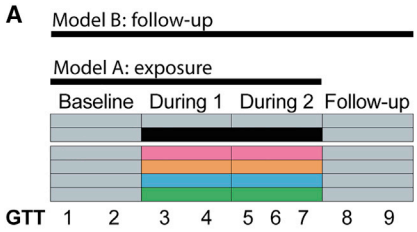
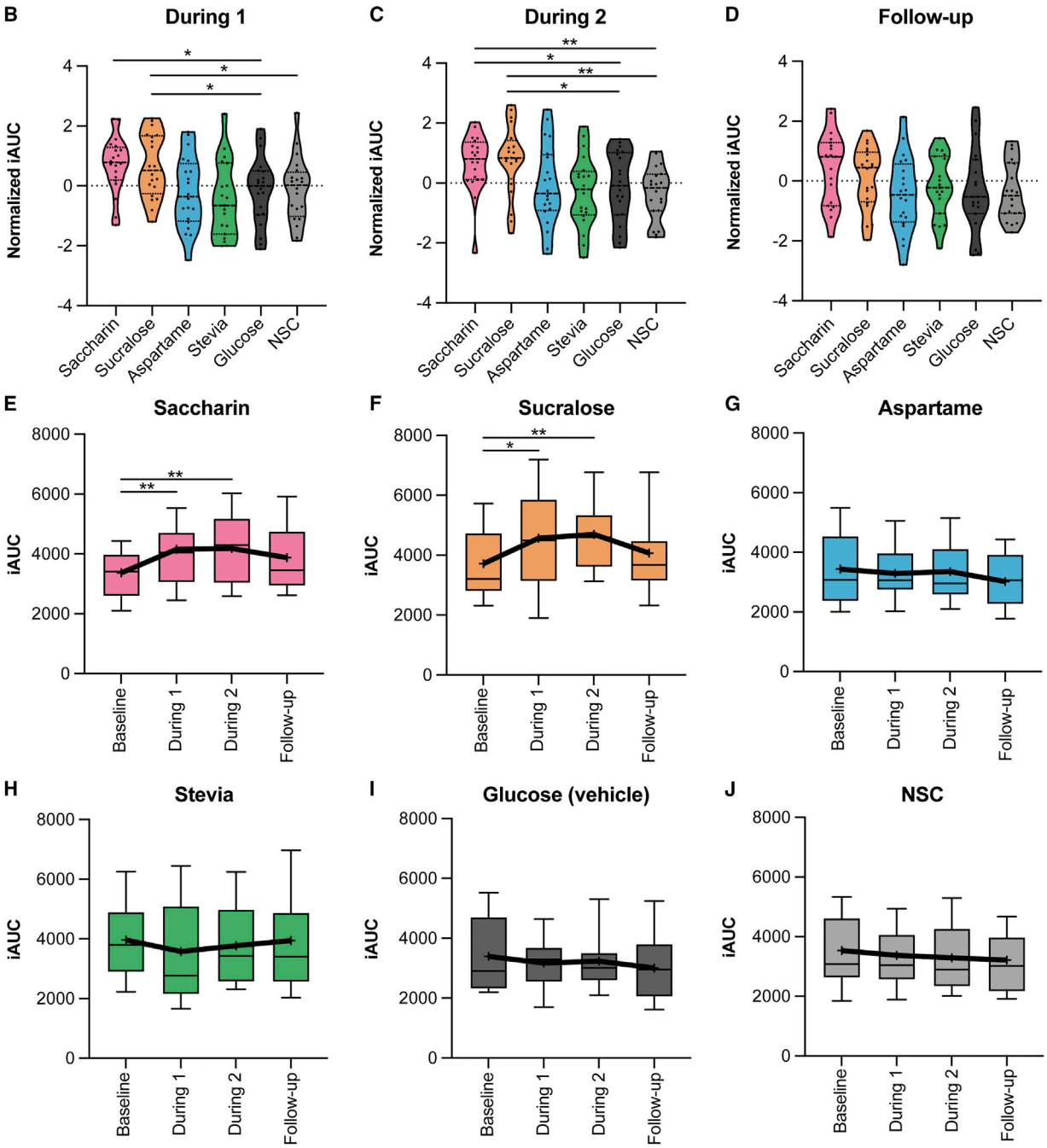


	Saccharin	Sucralose	Aspartame	Stevia	Glucose vehicle	NSC
Model A: exposure						
vs. glucose vehicle	**0.042**	**0.004**	0.99	0.57	-	0.78
vs. NSC	**0.018**	**0.001**	0.77	0.39	0.78	-
Model B: follow-up						
vs. glucose vehicle	0.18	0.051	0.99	0.26	-	0.93
vs. NSC	0.21	0.059	0.92	0.29	0.93	-



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and 2nd (p = 0.99 and 0.78, respectively) exposure weeks, or during follow-up (p = 0.32 and 0.99, respectively). No significant effect on glucose tolerance was observed in the glucose vehicle group (Figure 2I, week 1, p = 0.62, week 2, p = 0.85, follow-up, p = 0.33) or the NSC group (Figure 2J, p = 0.77, 0.41, and 0.11). The lack of significant effect in these control groups indicates that reduced glucose tolerance in the saccharin and sucralose groups is not a result of daily supplementation with a glucose-containing mixture (as glucose quantity is comparable between the vehicle and NNS groups, STAR Methods) or the experimental protocol, which was identical in all groups (i.e., nine GTTs performed during the 29 days trial in all participants). Taken together, these findings indicate that short-term consumption of sucralose and saccharin in doses lower than the ADI can impact glycemic responses in healthy individuals.

In addition to a standardized GTT, we sought to determine whether NNS supplementation also affected daily fluctuations in glucose levels as an additional clinically-relevant parameter of glucose homeostasis. The daily coefficient of variance (CoV) in glucose as derived from the CGM indicated higher variability in the saccharin (linear-mixed-effects regression calculated on all days p = 0.0003, Figure S1A) and stevia (p = 0.005, Figure S1B) groups, but not in the sucralose (Figure S1C) or aspartame (Figure S1D) groups, compared with NSC. However, this was likely due to lower variability in the NSC group (p = 0.07 versus glucose vehicle), and none of the NNS groups was significantly different than the glucose vehicle group (Figures S1A–S1D). As both saccharin and sucralose demonstrated a cohort-wide effect on glucose tolerance, we asked whether the elevated glycemia associated with their intake is due to NNS effects on glucagon-like peptide-1 (GLP-1) or insulin production. Although participants in the NNS and the glucose vehicle groups were all exposed to an equivalent amount of glucose, a significant increase in plasma insulin during exposure was noted in the glucose vehicle group (mean 7.27 mU L^{-1}, 0.14–14.4, two-way ANOVA & Dunnett p = 0.045, Figure S1E), which remained elevated on the last day of the trial (10.27 mU L^{-1}, 3.4–17.1, p = 0.004, Figure S1E) and during exposure in the stevia group (6.38 mU L^{-1}, 0.19–12.58, p = 0.043, Figure S1E). However, there were no significant changes in blood insulin in the saccharin, sucralose, aspartame, or NSC groups (Figure S1E). GLP-1 levels were not significantly altered in any of the groups (Figure S1F). None of the measured anthropometrics (BMI, waist and hip circumference, systolic and diastolic blood pressure, and resting heart rate) or blood markers (blood pressure, HbA1c, C-reactive protein, ALT, AST, and blood immune cell counts, see STAR Methods for full list) were significantly impacted (following Benjamini-Hochberg correction for multiple hypothesis testing) by NNS supplementation compared with the control groups (Table S1).

To determine whether supplementation with sweeteners affected participant nutrient intake or physical activity, participants in the study logged their meals and activities in real time throughout the 4-week trial period. Although all participants modestly reduced their energy intake throughout the trial, regardless of the group (Table S3), likely due to the established effect of heightened awareness (Robinson et al., 2015), there were no significant differences in nutrient (carbohydrates, sugar, fiber, protein, fat, and cholesterol) intake or physical activity between the groups (Table S3). It is therefore unlikely that the differences in glycemic responses stem from differential intake of calories or macronutrients or physical activity.

Non-nutritive sweeteners functionally modulate the human gut and oral microbiomes

One of the mechanisms through which NNS can affect human metabolism may involve alteration of the intestinal microbiome. Evidence for this mechanism stems mostly from animal models, whereas evidence in humans is limited and conflicted (Harrington et al., 2022). Importantly, as previous studies utilized 16S rDNA microbiome profiling, the effect of NNS on species-level abundance and functional capacity of the microbial community remains elusive. We therefore collected longitudinal stool samples from all participants throughout the baseline, exposure, and follow-up phases and performed shotgun metagenomic sequencing (n = 1,182 stool samples after quality filtration, STAR Methods).

Baseline stool microbiome composition and function were comparable between the NNS (aspartame, sucralose, saccharin, and stevia) and control groups (glucose vehicle, NSC, and PERMANOVA p > 0.05, Table S4). To determine whether NNS supplementation had an effect on the microbiome's temporal dynamics, we performed a trajectory analysis using M-product (Kilmer et al., 2021) based tensor component analysis (TCAM, Mor et al., 2022; STAR Methods) compared with the NSC group. This analysis was performed twice, using samples from individual sampling days as well as weekly averaged abundances per participant. A significant effect on the microbiome composition was observed in the sucralose (genus days PERMANOVA, p = 0.002, Figure 3A; genus weeks, p = 0.011, Figure S2A; species days, p = 0.033, Figure S2B; species weeks, p = 0.012, Figure S2C) and saccharin (genus days, p = 0.014, Figure 3B; species days, p = 0.018, Figure S2D) groups. All four NNS had a significant effect on microbiome

Figure 2. Saccharin and sucralose detrimentally affect glycemic response in humans

Oral glucose tolerance tests were performed before, during, and after exposure to saccharin, sucralose, aspartame, stevia, and glucose vehicle in an amount equivalent to bulking glucose in the NNS, or no supplement control (NSC). The incremental area under the glucose curve (GTT-iAUC) was calculated.

(A) Schematic and p values of two linear-mixed models comparing GTT-iAUCs of NNS and controls; model A included baseline and exposure GTTs, model B also included the follow-up GTTs.

(B–D) GTT-iAUC of each individual were normalized to their average baseline GTT-iAUC and compared between the groups during the (B) 1st and (C) 2nd week of exposure, or the (D) follow-up week after cessation. Each dot represents an individual; horizontal lines, quartiles.

(E–J) GTT-iAUCs of each individual in the (E) saccharin, (F) sucralose, (G) aspartame, (H) stevia, (I) glucose vehicle, or (J) NSC groups were averaged per-person during each week of the trial and compared with their baseline.

Black line, connects the means; horizontal lines, median;whiskers, 10–90 percentiles. *p < 0.05; **p < 0.01; repeated-measures two-way ANOVA & Dunnett. Related to Figures S1 and S8.

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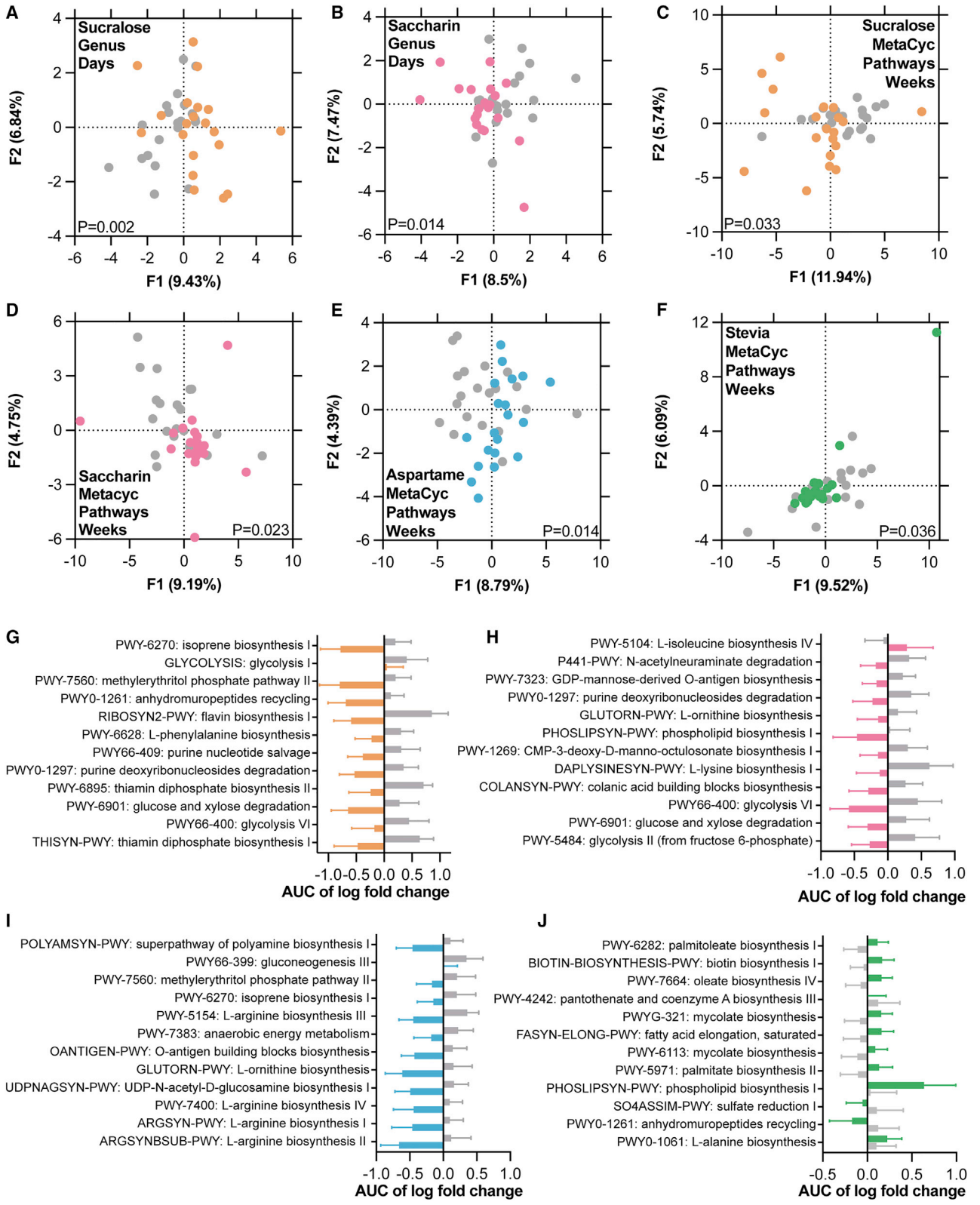

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function (sucralose weeks, p = 0.033, Figure 3C; saccharin weeks, p = 0.023, Figure 3D; saccharin days, p = 0.04. Figure S2E; aspartame weeks, p = 0.014, Figure 3E; aspartame days, p = 0.016, Figure S2F; stevia weeks, p = 0.036, Figure 3F; stevia days, p = 0.017, Figure S2G), as well as KEGG modules in sucralose (days, p = 0.015, Figure S2H). None of these microbiome features was significantly different between the glucose vehicle and the NSC groups (Figures S2I–S2O).

To determine the microbial features underlying these effects, we plotted the area under the log-fold change curve for the top loadings of each significant comparison. Several top loadings in the sucralose group were related to purine metabolism (Figure 3G). Top loadings for the saccharin group included pathways related to glycolysis and glucose degradation (Figure 3H). Many of the top loadings in the aspartame group were related to polyamines metabolism (Figure 3I). Several top loadings in the stevia group were related to fatty acid biosynthesis (Figure 3J). Collectively, these results suggest that dietary supplementation with NNS can impact the functional potential of the human microbiome in NNS-specific manners, with the most prominent effects on the fecal microbiome observed with sucralose.

Similarly, NNS distinctly impacted the oral microbiome (eight samples per participant throughout the trial, STAR Methods). The relative abundance of four metabolism-related KEGG pathways (Figures S3A–S3D) and three modules (Table S5) decreased during the 2nd week of exposure in the oral microbiome of the stevia group. Notable oral microbiome alterations ($p < 0.1$) in the other NNS groups include changes in relative abundances of six *Streptococcus* species in the sucralose group (Figure S3E; Table S6), reduced relative abundance of *Fusobacterium* in the saccharin group (Figure S3F; Table S5), and reduced abundance of *Porphyromonas* (Figure S3G) and *Prevotella nanceiensis* (Figure S3H) in the aspartame group (Table S5). There were no oral microbiome alterations with an FDR-corrected $p < 0.1$ in either control group (Table S6), and baseline oral microbiome composition and function were comparable between the NNS and control groups (PERMANOVA $p > 0.05$, Table S4).

