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Non-caloric artificial sweeteners and the microbiome: findings and challenges

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Non-caloric artificial sweeteners (NAS) are common food supplements consumed by millions worldwide as means of combating weight gain and diabetes, by retaining sweet taste without increasing caloric intake. While they are considered safe, there is increasing controversy regarding their potential ability to promote metabolic derangements in some humans. We recently demonstrated that NAS consumption could induce glucose intolerance in mice and distinct human subsets, by functionally altering the gut microbiome. In this commentary, we discuss these findings in the context of previous and recent works demonstrating the effects of NAS on host health and the microbiome, and the challenges and open questions that need to be addressed in understanding the effects of NAS consumption on human health.

Keywords: artificial sweeteners, diabetes, glucose intolerance, Microbiome

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Introduction

Almost a century has passed since the introduction of non-caloric artificial sweeteners (NAS) to our diet,[1] and today they are estimated to be consumed by 32% of adult Americans.[2] The number of food products supplemented by NAS is steadily increasing, as they are perceived and recommended by medical authorities as means of caloric and glycemic control while retaining a sweet taste.[3] Several NAS compounds have been FDA-approved and are generally considered safe.[3] However, several studies suggested that counterintuitive links may exist between NAS consumption and the same ailments of the metabolic syndrome they are meant to prevent, such as weight gain,[4,5] cardiovascular disease,[6,7] and type II diabetes mellitus.[8,9] Several physiological mechanisms have been suggested for these phenomena, such as stimulation of intestinal sugar absorption,[10] disruption of the ability of sweet taste to signal caloric consequences,[11,12] an increase in appetite[13] and impaired glycemic or insulin responses.[14] In contrast, other studies have shown NAS efficacy in weight control,[15-18] but most of these comparisons were made between individuals consuming NAS to those consuming caloric sweeteners, with only a few studies directly comparing consumption of NAS to avoidance of caloric and non-caloric sweetened products.[19] Another obstacle in drawing conclusions as to the physiological roles of NAS consumption is attributed to the difficulty in the interpretation of results due to reverse causality, that is, does NAS consumption causes metabolic derangements, or rather, NAS are consumed by individuals already suffering from overweight / high blood glucose levels. These general controversies in interpretations of animal and humans studies related to favorable and potentially harmful NAS effects on physiological parameters are beyond the scope of this review, with different views concisely described in reviews by Miller and Perez[20] and Swithers.[21]

In Search of Causality of NAS Effects: Animal Modeling of Metabolic Syndrome

Only few prospective interventional human studies address possible causal

effects of NAS on human metabolic homeostasis,[16] presumably due to difficulties in allocation of cohorts of healthy individuals who have not been previously exposed to NAS, and needs for robust stratification of potentially confounding factors such as genetics and lifestyle. Consequently, researchers have turned into animal models to study the effects of NAS on metabolism. Some examples of non-favorable NAS metabolic effects suggested in such models include works by Swithers and colleagues, who demonstrated weight gain in rats following consumption of saccharin,[12,22] acesulfame-potassium (AceK)[22] or stevia,[11] with saccharin also linked to increased adiposity.[12] Increased weight gain in rats consuming saccharin or aspartame was also reported by Bertoluci and colleagues,[23] while aspartame consumption was shown to increase weight gain and adiposity in mice, as reported by Al-Mohanna and colleagues[24] and by Shibata and colleagues,[25] respectively. Rats consuming sucralose were also shown by Schiffman and colleagues[26] to gain more weight, a finding that initiated intense debate.[27] In addition to weight gain, both saccharin and aspartame have been associated with impaired glucose homeostasis in mice,[24,28,29] and aspartame was also shown to induce hyperinsulinemia,[25] impaired insulin tolerance[24,28] and worsened atherosclerosis in genetically-susceptible ($ApoE^{-/-}$) mice.[30,31]

In contrast, Flatt and colleagues described anti-hyperglycemic and anti-hyperinsulinemic effects for saccharin in genetically obese (ob/ob) mice, coupled with attenuation of weight gain.[32] In fact, beneficial effects of NAS were at times described to co-occur with detrimental ones in some studies. Shibata and colleagues described anti-hyperglycemic effects of saccharin in mice that also featured increased adiposity and hyperinsulinemia,[25] and Shearer and colleagues recently reported beneficial effects for aspartame on weight gain in rats, co-occurring with aspartame-induced insulin resistance in the same aminals.[28] Altogether, as in human correlation studies, different works and models produced conflicting and at times opposing results. Many of these may stem from differences in methodologies, or from independent NAS effects on weight and glucose homeostasis. An alternative and previously unexplored possibility is that differences in microbiome composition and function, featured by different animals at different facilities, may have contributed to the variability in results and interpretations of these studies.