Microbiome features correlate with NNS impacts on the human glycemic response

We next sought to identify microbiome features that potentially contribute to the NNS effects noted on glycemic control and uncover putative host targets that may link these NNS-related functional microbiome alterations to an effect on the host. We initially focused on sucralose as this NNS is poorly absorbed and is more likely to interact with the intestinal microbiome. Furthermore, of the two NNS that significantly impacted glucose tolerance, the sucralose group displayed greater person-to-person heterogeneity, providing an opportunity to elucidate microbiome and metabolome contributions to personalized glycemic responses. First, we correlated the baseline abundance of stool bacterial genera and species, KEGG modules and pathways, and MetaCyc pathways with the GTT-iAUC measured during the 2nd week of sucralose exposure. We then assessed how the significantly correlated features (Pearson $p < 0.05$) changed throughout the trial (Table S6). Significant metagenomic results included those strictly appearing in the sucralose group (n = 20), although not being altered in the glucose (n = 20) and NSC (n = 20) control groups. The plasma metabolome for sucralose consumers was profiled at baseline (day 0) and after the 1st week of NNS supplementation (day 14). Significant metabolomic results included those strictly appearing in the sucralose group (n = 20), although not being altered in the glucose (n = 10) and NSC (n = 10) control groups (Table S6).

Baseline abundance of three bacterial species correlated with GTT-iAUC (Figure 4A). Functionally, the abundances of several purine biosynthesis pathways were positively associated with GTT-iAUC and gradually decreased during the trial. Mixed acid fermentation and the TCA cycle were also inversely correlated with GTT-iAUC; the abundances of these pathways increased during both exposure weeks and trended toward baseline during follow-up (Figure 4A). Nine metabolites significantly increased in plasma during sucralose supplementation and three decreased (FDR-corrected paired t test $p < 0.05$, Figures 4B–4D). No significant changes were noted following FDR correction in plasma metabolites in the glucose vehicle and NSC groups (Table S6). In line with the increased abundance of the TCA cycle pathway in the microbiome, the levels of iso-citrate and trans-aconitate, TCA cycle intermediates, increased in plasma during sucralose supplementation (Figure 4B). Levels of the amino acids serine, *N*-acetyl alanine, and aspartate, as well as the aspartate metabolite quinolinate, also increased during supplementation (Figure 4B). Two additional TCA cycle metabolites (citrate and fumarate) and several additional amino acids (cystine, lysine, and glycyl-L-valine) significantly increased during supplementation (before FDR correction, Table S6). In line with the reduction of microbial pathways related to purine metabolism, plasma levels of pseudouridine and uric acid were significantly reduced during sucralose supplementation (Figure 4D), as well as

Figure 3. NNS functionally modulate the gut microbiome

Stool microbiome samples were collected at pre-determined days and analyzed for bacterial composition (using Kraken2 & Bracken) and function (MetaCyc, as well as KEGG modules and pathways).

(A–F) Trajectory analysis ordination plots following tensor component analysis with M-product (TCAM), which tested if a group had a significant trajectory compared with the no supplement control group. TCAM was applied to the fold change from subject's baseline for each feature. This analysis was performed using samples from individual days as well as weekly averaged abundances per participant (indicated in panels).

(A) Bacterial genera in the sucralose group.

(B) Bacterial genera in the saccharin group.

(C–F) MetaCyc pathways in the (C) sucralose, (D) saccharin, (E) aspartame, or (F) stevia group.

(G–J) Top loadings in each MetaCyc comparison in the (G) sucralose, (H) saccharin, (I) aspartame, or (J) stevia groups.

(A–F) Hypothesis testing for trajectory analysis according to the PERMANOVA test.

(G–J) Bars, mean; error bars, SEM. NSC, no supplement control; Log. log2.

Related to Figures S2 and S3.

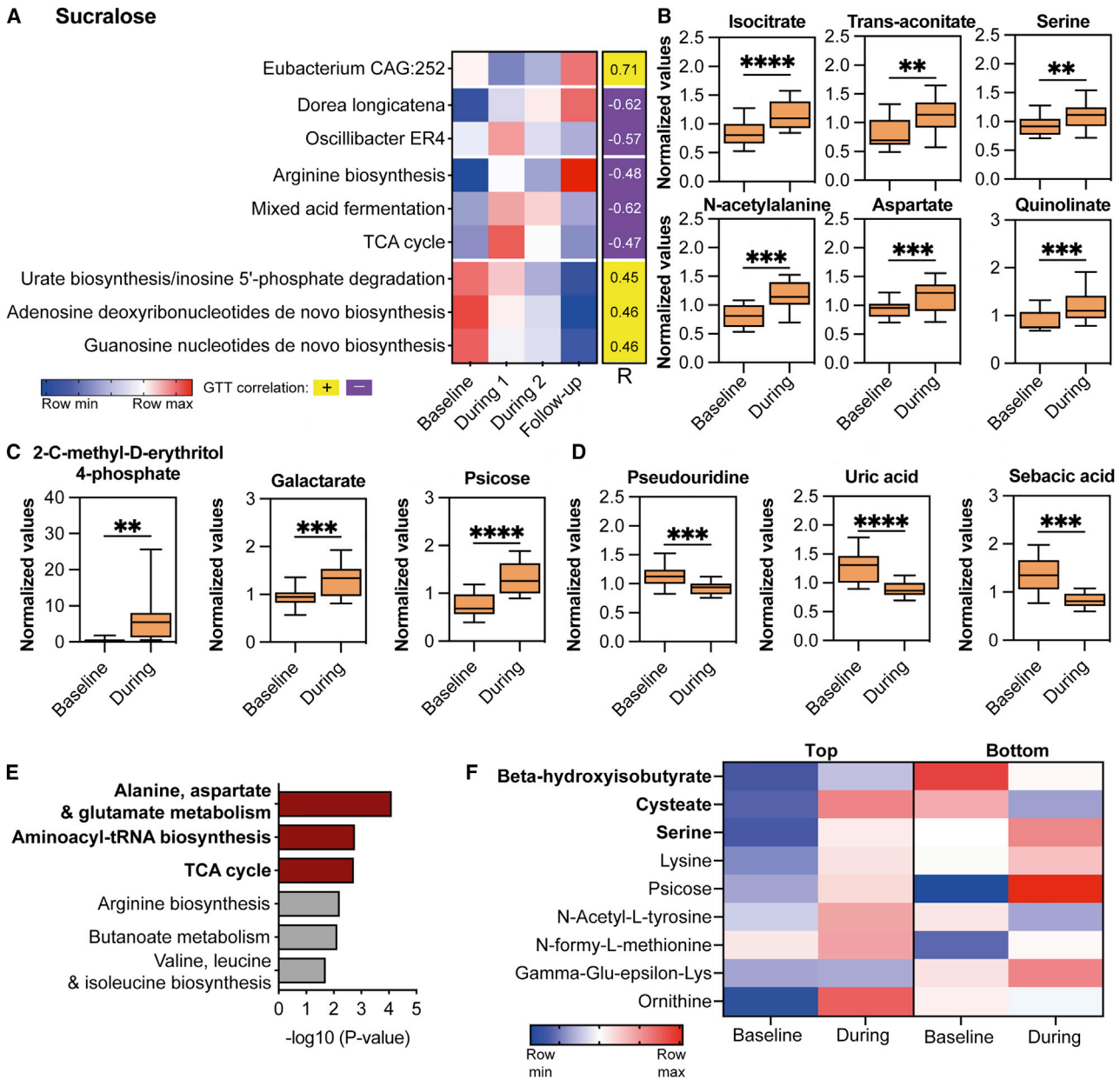

Figure 4. Microbial features and plasma metabolites are correlated with sucralose's effect on glycemic responses

(A) Per week abundance of bacterial species and KEGG and MetaCyc pathways significantly (Pearson $p < 0.05$) correlated at baseline with baseline-normalized per-person glycemic response in the 2nd week of exposure.

(B–D) Plasma metabolites significantly (FDR-corrected Student's t test $p < 0.05$) increased compared with baseline after the 1st week of exposure.

(E) Pathway enrichment based on metabolites in (B–D) and metabolites significantly correlated with GTT-iAUC. Pathways in bold are significant ($p < 0.05$) after FDR correction.

(F) Metabolites significantly different between the top and bottom five responders based on two-way ANOVA. Metabolites in bold are significant after FDR correction.

**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, FDR-corrected Student's t test. Horizontal lines, median; whiskers, 10–90 percentiles.

guanosine, 1-methylguanine, inosine, and paraxanthine (before FDR correction, Table S5).

In addition, 22 metabolites were significantly correlated with an increase in GTT-iAUC noted in the sucralose group, but not in the NSC or glucose vehicle group, including the SCFA propionate, butyrate, and valerate (before FDR correction, Table S6). Based on all of the above differentially abundant metabolites, we performed a pathway enrichment analysis which highlighted changes in amino acid metabolism and biosynthesis and the TCA cycle pathways to be associated with the impact

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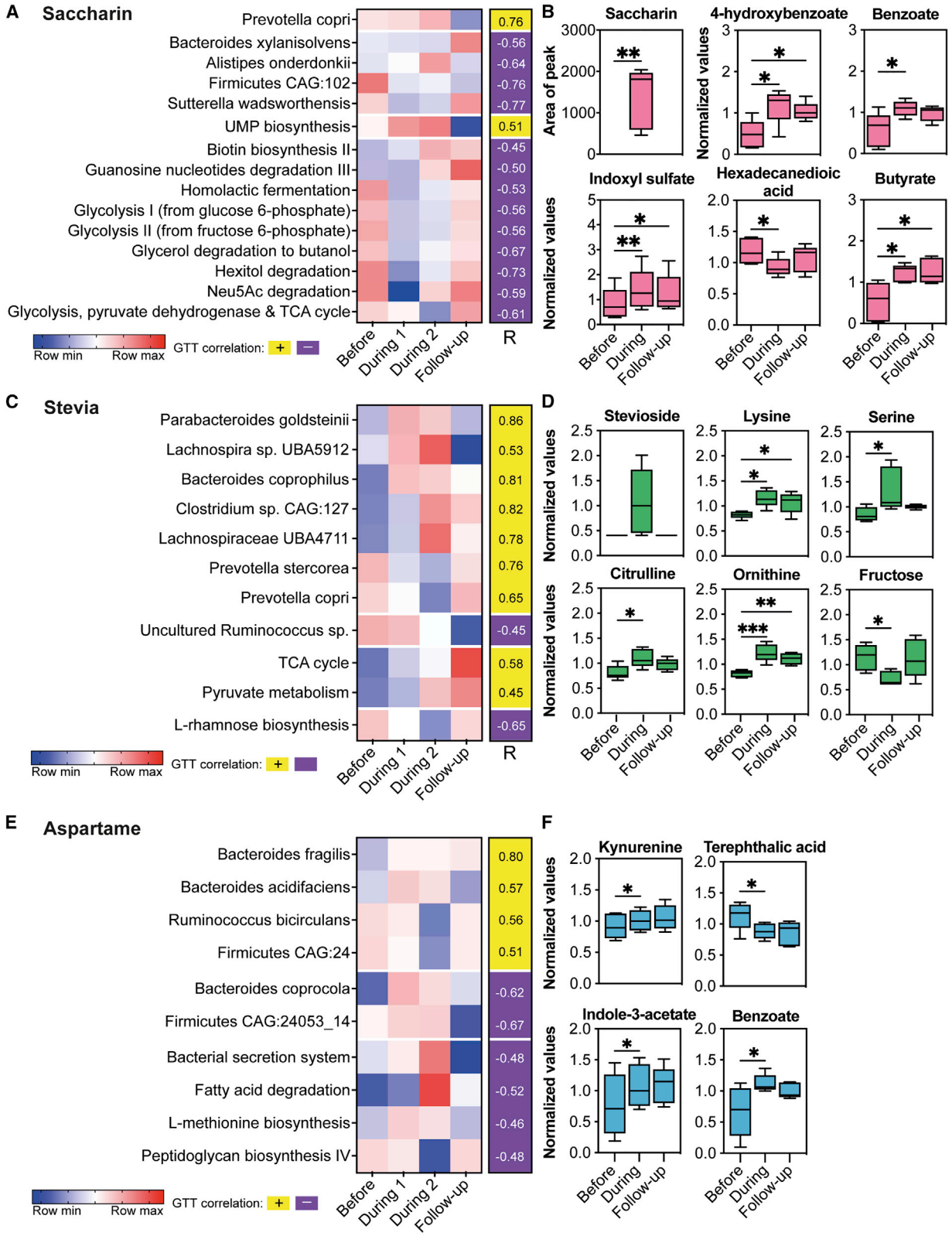

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of sucralose on glycemic control (FDR-corrected $p < 0.05$, Figure 4E). To identify metabolites that may mediate responsiveness to sucralose and those that are potentially involved in sucralose effect on glucose tolerance, we next compared the metabolomic profiles of the top and bottom five sucralose responders. Three metabolites were significantly different between top and bottom responders (FDR-corrected two-way ANOVA $p < 0.05$, Figure 4F): the ketone body beta-hydroxybutyrate, serine, and the cysteine derivate cysteate. All three were lowest in top sucralose responders at baseline and increased during supplementation. None of these metabolites was significantly different between the five top and bottom responders in the glucose and NSC control groups (Table S6).

We similarly correlated the abundances of baseline metagenomic features with GTT-iAUC in the saccharin, stevia, and aspartame groups, as well as metabolomic profiles in the top 5 responders in each group. Significant metagenomic results included those strictly appearing in any of the saccharin, aspartame or stevia groups, although not being altered in the glucose and NSC control groups. The plasma metabolome for sucralose consumers was profiled at baseline (day 0) , after the 1st week of NNS supplementation (day 14) and at end of the follow-up (day 28). Significant metabolomic results included those strictly appearing in the any of the saccharin, aspartame, or stevia groups, although not being altered in the respective glucose and NSC control groups. In the saccharin group, baseline levels of *Prevotella copri* and UMP biosynthesis were positively associated with GTT-iAUC and gradually increased during exposure, whereas baseline levels of *Bacteroides xylanisolvens* were negatively associated with GTT-iAUC and increased during exposure (Figure 5A). Many of the pathways negatively correlated with GTT-iAUC were related to glycolysis and glycan degradation (Figure 5A). Untargeted plasma metabolomics of the top five saccharin responders readily detected high levels of saccharin during exposure (Figure 5B). The plasma levels of indoxyl sulfate, a metabolite associated with vascular disease, increased during saccharin exposure (Figure 5B). Levels of the SCFA butyrate increased during the trial, whereas those of three long-chain fatty acids were reduced (Figure 5B; Table S5). In the stevia group, two *Prevotella* spp. were positively associated with GTT-iAUC, and reduced during exposure; *Bacteroides coprophilus*, *Parabacteroides goldsteinii*, and a *Lachnospira* spp., which were also positively associated with GTT-iAUC increased during both exposure weeks (Figure 5C). Stevioside was readily detected in plasma samples of the stevia group, exclusively during supplementation (Figure 5D). Levels of the amino acids serine and lysine increased during stevia supplementation (Figure 5D). Notably, two metabolites of arginine, ornithine and citrulline, also increased during exposure (Figure 5D). In the aspartame group, *B. fragilis* and *B. acidifaciens* were positively associated with GTT-iAUC, whereas *B. coprocola* had an inverse correlation (Figure 5E). Levels of kynurenine, a metabolite associated with diabetes, increased during aspartame consumption (Figure 5F). Collectively, human NNS supplementation induced distinct alterations in microbiome composition and function, as well as in distinct plasma metabolites, in each of the NNS-supplemented groups, which correlated with host glycemic responses. Notably, changes in abundance of many of the correlated microbiome or metabolome features start as early as the 1st week of exposure and revert to baseline during follow-up, suggesting that these bacterial species and functions may respond to the presence of NNS.

Causative personalized impacts of NNS-modulated microbiome on glycemic responses

To determine whether the aforementioned alterations in human microbiome configuration causally contribute to NNS-induced hyperglycemia, we colonized adult GF mice with stool microbiome collected either at the beginning of the trial (day 1), or on the last day of exposure (day 21), from all four NNS-supplemented and control groups (Figure S4A). In total, we transplanted microbiomes from 42 individuals, the four individuals in each group that had the most potent response (“top responders”), and the three that had the lowest response (“bottom responders”) in their respective groups. Statistically, each individual donor was strictly treated as a random effect in the LMM while modeling the glycemic response variable. Strikingly, for each of the four “top responders” in the saccharin and sucralose groups, mice humanized with the day 21 sample had a significantly higher glycemic response compared with mice that received the baseline sample from the same individual (saccharin top 1-t test $p = 0.0072$, top 2 $p = 0.025$, top 3 $p = 0.0046$, top 4 $p = 0.048$, Figure S4; sucralose top 1 $p = 0.025$, top 2 $p = 0.016$, top 3 $p = 0.043$, top 4 $p = 0.0031$, Figure S5), resulting in a significant group effect for both saccharin (Figure 6A, mixed-effects ANOVA $p < 0.0001$) and sucralose (Figure 6B, $p < 0.0001$). Microbiomes transferred from the last day of exposure of the four “top responders” to stevia and aspartame resulted in elevated glycemic response in recipient mice of three out of the four top-responding donors (stevia $p < 0.0001$, $p < 0.0001$, $p = 0.029$; aspartame $p = 0.012$, 0.048, and 0.015, Figure S6), resulting in a significant group effect for both (stevia $p < 0.0001$, Figure 6C; aspartame $p = 0.0022$, Figure 6D). Importantly, none of the day 21 stool microbiome samples transferred into GF mice from the four “top responders” in the glucose vehicle or the NSC groups (Figures 6E and 6F; Figure S7) resulted in a significant effect on glucose tolerance, compared with baseline microbiome samples transferred from the same individuals. To further elucidate the extent of the causative personalized NNS-mediated effect size, we transplanted baseline and day 21 microbiomes from the bottom three responders in each group into GF mice. Interestingly, in the saccharin group, the

Figure 5. Effects of saccharin, stevia, and aspartame on the microbiome correlate with glycemic response

(A, C, and E) Per week abundance of bacterial species and KEGG and MetaCyc pathways significantly (Pearson $p < 0.05$) correlated at baseline with baseline-normalized per-person glycemic response in the 2nd week of exposure, in the (A) saccharin, (C) stevia, or (E) aspartame group.

(B, D, and F) Plasma metabolites significantly (non-FDR-corrected one-way ANOVA & Dunnet $p < 0.05$) increased compared with baseline after the 1st week of exposure, in the (B) saccharin, (D) stevia, or (F) aspartame groups.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Horizontal lines, median; whiskers, 10–90 percentiles.

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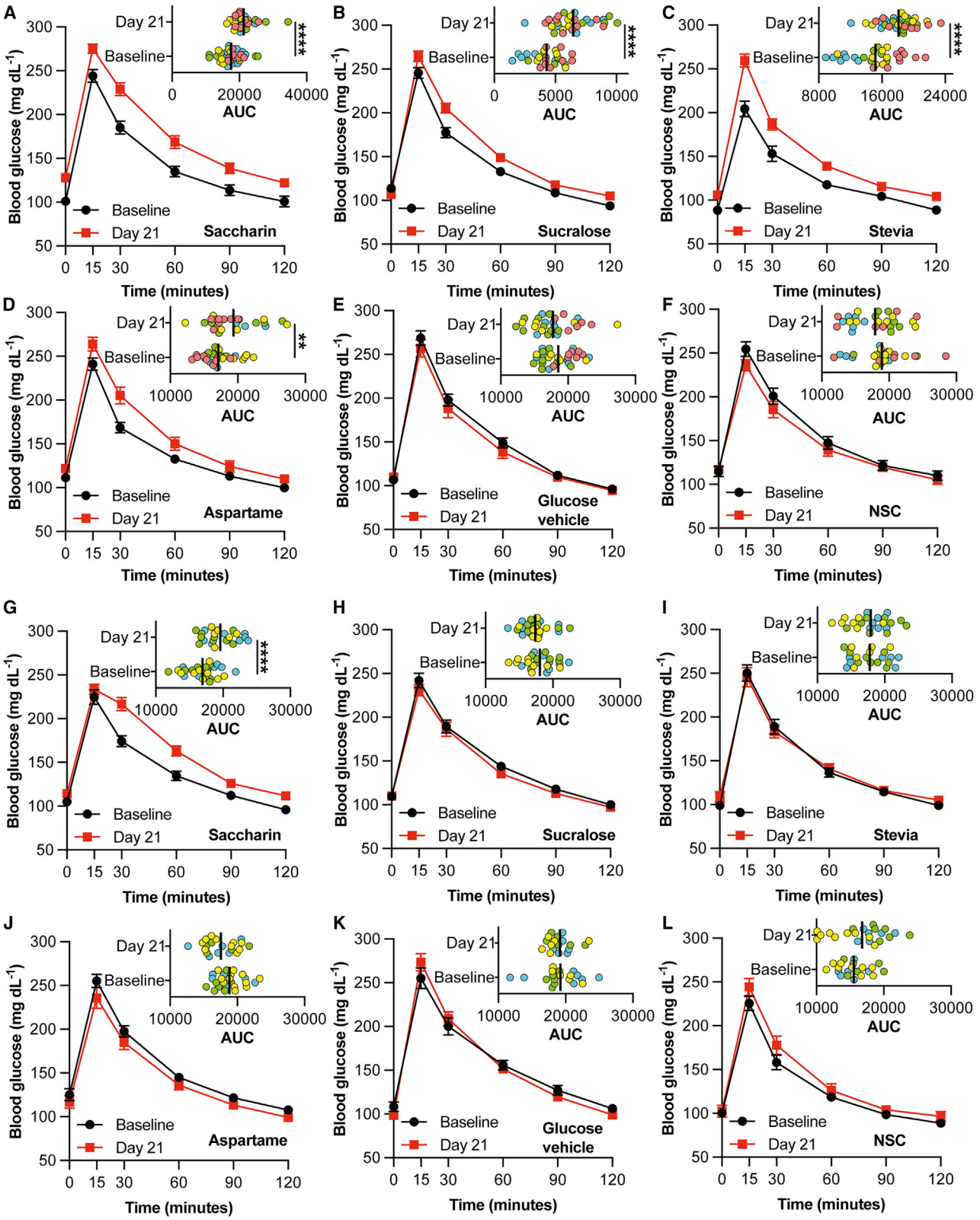

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last day of exposure microbiomes from the bottom three responders still elevated glycemic response in recipient GF mice (Figure 6G, p = 0.0003), in line with its strong cohort-wide elevated glycemia. No significant effects on glycemic response were observed with bottom responders from any of the other treatment (sucralose p = 0.23, Figure 6H; stevia p = 0.96, Figure 6I; aspartame p = 0.076, Figure 6J) or control (glucose vehicle p = 0.97, Figure 6K; NSC p = 0.058, Figure 6L) groups, suggesting a personalized microbiome-mediated impact in response to NNS exposure in these groups. Collectively, the glycemic responses in the humanized mice largely reflected those of their NNS-supplemented donors and serve as a likely causal link between NNS-related microbiome modulations and disrupted glycemic control.

Microbiome features correlate with person-specific variations in glycemic responses to NNS

Finally, we compared fecal microbiome features potentially differentiating human top NNS responders (n = 4 in each NNS) and bottom responders (n = 3 in each NNS). In human participants, KEGG pathways Bray-Curtis-based dissimilarity to baseline configurations trended to be higher in top responders throughout the trial in the sucralose (Figure S8A), stevia (Figure S8B), and aspartame (Figure S8C) groups and were initially higher in bottom responders but converged in the saccharin group (Figure S8D). Importantly, top and bottom "responders" were comparable in the glucose vehicle and NSC groups (Figures S8E and S8F). The fold change between baseline and the 2nd week of exposure of pathways related to glycolysis and TCA cycle was higher in sucralose top responders compared with bottom responders (Figure S8G). Pathways related to biosynthesis, degradation and metabolism of purines and pyrimidines increased in top stevia responders (Figure S8H). Pathways related to the urea cycle and its metabolites increased in top aspartame responders, whereas *Akkermansia muciniphila*, associated with metabolic health of the host (Cani et al., 2022), increased in bottom responders (Figure S8I). Degradation of the cyclic amide caprolactam increased in saccharin top responders, suggesting a possible potential for degradation of chemically related saccharin. Biosynthesis of the branched-chain amino acid isoleucine, associated with poorer metabolic health (Yu et al., 2021), also increased in top saccharin responders (Figure S8J).

To further exemplify such personalized microbiome differences, we compared the fecal microbiome configurations of GF mouse groups transplanted with microbiomes of top (n = 3) and bottom (n = 3) human sucralose responders (n = 3, Figure 7A), in which each of the top human donors and none of the bottom donors exhibited a significant difference in glycemic response between baseline and day 21 of the clinical trial (Figure S5). GF mouse recipients of microbiomes from top sucralose human responders on day 21 featured a distinct microbiome configuration compared with GF mouse recipients of microbiome from bottom sucralose human responders on day 21 (Rcpt: D21, PERMANOVA with donor as random effect PC3 p = 0.008, Figure 7B), in line with the differential glycemic responses noted in the human donors at day 21 time point (Figure S5). Total 22 species and 19 pathways were differentially abundant between mouse recipients of microbiomes from top and bottom human sucralose responders on day 21, of which only two were also different at baseline (pyrimidine biosynthesis and tRNA charging, Table S7). The putrescine biosynthetic pathway was overrepresented in mice conventionalized with top responders' day 21 samples ($p < 0.0001$); elevated plasma levels of putrescine were previously associated with type-2 diabetes (Fernandez-Garcia et al., 2019) and gestational diabetes (Liu et al., 2021).

Notably, transplantation of baseline microbiomes of top and bottom sucralose responders into GF mice resulted in comparable glucose tolerance in recipient mice (Rcpt: BL, Figure 7C). Nonetheless, microbiome configurations of mice receiving baseline pre-exposure microbiomes from human top sucralose responders were already significantly different from those of recipients of baseline microbiomes from human bottom sucralose responders. Microbiome composition of mouse recipients of baseline samples of top and bottom human responders was significantly separated on PC3 (PERMANOVA with donor as

Figure 6. NNS-associated alteration in human microbiome causally linked to hyperglycemia

7- to 9-week-old male Swiss-Webster germ-free mice were conventionalized by oral gavage with microbiome extracted from stool samples of the seven individuals that experienced the strongest effect on glucose tolerance (A–F, top four and G–L, bottom three) from each of the following groups: (A and G) saccharin, (B and H) sucralose, (C and I) stevia, (D and J) aspartame, (E and K) glucose vehicle, and (F and L) no supplement control. Magnitude of response is defined as the change in GTT-iAUC on the 2nd week of exposure compared with baseline. A glucose tolerance test was performed 7 days post-conventionalization. Experiments were conducted in pairs, in which two groups of mice received either the baseline sample or the last day of exposure (day 21) sample.

(A) Recipients of saccharin top responders' microbiomes: top 1 baseline N = 9, day 21 N = 9; top 2 N = 7 and 7; top 3 N = 7 and 7; top 4 N = 8 and 9.

(B) Recipients of sucralose top responders' microbiomes: top 1 N = 5 and 7; top 2 N = 6 and 6; top 3 N = 10 and 9; top 4 N = 6 and 7.

(C) Recipients of stevia top responders' microbiomes: top 1 N = 7 and 8; top 2 N = 6 and 9; top 3 N = 7 and 8; top 4 N = 9 and 8.

(D) Recipients of aspartame top responders' microbiomes: top 1 N = 9 and 8; top 2 N = 5 and 3; top 3 N = 8 and 8; top 4 N = 8 and 6.

(E) Recipients of glucose vehicle top responders' microbiomes: top 1 N = 8 and 7; top 2 N = 8 and 9; top 3 N = 6 and 6; top 4 N = 7 and 6.

(F) Recipients of NSC top responders' microbiomes: top 1 N = 8 and 8; top 2 N = 6 and 6; top 3 N = 6 and 7; top 4 N = 6 and 6.

(G) Recipients of saccharin bottom responders' microbiomes: bottom 1 N = 8 and 8; bottom 2 N = 9 and 9; bottom 3 N = 9 and 6.

(H) Recipients of sucralose bottom responders' microbiomes: bottom 1 N = 8 and 10; bottom 2 N = 8 and 8; and bottom 3 N = 9 and 8.

(I) Recipients of stevia bottom responders' microbiomes: bottom 1 N = 7 and 7; bottom 2 N = 9 and 9; and bottom 3 N = 6 and 7.

(J) Recipients of aspartame bottom responders' microbiomes: bottom 1 N = 6 and 6; bottom 2 N = 7 and 5; and bottom 3 N = 12 and 10.

(K) Recipients of glucose vehicle bottom responders' microbiomes: bottom 1 N = 7 and 6; bottom 2 N = 6 and 6; and bottom 3 N = 6 and 6.

(L) Recipients of NSC bottom responders' microbiomes: bottom 1 N = 6 and 7; bottom 2 N = 9 and 9; and bottom 3 N = 6 and 6.

The average of all donors is presented in the GTT panels. AUC insets - colors, respective donors in each group; GTT panels - symbols, mean; error bars, SEM. In AUC insets, lines, mean. ** $p < 0.01$; **** $p < 0.0001$, mixed-effects ANOVA with donor as random effect.

Related to Figures S4, S5, S6, and S7.

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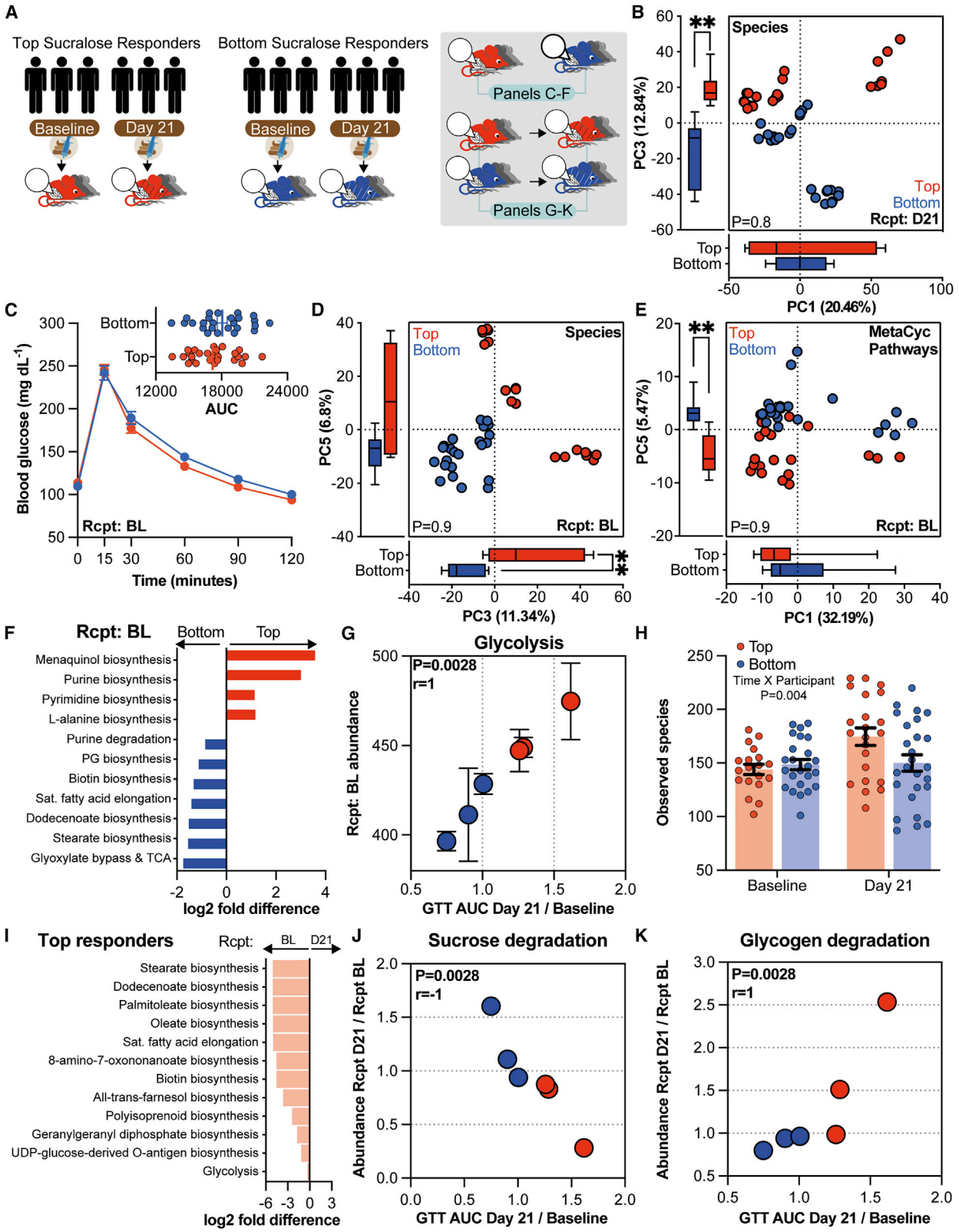