NAS and the Microbiome

NAS are synthetic compounds that are hundred of folds sweeter than sucrose, and thus can be used in small amounts with negligible added caloric value. Most NAS are excreted unchanged from the mammalian body, and therefore considered metabolically 'inert'[33-35] with no physiological effect exerted on the mammalian host. While these 2 notions form the foundation for the endorsement of NAS use, lack of NAS metabolism by the host does not rule out the possibility that these compounds may interact with the gut microbiome. In theory, such interaction may result in indirect yet profound microbial-induced consequences on the host, resulting in significant metabolic effects despite lack of eukaryotic recognition and metabolism of these compounds.

The Microbiome, a dense and diverse microbial ecosystem, inhabits our body from birth until death, and has been linked to multiple physiological roles and induction of susceptibility to many pathophysiological conditions.[36] The interaction of our diet with the microbiome and its consequences in promoting disease susceptibility is extensively researched. Both the composition[37] and function[38] of the microbiome are modulated and can be rapidly altered by diet,[39] with distinct diets (such as a diet rich in fat[40]) associated with distinct microbiomes. Conversely, distinct microbiome compositions and functions were determined to have a causal role in weight gain in mice and humans[41-43] and associated with propensity to type 2 diabetes[44,45] and metabolic syndrome.[46] Thus, the microbiome may serve as a hub channeling the effects of diet on the host's health and propensity to disease.[47,48] NAS, as commonly consumed dietary supplements, may be subjected to the same interactions with the microbiome and thus consequently exert their effects on the host.

The first report on NAS interactions with the microbiome by Anderson and Kirkland[49] dates to the early 1980 s, even prior to adequate availability of DNA sequencing techniques. In this report, saccharin was shown to alter aerobes-to-anaerobes ratio in the rat microbiome. Later studies by Schiffman and colleagues, used culturing techniques to characterize NAS effects on some commensal gut microbes, and suggested that sucralose consumption was associated with under-representation of several commensal members of the rat microbiome.[26] **Figure 1** summarizes previously published findings depicting, using several methodologies, associations between NAS exposure and alterations in complex microbiomes or cultured bacteria.[26,28,49-52] When considered in the context of known interactions between diet, the microbiome and health, these works indicate the importance of studying the microbiome as a potential mechanistic link between NAS consumption and its effects on human health. Nevertheless, it is difficult to draw conclusions from comparison of these findings, as they were obtained with diverse methods across different species. In addition, no causal role was sought or demonstrated between these NAS-associated microbiome alterations and possible effects on the host health. In our recent work,[53] we therefore set out to determine whether such causal role involving the microbiome could be determined upon NAS consumption, and whether it could influence metabolic homeostasis, using a variety of newly-introduced microbiome research tools including high-throughput next-generation sequencing techniques.

We began our study by supplementing the drinking water of mice with high doses of commercial formulations of saccharin, sucralose or aspartame. Surprisingly, after 11 weeks of exposure, each of the NAS-consuming mouse groups independently displayed marked glucose intolerance as compared to various controls, including water, sucrose or glucose consuming mice.[53] The latter control was especially important, as most commercial powdered NAS formulation involve a mixture of small amounts of NAS mixed into a larger