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random effect p = 0.008, Figure 7D), and function was separated on PC5 (p = 0.008, Figure 7E). The microbial features that potentially mediate these differences included 25 species and 27 pathways that are significantly (FDR-corrected linear-mixed model $p < 0.05$) differentially abundant between the two subsets (Table S7). Pathways related to menaquinol biosynthesis, previously associated with type 1 (Roth-Schulze et al., 2021) and type 2 diabetes (Balvers et al., 2021; Dash and Bataineh, 2021; Wu et al., 2020), were the most overrepresented features in mouse recipients of baseline microbiomes from top responders (p = 0.0006, Figure 7F). Pathways related to purine ($p < 0.0001$) and pyrimidine (p = 0.005) biosynthesis were more abundant in mouse recipients of baseline microbiomes of top responders, whereas purine degradation ($p < 0.0001$) was more abundant in recipients of baseline microbiomes from bottom responders (Figure 7F), in line with the correlation between purine biosynthesis and elevated glycemic response noted in the entire sucralose group (Figure 4A). Most pathways over-abundant in mouse recipients of baseline microbiomes from bottom responders were related to fatty acid biosynthesis (stearate biosynthesis p = 0.014, dodecanoate biosynthesis $p < 0.0001$, saturated fatty acid elongation p = 0.005, and phosphatidylglycerol biosynthesis p = 0.003) and their utilization for energy production and gluconeogenesis (glyoxylate bypass and TCA $p < 0.0001$) (Figure 7F). To identify microbial pathways that may predict the effect of sucralose on glucose tolerance, we correlated the baseline abundance of each pathway with the fold difference in the GTT AUC of recipient mice (day 21/baseline). Interestingly, the baseline abundance of the glycolysis pathway demonstrated a significant (FDR-corrected) and strong correlation with GTT-AUC (Spearman r = 1, p = 0.0028, Figure 7G; Table S7). Collectively, these results suggest that a unique pre-supplementation baseline microbiome configuration may contribute to the personalized responses noted upon subsequent exposure to sucralose.

To further identify microbiome functions potentially linked to alterations in glucose tolerance in recipient mice of top and bottom sucralose-responder human microbiomes, we compared microbiome profiles of mouse recipients of top and bottom human sucralose responders collected on day 21 with those in mice receiving baseline pre-exposure samples from the same top and bottom sucralose-responsive humans. Indeed, mice receiving day 21 microbiome samples from top responders presented higher alpha diversity compared with those receiving baseline microbiomes from the same top responders or compared with mice receiving day 21 microbiomes from bottom responders (linear-mixed effects regression, p = 0.004; Figure 7H). A comparison between the baseline and day 21 mouse recipient microbiomes revealed 13 pathways significantly altered in the top responders' group and none in the bottom responder mice. All significantly altered pathways were less abundant on day 21 compared with baseline (Figure 7I; Table S7). Pathways related to the biosynthesis of fatty acids, already less abundant in mouse recipients of baseline top responder microbiomes compared with mouse recipients of baseline bottom responder microbiomes, became undetectable in mice transplanted with day 21 top responder microbiome (p = 0.012). The fold increase of two pathways (day 21/baseline abundance) was significantly correlated with an increased glycemic response: sucrose degradation (Spearman $r = -1$, p = 0.0028, Figure 7J) and glycogen degradation (Spearman r = 1, p = 0.0028, Figure 7K). Taken together, these results suggest that the ability of the microbiome to respond to sucralose in altering host glucose tolerance may be mediated, at least in part, by the capacity of the bacteria to metabolize dietary and/or host-derived carbohydrates and utilize them for energy production. These results merit future causative validation in future studies.

DISCUSSION

Our work provides evidence of human microbiome responsiveness to NNS and its ability to transmit, in specific configurations, downstream effects on the host glucose tolerance. As such, and

Figure 7. Microbial functions linked to sucralose responsiveness and its effect on glucose tolerance

Fecal samples were collected from 7- to 9-week-old male Swiss-Webster germ-free mice 7 days post-conventionalization with microbiome extracted from stool samples of the three top and three bottom responders to sucralose. Magnitude of response is defined as the change in GTT-iAUC on the 2nd week of exposure compared with baseline.

(A) Schematic design of microbiome analyses in recipient GF mice.

(B) Principal components analysis of species-level (Bracken) composition in mice receiving day 21 samples from top and bottom responders. Horizontal lines, median; whiskers, 10–90 percentiles. ** $p < 0.01$, PERMANOVA.

(C) Replot for comparison of GTT (AUC in inset) of mice receiving baseline microbiome samples from top and bottom responders. GTT panels - symbols, mean; error bars, SEM. AUC insets - lines, mean.

(D) Principal component analysis of species-level (Bracken) composition in mice receiving baseline samples from top and bottom responders. Horizontal lines, median; whiskers, 10–90 percentiles. ** $p < 0.01$, PERMANOVA.

(E) Same as (D) but based on MetaCyc pathways.

(F) Pathways significantly (FDR-corrected linear-mixed model $p < 0.05$) differentially abundant ($\log_2$ fold difference > 1) between mice receiving top and bottom responder baseline samples. PG, phosphatidylglycerol; Sat, saturated.

(G) Spearman correlation of glycolysis pathway baseline abundance with fold difference in GTT-AUC of each of the conventionalized mouse groups. Circles represent the mean of the pathway abundance in all mice receiving a transplant from each donor, error bars, SEM.

(H) Alpha diversity (observed species) in mice receiving baseline and day 21 samples from top and bottom responders. Significance according to linear-mixed effect regression. Error bars, SEM.

(I) Same as (F), but top responders are compared on baseline and day 21; no pathways were overrepresented on day 21.

(J) Spearman correlation of sucrose degradation pathway fold change abundance (day 21/baseline) with fold difference in GTT-AUC of each of the conventionalized mouse groups.

(K) Same as (J) but glycogen degradation. Rcpt, recipient; BL, baseline; D21, day 21.

in contrast to the common notion suggesting that NNS are metabolically inert, these data suggest that the human gut microbiome may constitute a "responsiveness hub" enabling, in some individuals, the transmission of NNS effects on human physiology. Similarly, other "modern" food additives such as dietary emulsifiers (Chassaing et al., 2015; Tang et al., 2013), food preservatives (Tirosh et al., 2019), and colorants (He et al., 2021) have been suggested to impact the microbiome and, in some cases, mediate downstream host metabolic effects. Interestingly, although the small amount of vehicle glucose incorporated into the NNS sachets and consumed by all participants in the NNS groups was comparable to the amount of glucose consumed by participants in the vehicle group, plasma insulin levels rose during supplementation only in the stevia and glucose vehicle groups. These results suggest a possible blunting of glucose-stimulated insulin secretion, leading to elevated glycemia in participants consuming saccharin or sucralose (with glucose as a vehicle). Notably, coupling of NNS with a caloric sweetener was reported to result in a higher insulin response compared with NNS alone (Dalenberg et al., 2020). The putative impacts of NNS on insulin sensitivity with and without a carbohydrate moiety should be addressed in further studies under glucose challenge conditions.

Notably, all four tested NNS (saccharin, sucralose, aspartame, and stevia) significantly and distinctly altered the human intestinal and oral microbiome, as would be expected for these chemically diverse compounds. Such an effect was not observed in the two control groups. Sucralose (Olivier-Van Stichelen et al., 2019; Uebanso et al., 2017), saccharin (Serrano et al., 2021), and stevia metabolites (Wheeler et al., 2008) are found in stool of NNS-supplemented animals and humans, suggesting that direct interaction of these NNS with the intestinal microbiome is fully plausible. Sucralose is poorly absorbed, and thus, the majority of orally supplemented sucralose reaches the colon, and subsequently, most, but not all, is excreted unchanged in feces (John et al., 2000; Roberts et al., 2000; Sims et al., 2000; Wood et al., 2000). The metabolic fate of the remaining fraction is currently unknown, although sucralose-related metabolites of unknown function have been identified in feces and adipose tissue (Bornemann et al., 2018). Interestingly, inter-subject variability in fecal excretion was reported (Roberts et al., 2000; Sims et al., 2000; Sylvetsky et al., 2017c; Wood et al., 2000), potentially underlying heterogeneity in metabolic responses to sucralose. Saccharin is slowly absorbed from the gut to the bloodstream, and a minority of ingested saccharin (5%–15%) is excreted in feces, mostly unchanged (Ball et al., 1974; Renwick, 1985; Sweatman et al., 1981). The long absorption time and poor bioavailability support possible interactions with the microbiome. Degradation of steviol glycosides by gut bacteria is an established component of their metabolism (Magnuson et al., 2016), although some species may be more proficient than others in performing this task (Gardana et al., 2003), and thus, pre-exposure microbiome heterogeneity may conduce to differential responses to stevia. In contrast to sucralose, saccharin, and stevia, aspartame is metabolized by host enzymes in the proximal regions of the gastrointestinal tract (Magnuson et al., 2016). Thus, the mechanisms through which aspartame modulated the fecal microbiome of human participants in this study and in previous reports in animal models (Nettleton et al., 2020; Palmnäs et al., 2014) merit further study.

NNS may impact gut commensals through several direct and indirect mechanisms, some highlighted by our study's results. First, NNS may induce microbial growth inhibition, as was shown for cultured *E. coli* (Harpaz et al., 2018; Wang et al., 2018), pathogens (Sünderhauf et al., 2020), oral (Prashant et al., 2012), and environmental (Omran et al., 2013) bacteria, or of commensals of the rat cecal content (Naim et al., 1985) and human stool (Vamanu et al., 2019). *In vivo* studies similarly demonstrated a reduction in total fecal bacterial loads in animals treated with sucralose (Abou-Donia et al., 2008) or saccharin (Sünderhauf et al., 2020). The bacterial targets affected by NNS are not fully identified and potentially include disruption of quorum sensing (Bian et al., 2017c; Markus et al., 2021), triggering of SOS responses (Yu et al., 2021), increased membrane permeability (Yu et al., 2021), increased mutation frequency (Qu et al., 2017), inhibition of glucose/sucrose transport to the bacterial cell (Omran et al., 2013; Pfeffer et al., 1985), inhibition of sucrose enzymatic degradation or glucose fermentation (Omran et al., 2013; Pfeffer et al., 1985), and a reduction in the abundance of phosphotransferase system (PTS) genes involved in the transport of sugars to the bacterial cell, in microbiome cultures and in mice exposed to saccharin (Suez et al., 2014). In the current study, sucralose exposure resulted in reduced abundance of nucleotide biosynthesis genes, which might be linked to inhibited bacterial replication. In contrast, the abundance of genes related to mixed-acid fermentation and TCA cycle increased during sucralose supplementation. TCA metabolites were also elevated in plasma during sucralose supplementation, suggestive of possible microbiome contributions. Elevated plasma levels of TCA metabolites has been associated with impaired glycemic control (Fiehn et al., 2010; Guasch-Ferré et al., 2020).

In addition, some bacterial species may bloom in the presence of NNS (Palmnäs et al., 2014; Rodriguez-Palacios et al., 2018). Blooming may be mediated by an unoccupied niche, depleted of species inhibited by NNS. Alternatively, some gut bacteria can utilize NNS that reach the lower gut (e.g., saccharin and sucralose) as an energy or carbon source, thus gaining a growth advantage in their presence (Schiffman and Rother, 2013). Such mechanism was mainly studied to date in environmental bacteria (Labare and Alexander, 1995) or shown to exist in a single species in the presence of oxygen (Schleheck and Cook, 2003). One hint for such a mechanism in our study relates to the detection of several metabolites in the plasma of saccharin-supplemented individuals that may stem from saccharin degradation. Exploring such degradation capacities merits future studies in gut-residing human commensals. Notably, NNS could potentially alter the microbiome through indirect, host-mediated effects. These include interaction between NNS and sweet and/or bitter taste receptors in the gut and downstream effects on the microbiome (Turner et al., 2020) and possible effects of NNS on the immune system (Bian et al., 2017a, 2017c; Cheng et al., 2021; Martínez-Carrillo et al., 2019). These merit further study.

Collectively, our study suggests that commonly consumed NNS may not be physiologically inert in humans as previously contemplated, with some of their effects mediated indirectly through impacts exerted on distinct configurations of the human

microbiome. We stress that these results should not be interpreted as calling for consumption of sugar, which is strongly linked to cardiometabolic diseases and other adverse health effects (Malik and Hu, 2022; Vos et al., 2017). Unraveling molecular mechanisms and clinical consequences of NNS consumption on the human host and microbiome may enable to optimize dietary recommendations in preventing and treating hyperglycemia and its metabolic ramifications.

Limitations of the study

Limitations of our study include the inclusion of healthy, non-overweight, normoglycemic individuals, as NNS effects may differ between healthy and individuals with cardiometabolic diseases (Nichol et al., 2019), calling for further studies in these populations. In addition, the NNS in our study were administered as commercially available sachets containing a mixture of glucose and a given NNS. Microbiome and glycemic responses may differ when administered as commercial NNS sachets (containing carbohydrates as fillers) (Romo-Romo et al., 2018; Suez et al., 2014) in comparison with their purified forms (Ahmad et al., 2020a, 2020b; Kim et al., 2020; Serrano et al., 2021; Thomson et al., 2019). Indeed, Dalenberg et al. elegantly reported an adverse effect of short-term supplementation with sucralose on glycemic control, only when the NNS was coupled with a carbohydrate (Dalenberg et al., 2020). In line with this observation, we found that a combination of bulking glucose with saccharin or sucralose, but not glucose alone, resulted in an impaired glycemic response, whereas elevated plasma insulin was only observed in individuals supplemented with glucose alone or stevia. Notably, plasma insulin was not measured under fasting conditions, limiting the interpretation of these results. However, if such formulation distinction is true, NNS impacts should be compared in future controlled trials between consumers of carbohydrate-rich and carbohydrate-restrictive diets for their potential differential effects on human metabolic physiology. Of note, longer exposure periods (4–10 weeks) to pure NNS were suggested by some studies to negatively impact metabolic health even in the absence of a carbohydrate additive (Bueno-Hernández et al., 2020; Higgins and Mattes, 2019; Lertrit et al., 2018; Méndez-García et al., 2022). As such, a longer exposure period than the one utilized in our study may be required to fully assess the potential health ramifications mediated by the altered microbiome upon consumption of different NNS. Likewise, the NNS doses tested in our study were 240 mg (aspartame, ~8% ADI), 180 mg (saccharin, ~20% ADI), 102 mg (sucralose, 34% ADI), and 180 mg (stevia, ~75% ADI). Future studies may determine whether even lower doses may differentially impact the microbiome and host glycemic responses.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2022.07.016.

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AUTHOR CONTRIBUTIONS

J.S. and E.E. conceived the study and designed the intervention; directed the human trial and data collection; designed, performed, analyzed, and interpreted experiments and computational analysis; and wrote the manuscript. Y.C. headed and performed all computational analyses, analyzed and interpreted the results, wrote the manuscript, and equally contributed to the study.

R.V.-M. and U.M. performed computational analyses and provided essential tools and insights. M.D.-B. performed sample processing and next-generation DNA sequencing. S.F., H.S., A.L., R.L., and M.H. performed and assisted in experiments and sample processing. N.Z. participated in study design and protocol development. A.B., M.Z., R.B.-Z.B., S.E.-M., A.M., R.F., and O.S. coordinated the randomized-controlled trial, including data and sample collection; and recruiting, training, and following up on participants. S.M. and M.I. performed metabolomics experiments. N.S. and A.H. supervised all GF experiments. C.K.S.-T. contributed key insights and tools. E.S. and E.E. co-supervised the study.

DECLARATION OF INTERESTS

E.S. is a scientific co-founder of DayTwo. E.E. is a scientific co-founder of DayTwo and BiomX, and a paid consultant to Hello Inside and Aposense. E.E. is a member of the Cell scientific advisory board.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Insulin ELISA	Crystal Chem	Cat #90095
GLP-1 ELISA	Crystal Chem	Cat #81506
Chemicals, peptides, and recombinant proteins		
D(+)-Glucose monohydrate (for GTT in mice)	J.T. Baker	113
Commercial NNS: saccharin		N/A
Glucose for human trial	Floris	N/A
Commercial NNS: sucralose		N/A
Commercial NNS: aspartame		N/A
Commercial NNS: stevia		N/A
Critical commercial assays		
NextSeq 500/550 High Output v2 kit (75 cycles), for Metagenome shotgun sequencing	Illumina	Cat# 20024906
DNeasy PowerLyzer PowerSoil Kit	QIAGEN	Cat# 20-27100-12-EP
Experimental models: Organisms / strains		
Germ-free Swiss-Webster males 7-9 weeks of age	Weizmann Institute of Science	N/A
Deposited data		
Stool and oral metagenomics sequencing	PRJEB47383	N/A
Software and algorithms		
Code package for TCAM analysis	https://github.com/UriaMorP/mprod_package	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eran Elinav (Eran.Elinav@Weizmann.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Metagenomics sequencing data have been deposited at the European Nucleotide Archive (ENA) and are publicly available as of the date of publication (accession number PRJEB47383). Accession number is also listed in the key resources table. The code package for the TCAM analysis can be found in the Github repository https://github.com/UriaMorP/mprod_package. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Human study design

This was an open-label, multi-arm RCT that assessed the effects of short-term supplementation of non-caloric sweeteners on the microbiome, glucose tolerance, and additional health parameters, in healthy adults. The primary outcome was blood glucose levels, measured during standardized glucose tolerance tests (GTT) using a continuous glucose monitor (CGM). Secondary outcomes included microbiome readouts in stool and oral samples, and additional anthropometrics. The study protocol and associated procedures were approved by Weizmann Institute of Science Bioethics and Embryonic Stem Cell Research oversight committee (IRB approval number 170-2), and reported to http://clinicaltrials.gov/, Registration Number NCT03708939. Written informed consent was obtained from all subjects.

Inclusion and exclusion criteria

Eligible participants were males and females aged 18–70 able to provide informed consent and operate a glucometer and a smartphone. We excluded participants who consumed any quantity of NNS-containing foods or beverages in the six months prior to the trial initiation using an online custom-made food frequency questionnaire, further validated over the phone by a Clinical Research Associate (CRA). Participants were required to provide their consumption frequency (never; once a month; more than once a month; once a week; more than once a week; once a day; more than once a day) for each of the following NNS-containing products on the Israeli market: carbonated diet drinks; sugar-free energy drinks; non-carbonated diet drinks (diet iced tea, diet fruit juices, diet syrups); diet yogurt; sugar-free chewing gum; protein powders/gainers; sugar-free ice-cream; diet/low/sugar-free cookies, cakes, pastry; low/sugar-free jam, marmalade; sachets/tablets/drops of non-nutritive sweeteners; low/sugar-free ketchup or other sauces; sugar-free halva; diet bread / pita bread; diet syrups (chocolate, maple…); sugar-free chocolate; low/sugar-free cereals; any product labeled as ''diet / low / sugar-free''. Additional exclusion criteria included: (i) pregnancy or fertility treatments; (ii) breastfeeding (including baby to breast and bottle feeding mother's expressed breast milk); (iii) usage of antibiotics or antifungals within three months prior to participation; (iv) BMI < 18 or > 28; (v) pre-diagnosed type 1 or type 2 diabetes mellitus or treatment with anti-diabetic medication; (vi) chronically active inflammatory or neoplastic disease in the three years prior to enrollment; (vii) chronic gastrointestinal disorder, including inflammatory bowel disease and celiac disease; (viii) active neuropsychiatric disorder; (ix) myocardial infarction or cerebrovascular accident in the six months prior to participation; (x) coagulation disorders; (xi) chronic immunosuppressive medication usage; (xii) alcohol or substance abuse; (xiii) bariatric surgery; (xiv) phenylketonuria excluded randomization to the aspartame group. Screening for individuals meeting the aforementioned criteria was achieved using an online questionnaire, validated over the phone by a CRA or a medical doctor. The most frequent exclusion criterion was NNS consumption (Figure 1B).

Participants

Between 2018-2020, a total of 131 eligible participants were invited in groups of 4-12 to an initiation meeting at the Weizmann Institute of Science, during which they received full details regarding the study aims, protocol, and risks. After the meeting, all 131 individuals consented to participate, filled an informed consent form, and were randomized to six intervention arms: aspartame, sucralose, saccharin, stevia, glucose vehicle, or no supplement control (see the Consolidated Standards of Reporting Trials (CONSORT) flow diagram in Figure 1B). Seven participants decided to prematurely terminate their participation for the following reasons (Figure 1B): positive result in a pregnancy test conducted during the baseline week (N=1, saccharin); dislike for the taste of the NNS (N=1, sucralose); nausea after performing GTTs (N=1, NSC); pain and minor bleeding during sensor insertion (N=1, stevia); difficulty with adherence to the study protocol (N=3, two in the glucose vehicle group and one in the NSC group). All participants withdrew prior to day 14, thus an intention-to-treat analysis was not feasible. Four additional participants were excluded from the analysis after the trial was completed due to insufficient data for the primary outcome, as follows: mishandling of the CGM resulting in no recorded glucose events (N=1, stevia); less than four valid recorded GTTs (out of nine, see GTT exclusion criteria below, N=3, one each in the stevia, glucose, and NSC groups).

Cohort details

A total of 120 participants were included in the analysis, of which 65% were female, median age 29.95 (IQR 26.93-35.23). No baseline differences were found between the groups in the following parameters (Table S1): Weight, BMI, waist-hip ratio, %HbA1c, CRP, total cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, heart rate, ALT, AST (Kruskal-Wallis); dietary habits, smoking (Chi-square).

Animals

Germ-free mice were used to causally link between microbiome of NNS-consuming humans and glucose tolerance. All mice were Swiss-Webster WT adult (seven- to nine-week-old) males and served as recipients for fecal microbiome transplants from human donors. All animal studies were approved by the Weizmann Institute of Science Institutional Animal Care and Usage Committee (IACUC), application number 13250419-3.

METHOD DETAILS

Human trial experimental procedures

During an introductory meeting at the Weizmann Institute, consenting individuals were connected to a continuous glucose monitor (CGM; FreeStyle Libre, Abbott) and anthropometric, blood pressure and heart-rate measurements were taken by a Clinical Research Associate (CRA) or a certified nurse, as well as a non-fasting blood test used for the blood works detailed in Table S1, as well as for insulin and GLP-1 using commercial ELISA kits (Crystal Chem). This was considered as day 0 (Figure 1). Participants arrived for two additional identical sessions on days 14 and 28, which was the last day of the trial. The CGM was replaced on day 14 according to the manufacturer's instructions. Participants were provided with a kit to sample their microbiome and perform glucose tolerance tests at home on pre-determined days. On days 8-21, participants in all groups except NSC consumed six commercially available sachets of NNS (with glucose as a vehicle bulking agent) or the equivalent amount of glucose daily, dissolved in water. NNS and glucose were provided by the researchers during the introductory meeting. Throughout the 28 days of the trial, participants were instructed to record all daily activities, including standardized and real-life meals, in real-time using their smartphones; meals were recorded with exact components and weights. For oral microbiome profiling, participants serially swabbed their buccal cavity in the morning on

pre-determined days during the trial (eight samples per participant), following tooth brushing, but before consumption of food or usage of mouthwash.

Non-nutritive sweeteners supplementation in humans

Participants were supplied with sachets of commercial formulations of NNS used for sweetening hot beverages, all containing glucose as a bulking agent. Common, commercially available sachets were chosen in order to resemble real-life intake and maximize adherence to the protocol and acceptability of the supplement. The distinct flavor of NNS and glucose rendered blinding unfeasible. Participants consumed six sachets a day during the exposure period, resulting in the following daily doses: aspartame 0.24g & 5.76g glucose, saccharin 0.18g & 5.82g glucose, sucralose 0.102g & 5.898g glucose, stevia (steviol glycosides) 0.18g & 5.82g glucose. These doses are below the acceptable daily intakes, correspondingly: 50mg/kg, 15mg/kg, 5mg/kg, 4mg/kg. The glucose vehicle group was supplemented with 5g of glucose daily. All groups received the same number of sachets per day to allow comparison to the glucose vehicle group. Participants were instructed to consume two sachets of NNS dissolved in water three times during the day: morning, afternoon, and evening. Drinking of NNS was allowed either with or without meals. Participants recorded intake in real-time using a dedicated smartphone app. A CRA reviewed the logs daily to guarantee adherence to the protocol.

Glucose tolerance tests in humans

Participants were provided with 50 g of glucose (Floris) to perform GTT at home, with the following instructions: (A) at least seven and no more than fourteen hours without any food, supplements, physical activity or any drink other than water prior to GTT initiation; (B) the entire amount of glucose should be dissolved in a cup of water without any supplements and consumed in less than two minutes; (C) no physical activity, food or drinks other than water allowed during two hours after initiation; (D) GTT initiation should be recorded on the smartphone app, and data from the glucose sensor should be downloaded frequently to the reader. Participants received text message reminders on the days of the GTT. Out of 1080 expected GTTs, 5.5% (n=60) were not performed (n=23) or performed but data were not recorded (n=37). Sensor malfunctions resulted in the loss of 1% of GTTs (n=11). In addition, 2.6% of GTTs (n=28) were excluded from the analysis for not meeting the aforementioned requirements, as follows: food consumed during GTT (n=14), physical activity performed during GTT (n=5), fasting exceeds fifteen hours (n=4), consumption of glucose took >10 minutes (n=3), participant took a glucose-lowering medication (n=2). There were no significant differences (Chi-square) in the number of missing/excluded GTTs between phases (P=0.86) or treatment groups (P=0.61).

CGM Coefficient of variance (CoV) analysis

Daily CoV was evaluated for each participant by dividing the daily standard deviation, considering all glucose measurements of the day, by the daily average. The significance of the trajectory changes in CoV was tested using LMER with participants as random effect and testing for the interaction between time and groups.

Adverse events

Few minor adverse events were reported (n=5), all during the baseline week prior to supplementation with NNS: diarrhea after first GTT (n=2, one in the aspartame group and the other in the NSC group); common cold-like symptoms for several days without need for medical care (n=2, sucralose and saccharin); mild transient pain in the CGM insertion site (n=1, aspartame).

Diet and activity logs analysis

Throughout the trial period, participants logged the following activities on an in-house developed smartphone application (Zeevi et al., 2015): sleep and wakeup time, physical activity (type, duration and intensity), meals, snacks, and drinks (ingredients and quantities), and medications. Participants were monitored at near-real-time for compliance in recording food and activity and contacted by phone as needed. To limit confounding of the dietary analysis by poor or insufficient logging of meals, we included in the final analysis only participants that had at least 20 days with at least 1,000 kcal logged per day; the number of analyzed individuals was not significantly different between the groups.

Glucose tolerance tests in conventionalized germ-free mice

Germ-free Swiss-Webster WT adult (seven to nine-week-old) male mice served as recipients for fecal microbiome transplants and were housed in sterile isolators (Park Bioservices). Fecal samples from human donors were frozen immediately after collection and were stored in a -80C freezer prior to processing. Two hundred mg of frozen stool was resuspended in 5 ml of sterile PBS under anaerobic conditions (Coy Laboratory Products, 75% N_2, 20% CO_2, 5% H_2), vortexed for 3 min and allowed to settle by gravity for 2 min. The anaerobic homogenate was transferred to the animal facility in airtight Hungate tubes placed in anaerobic pouches (GasPak™ EZ Anaerobe Pouch System). Transplant into recipient mice was achieved by gavage with 200 μl of the supernatant. Mice were maintained on normal chow diet and water throughout the experiment. Mice were kept in iso-cages with sufficient food and water and were not handled until a glucose tolerance test was performed seven days post-conventionalization. Transplantation efficacy was determined by Bray-Curtis dissimilarity of each recipient mouse to its corresponding human donor, and was comparable between mouse recipients of top and bottom sucralose responder microbiomes and between mouse recipients of baseline and day 21 microbiomes. For **glucose tolerance tests**, mice were fasted for 6 h during the light phase, with free access to water.

Blood from the tail vein was used to measure glucose levels using a glucometer (Bayer) immediately before and 15, 30, 60, 90 and 120 min after oral gavage feeding with 40 mg glucose (J. T. Baker). In all experiments, each experimental group consisted of at least two cages to minimize cage-effects. To ensure sterility of the mice for this primary readout, additional metabolic outputs that require baseline measurements (e.g., weight) were not recorded.

Shotgun metagenomic sequencing

Illumina libraries were prepared using a Nextera DNA Library Prep kit (Illumina, 20034198) according to the manufacturer's protocol and sequenced on an Illumina NextSeq platform with a read length of 75 bp (single-end) for all samples.

Metagenomic analysis of stool and oral microbiome samples

For human microbiome samples, data from the Illumina NextSeq sequencer were converted to fastq files with bcl2fastq, resulting in 1222 stool samples and 735 oral samples (9948552 ± 3440344 average and standard deviation of reads per sample). Sequences were then QC trimmed using Trimmomatic (Bolger et al., 2014) with parameters PE -threads 10 -phred33 ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 SLIDINGWINDOW:4:20 MINLEN:50 and host sequences were removed using KneadData with default parameters using the hg19 reference. We then subsampled all the samples to 1M sequences for stool samples and 250,000 for oral samples, removing all samples below this threshold and retaining 1182 stool and 713 oral samples. We removed 40 additional oral samples due to contamination (defined as abundance of >1% for six or more of the top ten abundant species in stool). Kraken2 (Wood et al., 2019) was used for taxonomic analysis with a pre-built index database (Méric et al., 2019) and Bracken (Lu et al., 2017) was applied to estimate genus and species abundances. For functional annotations, we used both HUMAnN2 (Franzosa et al., 2018) as well as an in-house analytic pipeline. HUMAnN2 was used with the uniref90 (Suzek et al., 2007) as the protein database and Chocophlan (Franzosa et al., 2018) as the nucleotide database; the path abundance unstratified output with MetaCyc (Caspi et al., 2018) annotations was taken. The in-house pipeline consists of the following steps: first, we use diamond (Buchfink et al., 2021) against the human gut IGC (Li et al., 2014) reference which was filtered to contain only KEGG (Kanehisa and Goto, 2000) annotated entries with parameters –max-hsps 1 -k 1 -e 0.0001. We then sum all the hits for each gene divided by its length and grouped to KEGG Orthologs and relative abundance is calculated. Pathways and Modules were computed using EMPANADA (Manor and Borenstein, 2017). For dimensionality reduction of longitudinal microbiome data, participant microbiome trajectories were computed as the fold change from the baseline measurements averages, followed by application of M-product based Tensor Component Analysis (TCAM). Mouse samples were processed in a similar manner, with a total of 91 stool samples, and using the mm10 mouse genome reference. Mice samples were subsampled to 2.5M reads.