amount of caloric sugar. Indeed, as also shown in **Figure 2** from one of our repetitions, in which each of the commercial NAS was compared to a glucose control, enhanced glucose intolerance in mice drinking either saccharin, sucralose or aspartame was noted as early as 8 weeks following initiation of NAS consumption and culminating after 11 weeks of exposure. This phenotype seemed to be microbiome-related, as 2 different antibiotics regimens, targeting Gram-positive or Gram-negative bacteria, abrogated the NAS-induced glucose intolerance.[53] To further study the possible NAS effects on the microbiome, we focused on saccharin. To determine whether the effects noted with commercial saccharin can be shown for its pure form and at varying dietary conditions, we fed mice with a high-fat diet (HFD, 60% kcal from fat) and supplemented their drinking water with the same commercial saccharin regimen given to lean mice, or pure saccharin at an ADI-matched dose. Indeed, we determined that saccharin, at all of these conditions, including different diets, lower doses and purified form, could exacerbate glucose intolerance. Furthermore, as with NAS consumed in lean mice, antibiotic treatment ameliorated the exacerbated glucose intolerance induced in obese mice by either pure[53] or commercial saccharin (**Fig. 3**). The metabolic phenotype was not mouse-strain specific, as a similar hyperglycemic effect observed in C57 Bl/6

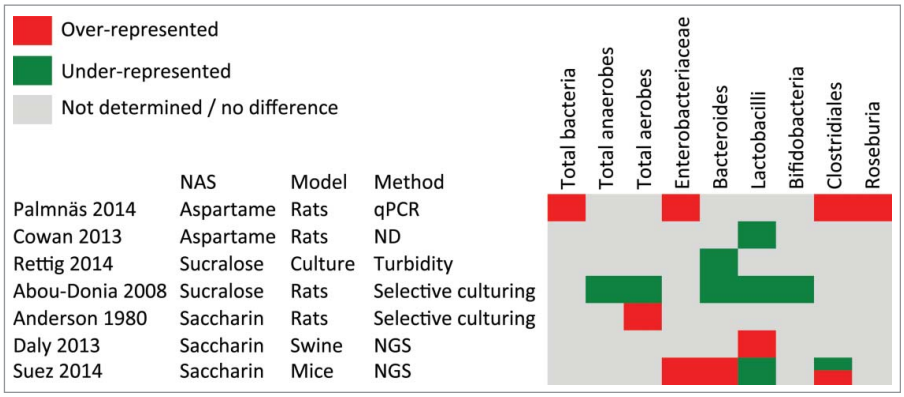

Figure 1. NAS-Bacteria interactions. Summarized are studies (referenced in the main text) describing effects of NAS on members of the microbiome or bacteria in culture. ND, no data; NGS, Next-generation sequencing.