Untargeted metabolomics

Metabolite extraction

Extraction and analysis of lipids and polar/semipolar metabolites was performed as previously described (Malitsky et al., 2016; Zheng et al., 2015) with some modifications: 90μl of serum were extracted with 1 mL of a pre-cooled (−20C) homogenous methanol:methyl-tert-butyl-ether (MTBE) 1:3 (v/v) mixture, containing following internal standards: 0.1 $\mu g^{*}mL^{-1}$ of Phosphatidylcholine (17:0/17:0) (Avanti), 0.4 $\mu g^{*}mL^{-1}$ of Phosphatidylethanolamine (17:0/17:0, 0.15 $nmol^{*}mL^{-1}$ of Ceramide/Sphingoid Internal Standard Mixture I (Avanti, LM6002), 0.0267 μg/mL d5-TG Internal Standard Mixture I (Avanti, LM6000) and 0.1 $\mu g^{*}mL^{-1}$ Palmitic acid-13C (Sigma, 605573). The tubes were vortexed and then sonicated for 30 min in ice-cold sonication bath (taken for a brief vortex every 10 min). Then, UPLC-grade water: methanol (3:1, v/v) solution (0.5 mL) containing internal following standards: C13 and N15 labeled amino acids standard mix (Sigma) was added to the tubes followed by centrifugation. The upper, organic phase was transferred into 2 mL Eppendorf tube. The polar phase was re-extracted as described above, with 0.5 mL of MTBE. Both organic phases were combined and dried in speedvac and then stored at −80C until analysis. For analysis, the lower, polar phase was used for polar and semipolar metabolite analysis was lyophilized and resuspended in 200 μL Methanol:DDW (50:50).

LC-MS for semipolar metabolites processing

Metabolic profiling of semipolar phase was performed using Waters ACQUITY UPLC system coupled to a Vion IMS QTof mass spectrometer (Waters Corp., MA, USA). The LC separation was as previously described (Itkin et al., 2011) with minor alterations. Briefly, the chromatographic separation was performed on an ACQUITY UPLC BEH C18 column (2.1×100 mm, i.d., 1.7 μm) (Waters Corp., MA, USA). The mobile phase A consisted of 95% DDW and 5% acetonitrile, with 0.1% formic acid; mobile phase B consisted of 100% acetonitrile with 0.1% formic acid. The column was maintained at 35C, and the flow rate of the mobile phase was 0.3 $mL^{*}min^{-1}$. Mobile phase A was initially run at 100%, and it was gradually reduced to 72% at 22 min, following a decrease to 0% at 36 min. Then, mobile phase B was run at 100% until 38 min; then, mobile phase A was set to 100% at 38.5 min. Finally, the column was equilibrated at 100% mobile phase A until 40 min. MS parameters were as follows: the source and de-solvation temperatures were maintained at 120C and 350C, respectively. The capillary voltage was set to 2 kV at negative ionization mode; cone voltage was set for 40 V. Nitrogen was used as de-solvation gas and cone gas at the flow rate of 700 $L^{*}h^{-1}$ and 50 $L^{*}h^{-1}$. The mass spectrometer was operated in full scan $HDMS^{E}$ negative or positive resolution mode over a mass range of 50–2000 Da. For the high-energy scan function, a collision energy ramp of 20–80 eV was applied; for the low energy scan function – 4 eV was applied. Leucine-enkephalin was used as a lock-mass reference standard.

Semipolar compounds identification and data processing

LC-MS data were analyzed and processed with UNIFI (Version 1.9.4, Waters Corp., MA, USA). The putative identification of the different semipolar species was performed by comparison accurate mass, fragmentation pattern and ion mobility (CCS) values to in-house made semipolar database, when several compounds were identified vs. standards, when available.

LC-MS polar metabolite analysis

Metabolic profiling of polar phase was done as previously described (Zheng et al., 2015) with minor modifications described below. Briefly, analysis was performed using Acquity I class UPLC System combined with mass spectrometer Q Exactive Plus Orbitrap™ (Thermo Fisher Scientific) which was operated in a negative ionization mode. The LC separation was done using the SeQuant ZicpHilic (150 mm × 2.1 mm) with the SeQuant guard column (20 mm × 2.1 mm) (Merck). The Mobile phase B: acetonitrile and Mobile phase A: 20 mM ammonium carbonate with 0.1% ammonia hydroxide in DDW: acetonitrile (80:20, v/v). The flow rate was kept at 200 μL^* min^{-1} and gradient as follow: 0-2 min 75% of B, 14 min 25% of B, 18 min 25% of B, 19 min 75% of B, for 4 min, 23 min 75% of B. For metabolites normalization, peak areas of metabolites were divided by summed relative abundances of internal standards (labeled amino acids). Further normalization was carried out by dividing each metabolite with the median value of that metabolite, as it was shown to produce the most accurate results (Wulff and Mitchell, 2018). Statistical tests were employed using repeated measures one way ANOVA for testing of three time points, and by paired t-test for the two time point analysis (for the sucralose and control groups), FDR-BH correction was applied. For pathway analysis P-value significant metabolites of the sucralose group were run in the MetaboAnalyst pipeline (Pang et al., 2021) using the hypergeometric test and the KEGG (*Homo Sapiens*) database.

Polar metabolites data processing

The data processing was done using TraceFinder (Thermo Fisher Scientific), when detected compounds were identified by accurate mass, retention time, isotope pattern, fragments and verified using in-house-generated mass spectra library.

QUANTIFICATION AND STATISTICAL ANALYSIS

The sample size for the RCT was calculated to have a power of >80% to detect a 30% increase in glucose tolerance (GTT AUC) with a probability of a type I error (α) of 0.05. This value is based on our preliminary trial, in which we observed an average increase in GTT AUC of 1000 units in saccharin-supplemented individuals (Suez et al., 2014). In all linear mixed-effects models, participants were considered as the random effect, reflected as donors in the mouse modeling. In the mouse modelling, we used either responsiveness (top, bottom) or time point (baseline, day 21) as the response variable, while the bacterial feature (gene, pathway, or bacterial species) was used as the explanatory variable. PERMANOVA was used with the stratification of participants when performing the random permutations. For the correlation analysis, we used the average of all mice relating to the same donor and time point to handle repeated samples. Statistical tests were performed in PRISM (V 9.2), R and Python. The tests used in each analysis are indicated in the main text. Types of center and dispersion measures are indicated in the figure legends. PRISM was used as the primary tool for statistical analysis, apart from the microbiome linear mixed modelling (R, lmer package) and microbiome PERMANOVA and univariate testing (Python). The AUC of log fold change was computed only for the topmost informative features detected by TCAM in primary PCs. The computation was performed by taking the cumulative sum of the $\log_2$ fold change from baseline values of each feature, unrelated to the TCAM algorithm computation.

Data integrity check

Figure and supplementary figure panels were checked for data integrity using the Proofig pipeline, https://www.proofig.com.

Supplemental figures

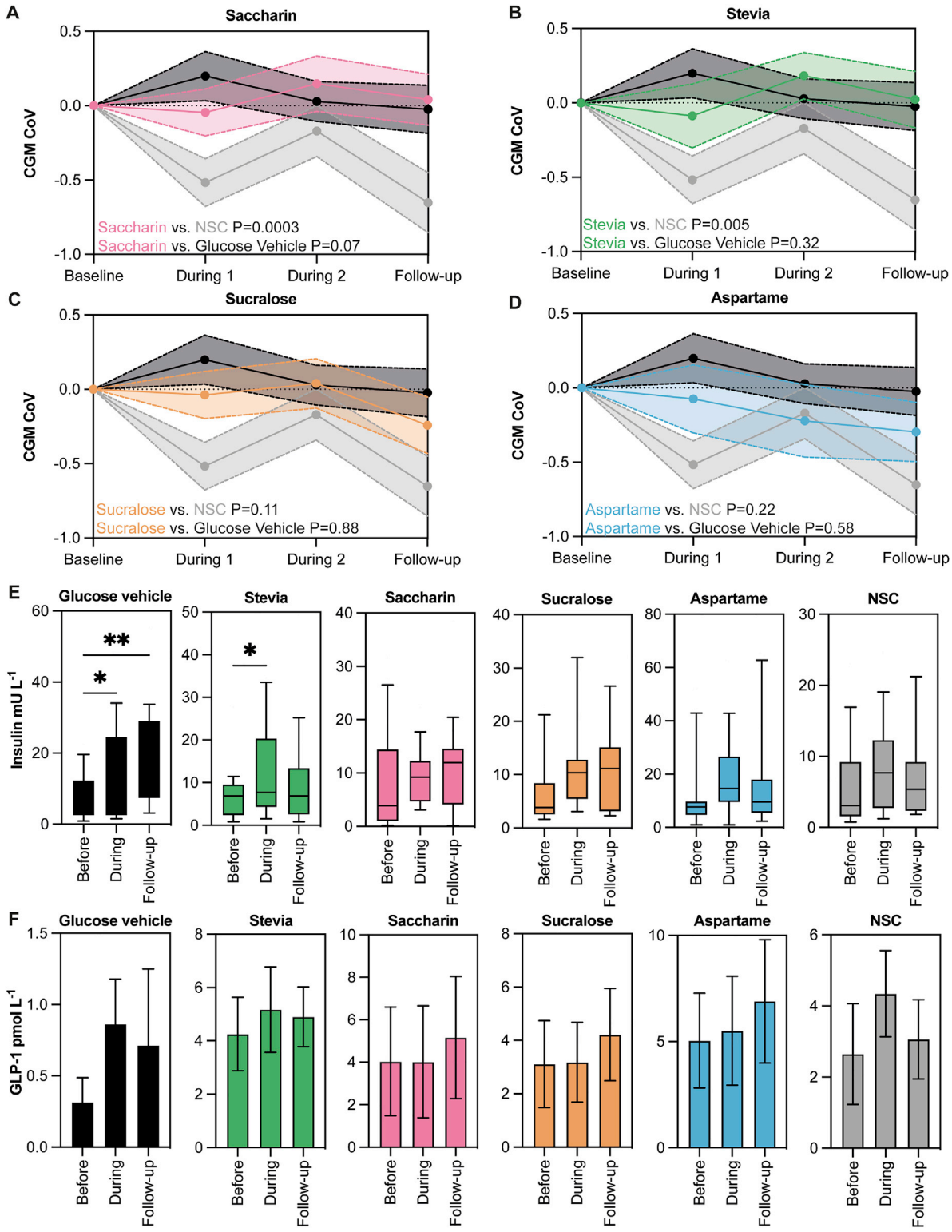

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Figure S1. Effect of saccharin and sucralose on additional measurements of glycemic control, related to Figure 2

(A–D) Interstitial glucose levels were monitored continuously throughout the trial using a continuous glucose monitor (CGM). The coefficient of variance (CoV) was calculated as a measurement of blood glucose fluctuations, in the glucose vehicle and NSC groups compared with (A) saccharin, (B) stevia, (C) sucralose, or (D) aspartame. Symbols, mean; error bars, SEM; Significance according to linear mixed effects regression.

(E and F) Blood samples were collected on the 1st and last day of the trial, and after 1 week of supplementation. Levels of non-fasting plasma (E) insulin and (F) glucagon-like peptide-1 (GLP-1) were measured using ELISA.

* $p < 0.05$; ** $p < 0.01$; two-way ANOVA and Dunnett. Horizontal lines, median; whiskers, 10–90 percentiles. NSC, no supplement control.

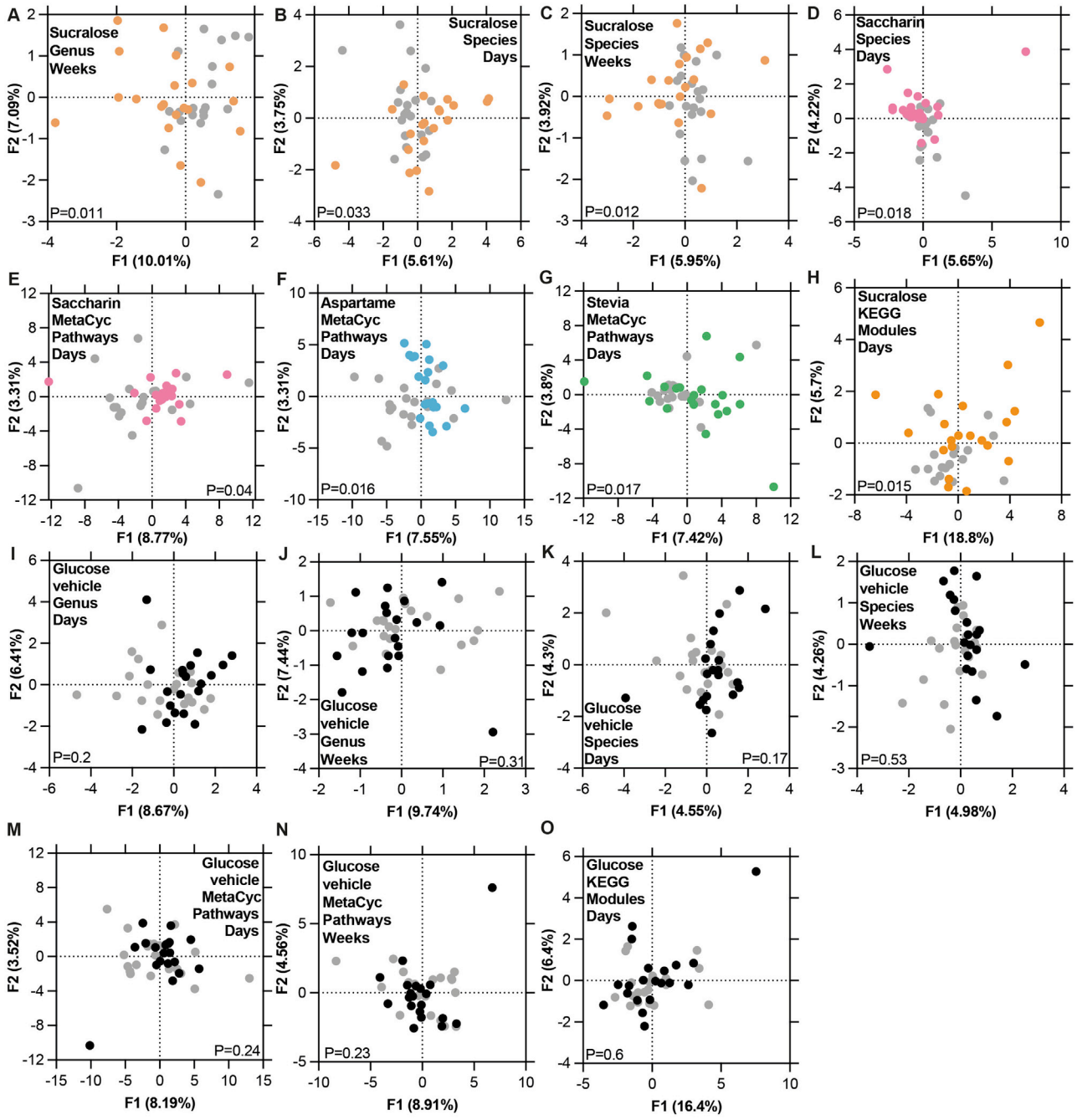

Figure S2. Effect of NNS and controls on the microbiome, related to Figure 3

Stool microbiome samples were collected at pre-determined days and analyzed for composition and function.

(A–H) Trajectory analysis ordination plots following tensor component analysis using M product (TCAM), which tested if a group had a significant trajectory compared with the no supplement control group. TCAM was applied to the fold change from baseline for each feature.

(A) Bacterial genera in the sucralose group.

(B and C) Bacterial species in the sucralose group stratified by (B) days or (C) weeks.

(D) Bacterial species in the saccharin group.

(E–G) MetaCyc pathways in the (E) saccharin, (F) aspartame, or (G) stevia group.

(H) KEGG modules in the sucralose group.

(I–O) Comparison of the glucose vehicle group to the no supplement control: (I and J) genus, (K and L) species, (M and N) MetaCyc pathways, or (O) KEGG modules. Significance of the trajectory analysis according to PERMANOVA.