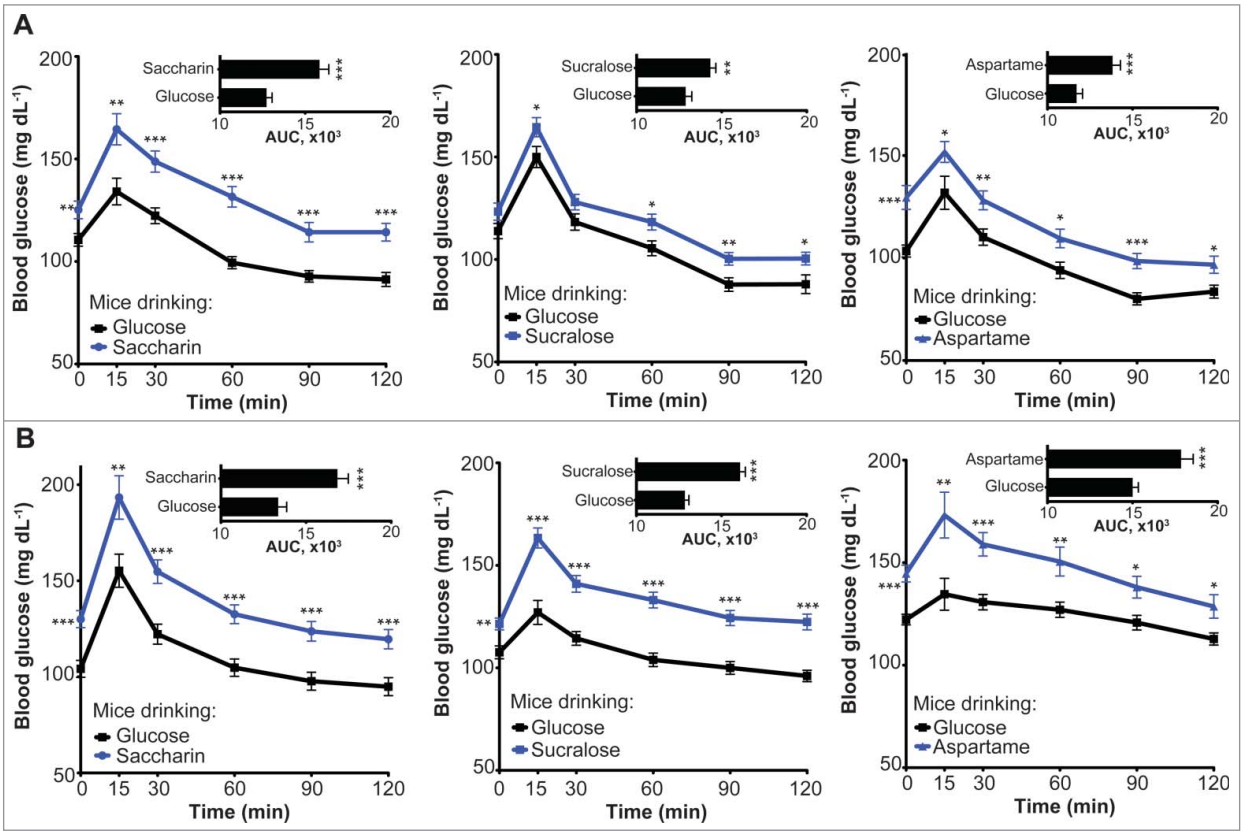

Figure 2. Impaired glycemic response in NAS consuming mice. Oral glucose tolerance test (OGTT) and area under the 2-hour blood glucose response curve (AUC) in normal-chow-fed mice drinking commercial NAS (N = 20–25) or glucose (N = 15–25) for (A) 8 weeks or (B) 11 weeks. Symbols (OGTT) or horizontal lines (AUC), mean; error bars, s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Unpaired two-sided Student *t-test*.

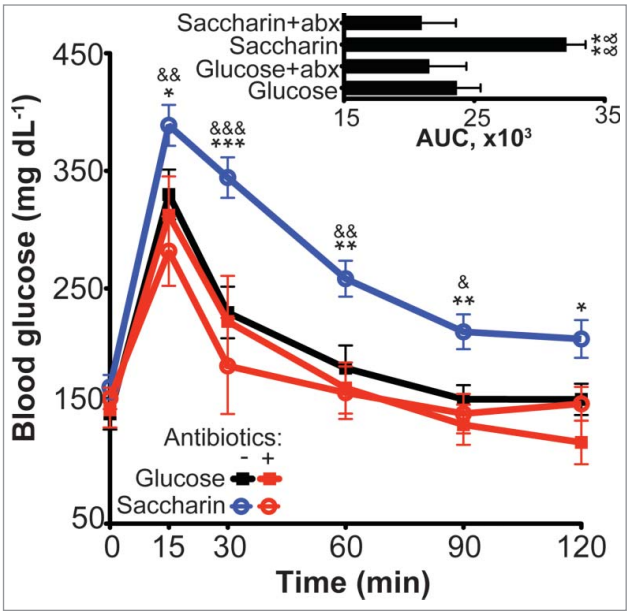

Figure 3. Antibiotics ameliorate saccharin-exacerbated glucose intolerance. Oral glucose tolerance test (OGTT) and area under the 2-hour blood glucose response curve (AUC) in high-fat diet-fed mice drinking commercial saccharin (N = 15) or glucose (N = 15) for 9 weeks, with a subset of each group (N = 5) supplemented with ciprofloxacin and metronidazole starting from week 5. Symbols (OGTT) or horizontal lines (AUC), mean; error bars, s.e.m. *, saccharin vs. glucose, and saccharin vs. saccharin-abx; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Unpaired two-sided Student *t-test*.

mice was attributed to saccharin in obese outbred Swiss-Webster mice.