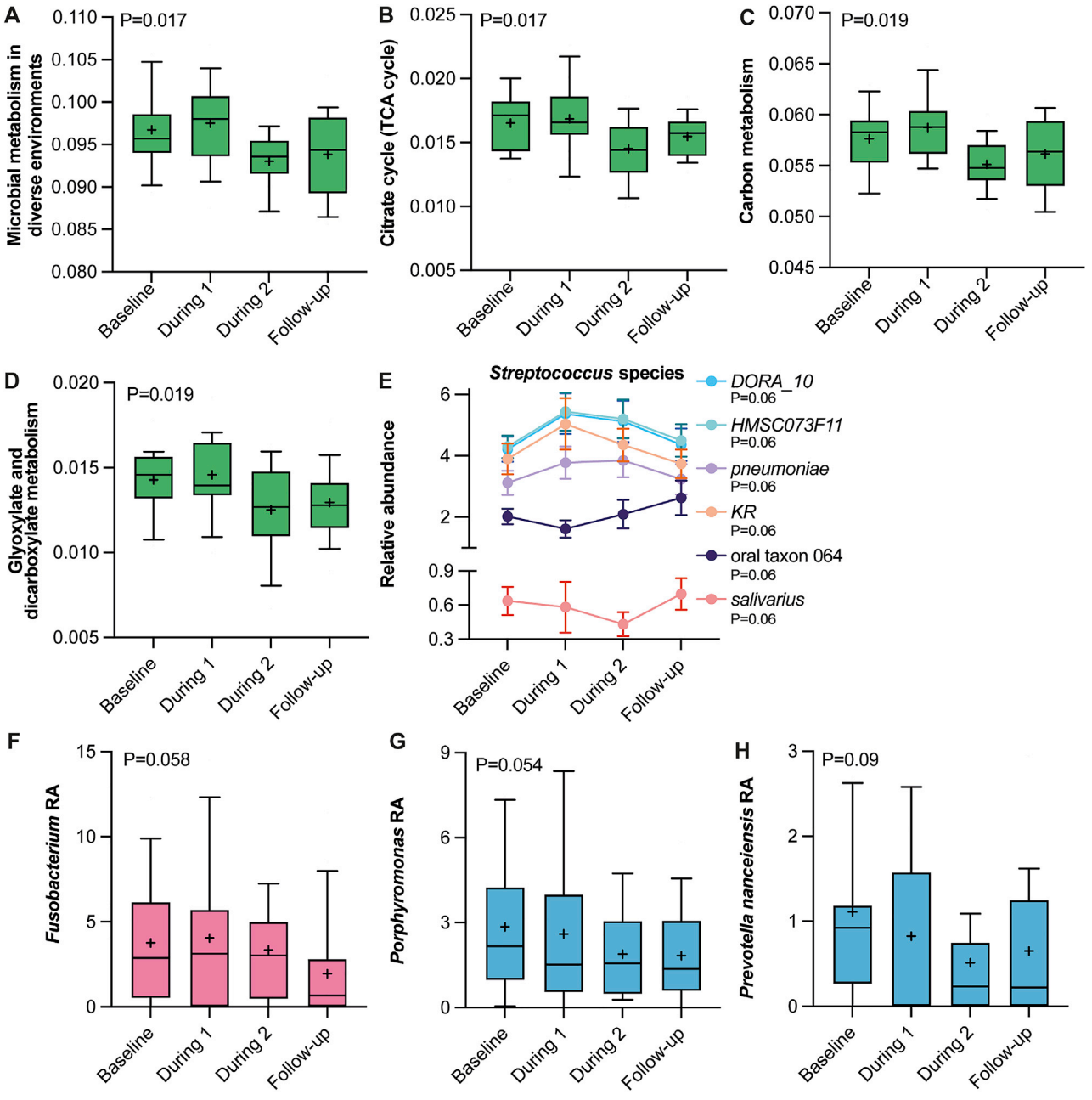

Figure S3. NNS modulate the oral microbiome composition and function, related to Figure 3

Relative abundances of features altered during exposure to NNS compared with baseline (FDR-corrected Friedman $p < 0.1$).

(A–D) KEGG pathways in the stevia group.

(E–H) (E) *Streptococcus* species in the sucralose group (F) *Fusobacterium*, saccharin, (G) *Porphyromonas*, aspartame, and (H) *Prevotella nanceiensis*, aspartame.

* $p < 0.05$; ** $p < 0.01$; two-way ANOVA and Dunnett. Horizontal lines, median; whiskers, 10–90 percentiles. NSC, no supplement control.

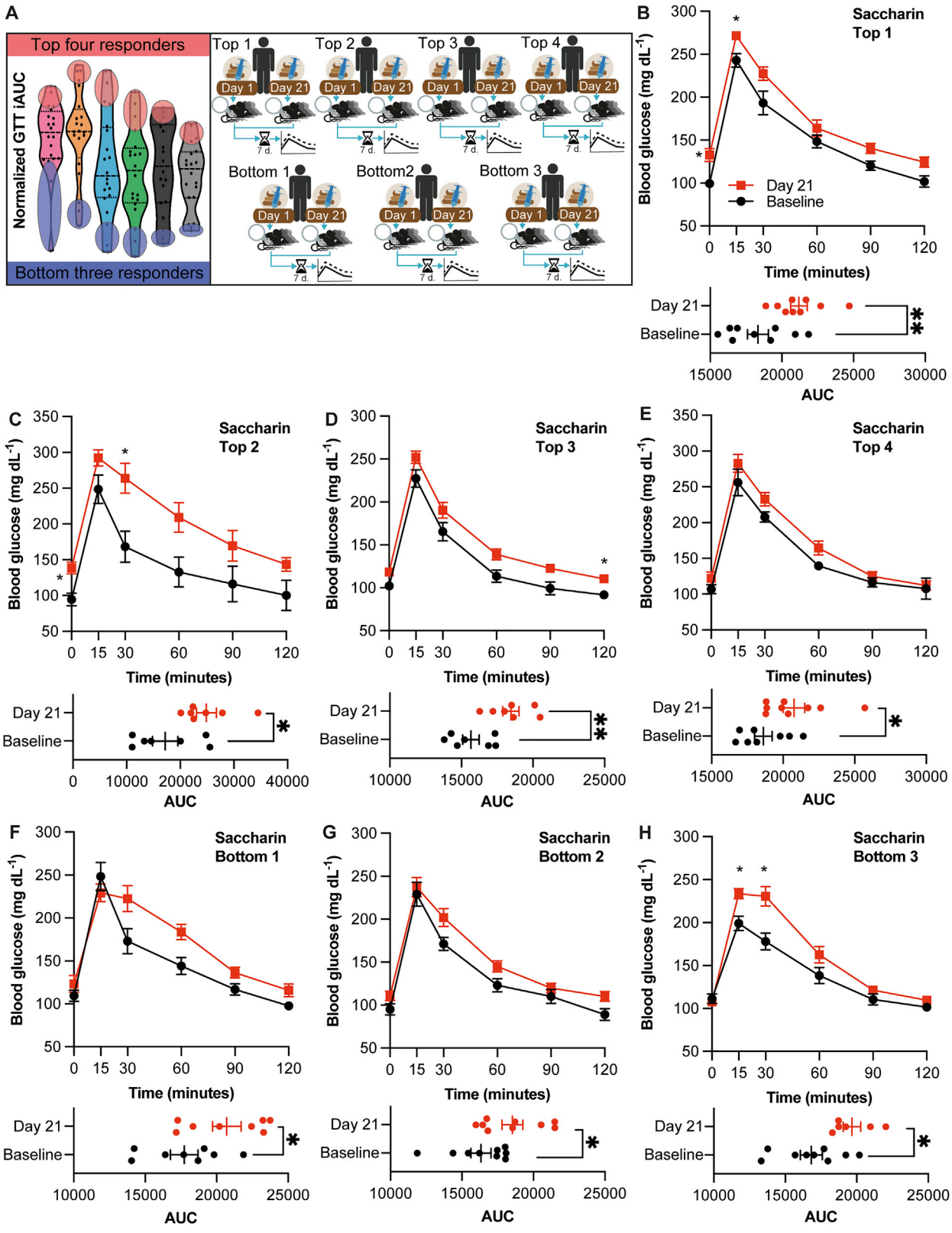

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Figure S4. Microbiomes of saccharin consumers causally linked to elevated glycemic response in germ-free mice, related to Figure 6

(A) Experimental design in conventionalized GF mice, related to Figure 6. The top four and bottom three responders in each of the six groups were defined following normalization to baseline of GTT-iAUCs during the 2nd week of exposure. From each of these 42 individuals, fecal samples from baseline and the last day of exposure (day 21) were used to conventionalize GF mice. A GTT was performed on recipient mice 7 days post colonization.

(B–H) Groups of age-matched germ-free male Swiss-Webster mice were transplanted with stool microbiomes taken during baseline and the last day of exposure to saccharin from the (B–E) top and (F–H) bottom glycemic responders (Figure 2). A glucose tolerance test was performed 6 days post-transplant (plotted with AUC).

(B) Recipients of top 1 microbiome: baseline N = 9, day 21 N = 9.

(C) Recipients of top 2 microbiome: baseline N = 7, day 21 N = 7.

(D) Recipients of top 3 microbiome: baseline N = 7, day 21 N = 7.

(E) Recipients of top 4 microbiome: baseline N = 8, day 21 N = 9.

(F) Recipients of bottom 1 microbiome: baseline N = 8, day 21 N = 8.

(G) Recipients of bottom 2 microbiome: baseline N = 9, day 21 N = 9.

(H) Recipients of bottom 3 microbiome: baseline N = 9, day 21 N = 6.

*$p < 0.05$; **$p < 0.01$; two-way ANOVA and Dunnett (in GTT panels) or Student's t test (in AUC panels). Lines (AUC) and symbols (GTT), mean; error bars, SEM.

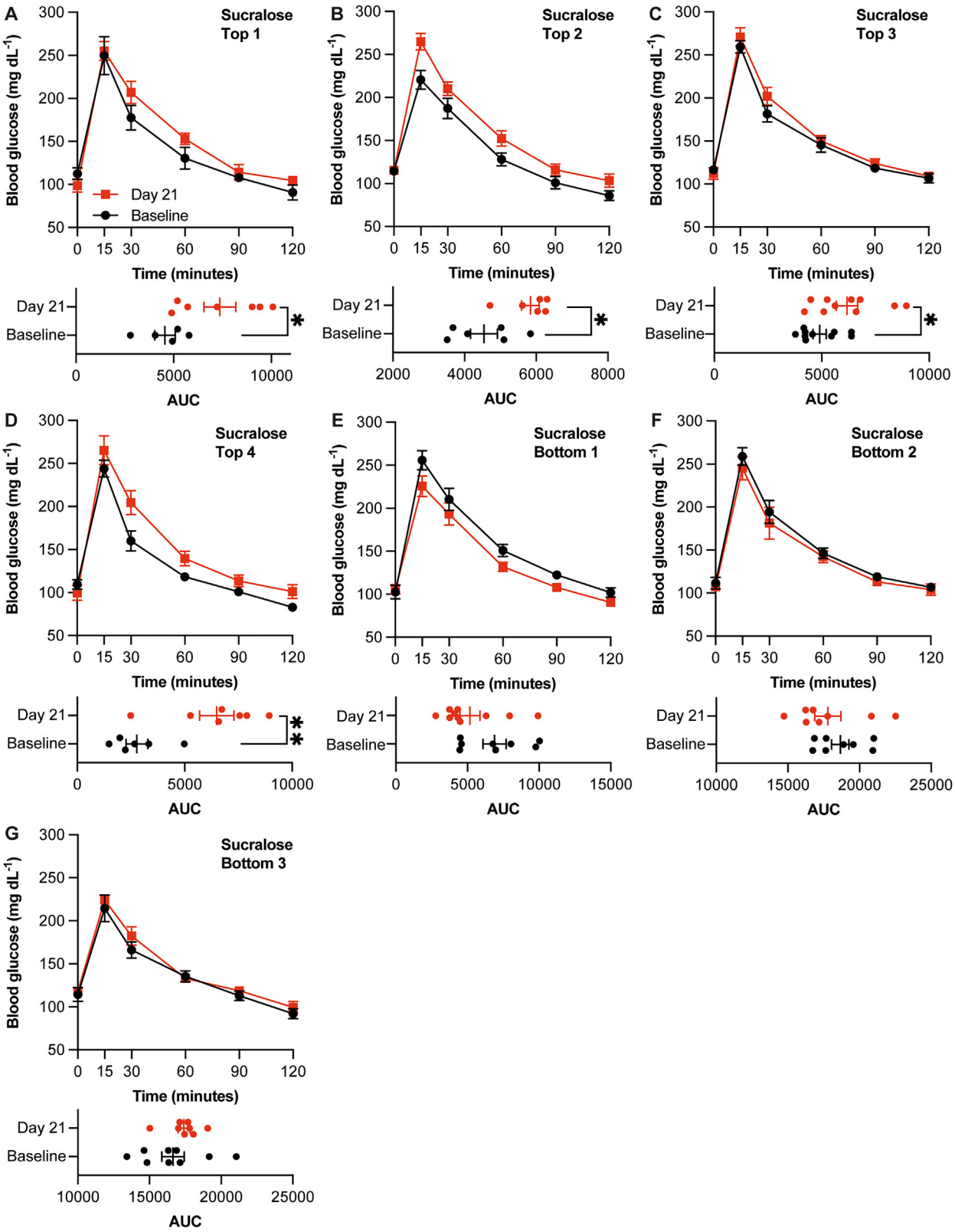

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Figure S5. Microbiomes of sucralose responders causally linked to elevated glycemic response in germ-free mice, related to Figure 6

(A–G) Groups of age-matched germ-free male Swiss-Webster mice were transplanted with stool microbiomes taken during baseline and the last day of exposure to sucralose from the (A–D) top and (E–G) bottom glycemic responders (Figure 2). A glucose tolerance test was performed 6 days post-transplant (plotted with AUC).

(A) Recipients of top 1 microbiome: baseline N = 5, day 21 N = 7.

(B) Recipients of top 2 microbiome: baseline N = 6, day 21 N = 6.

(C) Recipients of top 3 microbiome: baseline N = 10, day 21 N = 9.

(D) Recipients of top 4 microbiome: baseline N = 6, day 21 N = 7.

(E) Recipients of bottom 1 microbiome: baseline N = 8, day 21 N = 10.

(F) Recipients of bottom 2 microbiome: baseline N = 8, day 21 N = 8.

(G) Recipients of bottom 3 microbiome: baseline N = 9, day 21 N = 8.

* $p < 0.05$; ** $p < 0.01$; Student's t test. Lines (AUC) and symbols (GTT), mean; error bars, SEM.

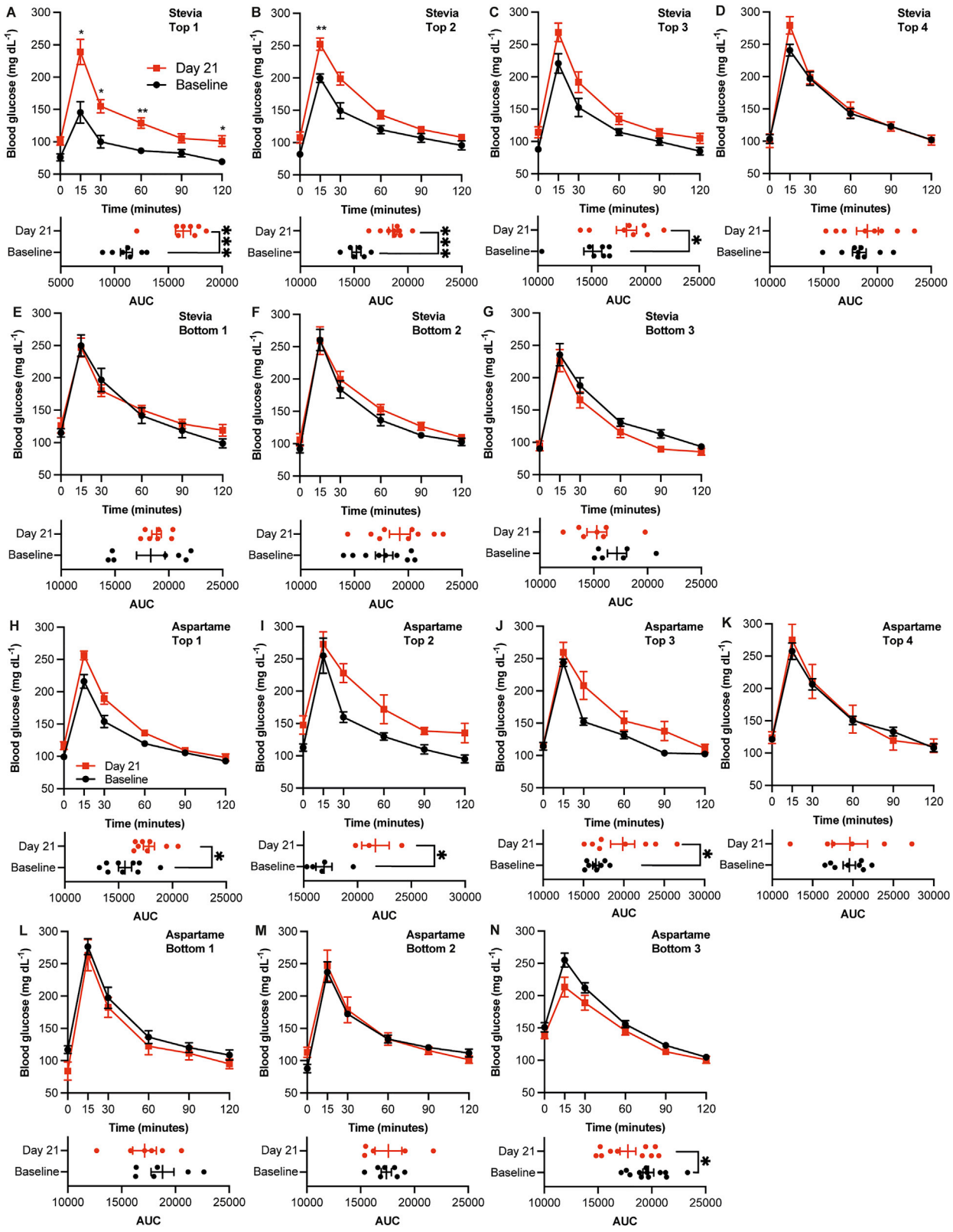

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Figure S6. Microbiomes of stevia and aspartame responders causally linked to elevated glycemic response in germ-free mice, related to Figure 6

(A–N) Groups of age-matched germ-free male Swiss-Webster mice were transplanted with stool microbiomes taken during baseline and the last day of exposure to (A–G) stevia or (H–N) aspartame from the (A–D and H–K) top and (E–G and L–N) bottom glycemic responders (Figure 2). A glucose tolerance test was performed 6 days post-transplant (plotted with AUC).