In agreement with the experiments with antibiotics, next generation sequencing of the microbiome indicated that mice drinking saccharin had distinct compositions from controls. This distinct microbiome was characterized by enrichment of taxa belonging to the *Bacteroides* genus or the *Clostridiales* order, with under-representation of *Lactobacilli* and other members of the *Clostridiales*. Several of the bacterial taxa that changed following saccharin consumption were previously associated with type 2 diabetes in humans.[44,45] While microbiome alterations can be directly related to glucose intolerance, they may also be a "passenger" effect of NAS consumption, irrelevant to the phenotype. Thus, to establish causality, we transplanted fecal microbiomes from mice drinking pure or commercial saccharin into germ-free mice. These recipient mice are entirely devoid of any microbial presence of their own, and thus serve as an excellent tool to study the effects of transplanted microbial communities on various phenotypes. Mice recipients of saccharin-associated microbiomes developed glucose intolerance and their microbiomes reflected many of the changes observed in the donors, as compared to those receiving microbiomes of control mice. Furthermore, in order to determine whether saccharin directly affected the microbiome, we cultured microbiomes from naive mice under anaerobic conditions in the presence of saccharin. Under these "host-free" conditions, saccharin altered the composition of the microbiome, which, upon transplant to germ-free mice, induced glucose intolerance. Shotgun sequencing of the entire genetic makeup of the microbiome revealed that saccharin-associated microbiomes were functionally distinct than those of control mice, with enrichment of many pathways previously reported in obese/diabetic mice and humans, as is described below.

Finally, we examined whether similar effects of NAS on the microbiome could be documented in human individuals. In our ongoing personalized nutrition study, we follow a large cohort of healthy non-diabetic individuals by monitoring their diets and performing continuous glucose measurements, coupled with an assessment of multiple clinical parameters. We analyzed the possible associations between NAS consumption, microbiome composition and metabolic outcomes in 381 of these participants, and showed that NAS consumption not only associates with various clinical parameters such as BMI, blood pressure, HbA1 C% and fasting glucose levels, but also with the presence of certain taxa, including expansion of the Actinobacteria phylum, the *Enterobacteriales* order, and of various taxa from the *Clostridiales* order. We then aimed to assess, in a preliminary small-scale study, whether NAS and their associated microbiomes could have causal roles in affecting glucose metabolism in humans. To this aim, we performed a small-scale intervention study, and demonstrated that supplementation of regular diet with the upper limit of daily saccharin doses (5 mg kg^{-1} d^{-1}) led to elevated glycemic response in 4 of the 7 volunteers ('responders'), while no response was noted in the other 3 participants. Poorer glycemic responses in the 4 responders were associated with microbiome alterations, that when transplanted into germ-free mice replicated the glycemic responses of their human donors. Furthermore, microbiome compositions from responders and non-responders were distinct even prior to exposure to saccharin. This suggests that the gut microbiome composition of an individual may be indicative of his susceptibility and personalized response to NAS or other food related compounds. Thus, this serves as a special case of "personalized nutrition" and suggests that the microbiome should be considered an important 'player' when attempting to design individually tailored health-maintaining diets.

Challenges: Toward Mechanistic Understanding of NAS Effects

The aforementioned studies, including ours, suggest that NAS may effect the microbiome composition and function, which in turn may affect host metabolic homeostasis in subsets of individuals and in specific contexts. More generally, these studies represent an example of how food

ingredients and additives may drive personalized effects on host physiology and its tendency for the development of multifactorial disorders, through effects exerted on its commensal gut microbial composition and function. In relation to NAS, our results suggest in both mice and humans, that the mammalian host may not be inert to their effects. Our results also highlight many interesting and important open questions that need to be explored and addressed in further studies.