(A) Recipients of stevia top 1 microbiome: baseline N = 7, day 21 N = 8.

(B) Recipients of stevia top 2 microbiome: baseline N = 6, day 21 N = 9.

(C) Recipients of stevia top 3 microbiome: baseline N = 7, day 21 N = 8.

(D) Recipients of stevia top 4 microbiome: baseline N = 9, day 21 N = 8.

(E) Recipients of stevia bottom 1 microbiome: baseline N = 7, day 21 N = 7.

(F) Recipients of stevia bottom 2 microbiome: baseline N = 9, day 21 N = 9.

(G) Recipients of stevia bottom 3 microbiome: baseline N = 6, day 21 N = 7.

(H) Recipients of aspartame top 1 microbiome: baseline N = 9, day 21 N = 8.

(I) Recipients of aspartame top 2 microbiome: baseline N = 5, day 21 N = 3.

(J) Recipients of aspartame top 3 microbiome: baseline N = 8, day 21 N = 8.

(K) Recipients of aspartame top 4 microbiome: baseline N = 8, day 21 N = 6.

(L) Recipients of aspartame bottom 1 microbiome: baseline N = 6, day 21 N = 6.

(M) Recipients of aspartame bottom 2 microbiome: baseline N = 7, day 21 N = 5.

(N) Recipients of aspartame bottom 3 microbiome: baseline N = 12, day 21 N = 10.

*$p < 0.05$; **$p < 0.01$;***$p < 0.001$; two-way ANOVA and Dunnett (in GTT panels) or Student's t test (in AUC panels). Lines (AUC) and symbols (GTT), mean; error bars, SEM.

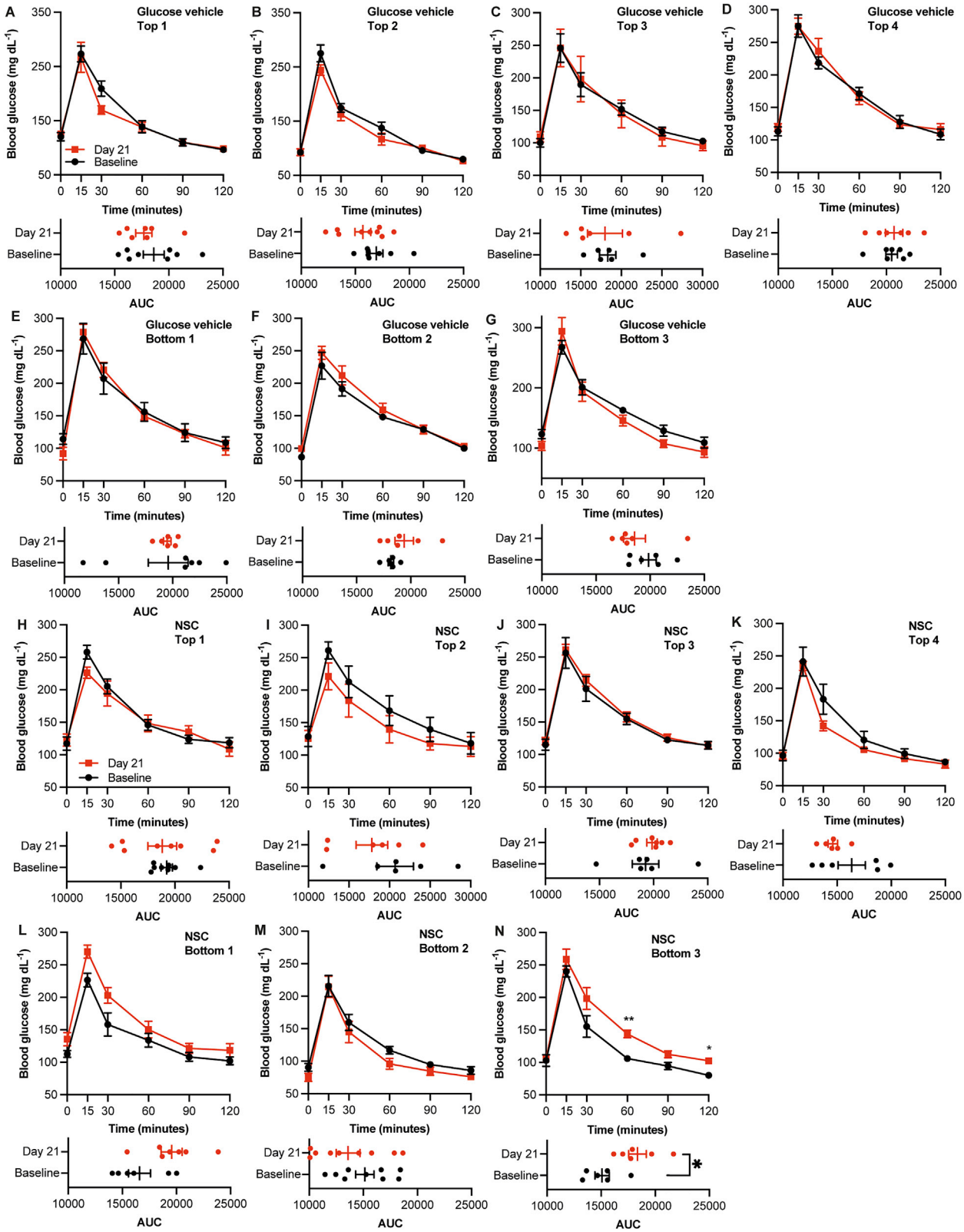

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Figure S7. Microbiomes of the glucose vehicle or no supplement control groups do not elevate glycemic response in germ-free mice, related to Figure 6

(A–N) Groups of age-matched germ-free male Swiss-Webster mice were transplanted with stool microbiomes taken during baseline and the last day of exposure to (A–G) glucose vehicle or (H–N) NSC from the (A–D and H–K) top and (E–G and L–N) bottom glycemic responders (Figure 2). A glucose tolerance test was performed 6 days post-transplant (plotted with AUC).

(A) Recipients of glucose vehicle top 1 microbiome: baseline N = 8, day 21 N = 7.

(B) Recipients of glucose vehicle top 2 microbiome: baseline N = 8, day 21 N = 9.

(C) Recipients of glucose vehicle top 3 microbiome: baseline N = 6, day 21 N = 6.

(D) Recipients of glucose vehicle top 4 microbiome: baseline N = 7, day 21 N = 6.

(E) Recipients of glucose vehicle bottom 1 microbiome: baseline N = 7, day 21 N = 6.

(F) Recipients of glucose vehicle bottom 2 microbiome: baseline N = 6, day 21 N = 6.

(G) Recipients of glucose vehicle bottom 3 microbiome: baseline N = 6, day 21 N = 6.

(H) Recipients of NSC top 1 microbiome: baseline N = 8, day 21 N = 8.

(I) Recipients of NSC top 2 microbiome: baseline N = 6, day 21 N = 6.

(J) Recipients of NSC top 3 microbiome: baseline N = 6, day 21 N = 7.

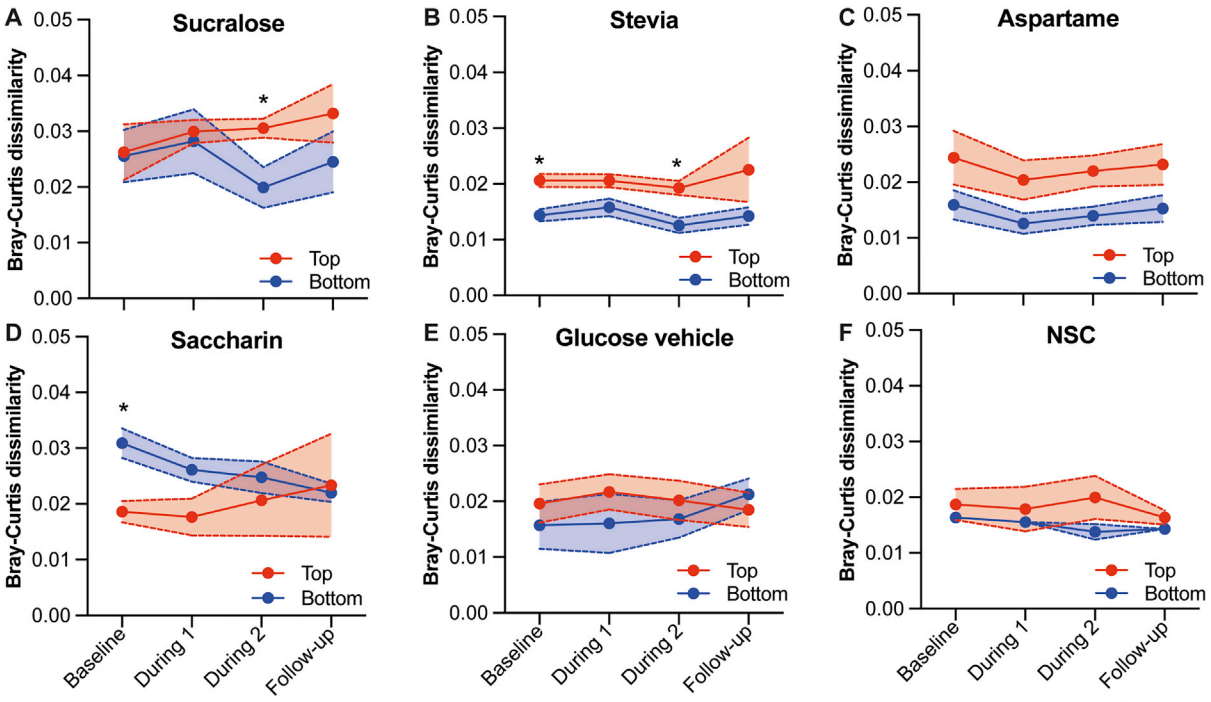
(K) Recipients of NSC top 4 microbiome: baseline N = 6, day 21 N = 6.

(L) Recipients of NSC bottom 1 microbiome: baseline N = 6, day 21 N = 7.

(M) Recipients of NSC bottom 2 microbiome: baseline N = 9, day 21 N = 9.

(N) Recipients of NSC bottom 3 microbiome: baseline N = 6, day 21 N = 6.

*$p < 0.05$; two-way ANOVA and Dunnett (in GTT panels) or Student's t test (in AUC panels). Lines (AUC) and symbols (GTT), mean; error bars, SEM.



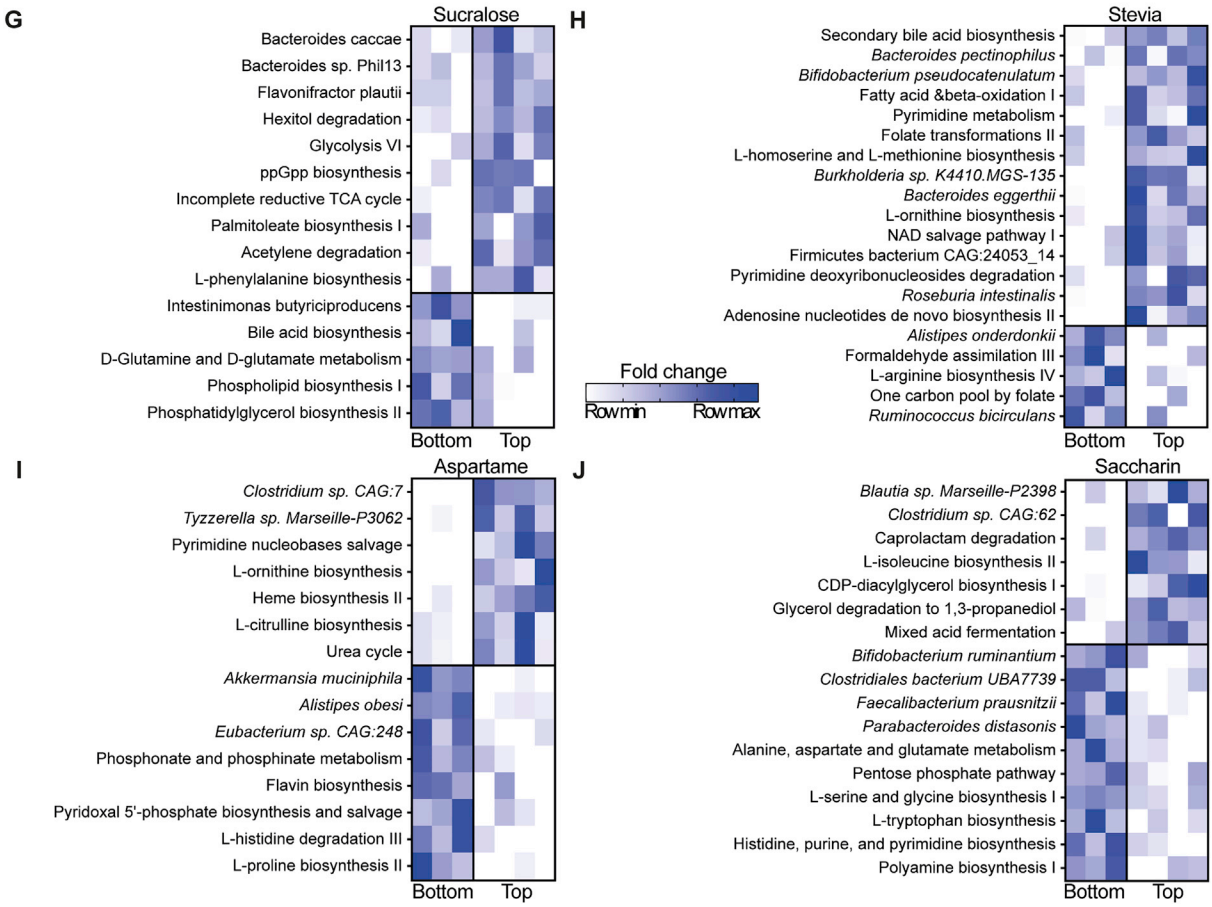


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Figure S8. Microbiome dissimilarities between NNS top and bottom glycemic responders, related to Figure 2

The microbiome profile of the top four glycemic responders in each group was compared to its bottom three. Bray-Curtis dissimilarities were computed between all of the samples of a participant to their baseline samples, before averaging the values within a given week.

(A–F) Bray-Curtis dissimilarity to baseline (2–3 samples per participant) based on KEGG pathways in the (A) sucralose, (B) stevia, (C) aspartame, (D) saccharin, (E) glucose vehicle, and (F) no supplement control groups.

(G–J) Features whose fold change between baseline to 2nd week of exposure is highly variable between top and bottom responders in the (G) sucralose, (H) stevia, (I) aspartame, or (J) saccharin groups.

*$p < 0.05$; Student's t test. Symbols, mean; error bars, SEM. NSC, no supplement control.