At the start of our study, we observed that high dose administration of 3 different sweeteners to mice induced a disturbance in glucose homeostasis. We then mainly focused on saccharin, which featured a similar effect in obese mice, in ADI-matched concentrations of its pure form (rather than the glucose-saccharin commercial mix) and demonstrated that it induces the glucose intolerance phenotype through characteristic effects, at a variety of doses and formulations, on the gut microbiome composition and function. Further studies will need to decipher the mechanisms driving the metabolic consequences of sucralose and aspartame use in mice, and whether they are similar or distinct from those noted for saccharin. Similarly, the dose and regimen effects of these and other NAS and their relevance to human consumption habits and microbiome composition merit further studies. Interestingly, Shearer and colleagues recently demonstrated that for rats, aspartame consumption was associated with a marked impairment in both fasting blood glucose and insulin tolerance.[28] These effects were reached in aspartame doses that were much lower than the ones used for this NAS in our studies, which are estimated to be well within the human ADI range. Notably, changes in the gut microbiome and metabolites in this study partially mirrored the ones noted in our studies (**Fig. 1**), including a marked increase in systemic levels of short chain fatty acid levels, compounds secreted by the microbiome and previously shown to be increased in obesity[54] and to promote gluconeogenesis.[55] Other sweeteners, not examined by our study, were suggested to have effects on the host. Short term stevia consumption in rats was suggested to be associated with weight gain,[11] in a yet unknown mechanism. Similarly interesting in that regard is another group of sugar substitutes, sugar alcohols such as xylitol, mannitol and sorbitol, that are added as supplements to numerous foods and have been recently suggested to interact with the gut microbiome.[56]

While the ability of diet to affect the microbial community is established,[39] it is less understood how the dietary components promote the differential bloom or suppression of certain taxa. In the case of NAS, one may consider a direct effect, in which bacteria that can metabolize NAS as energy source thrive, while others may experience toxicity. Such a direct effect is indeed plausible in the case of saccharin and sucralose, as both are largely not metabolized by the host's body[35,57] and have been previously demonstrated to affect the growth of certain bacteria.[58-60] Our findings suggest such direct effect of saccharin on the microbiome, and functional analysis of the saccharin-associated metagenome indicated that several pathways involved in metabolism of heterocyclic compounds were enriched, suggesting that saccharin exposure may have been associated with expansion of bacteria capable of utilizing it. It remains to be determined whether sucralose also directly affects the microbiome. As for aspartame, its utilization by bacteria has been reported,[61] even though aspartame is metabolized by the host.[62] It may be possible that the products of aspartame degradation affect the microbiome,[63] or alternatively that other, indirect mechanisms, are involved.

Equally interesting are the downstream microbiota effects on host metabolism. Functional alterations of the microbial community have been described in the various conditions that comprise the metabolic syndrome, including obesity[41] and diabetes mellitus.[44] In saccharin drinking mice, we described enrichment of multiple pathways associated with metabolic syndrome in mice and humans, including metabolism of sugars and sphingolipids and biosynthesis of lipopolysaccharide (LPS) and folate. The higher levels of glycan degradation products (SCFA) noted in our studies and others in NAS consuming animals,[28] may serve as energy source for the host, or as signaling molecules or substrates for gluconeogenesis, liponeogenesis and cholesterol synthesis.[54] Of the under-represented pathways, saccharin metagenomes were associated with reduction in genes of phosphotranferase systems (PTS), involved in the transport of sugars to the bacterial cell. Saccharin has been previously reported to inhibit anaerobic fermentation of glucose,[58] and it is possible that reduced transport of glucose contributes to this inhibition. It is important to note that in our study, not all pathways were similarly altered when comparing mice drinking saccharin on a high vs. normal fat diet. Thus, despite resulting in a similar phenotype (microbiota-dependent glucose intolerance), it is likely that multiple bacterial functions may contribute to the metabolic derangements. This may also be the case when functional analysis is performed on other NAS. Likewise from the host side, further metabolic analysis including clamp studies will potentially enable to decipher whether the microbiome-induced impaired glucose tolerance stems from impaired pancreatic or peripheral function, or a combination of the two.

Finally, the full extent to which NAS may affect the human microbiome and consequently human health merits further studies in form of prospective blinded clinical trials. As such, it would be interesting to delineate in larger cohorts than our small-scale prospective human trial what is the actual fraction of saccharin 'responders' in the general human population, and whether similar effects are to be expected upon consumption of other NAS and at lower doses. Furthermore, long-term effects of NAS consumption on human metabolic homeostasis merit further prospective elucidation. Equally interesting and important would be the study of the effects of human NAS consumption on the compositional and functional alterations of the microbiome, and whether such microbial changes could be employed to predict the eventual response to NAS or other food supplements even before their consumption. An important issue to be explored in future studies relates to the reversibility of microbiome and metabolic effects upon cessation of NAS consumption in 'responders'. Of note, in our preliminary study, microbiomes of 2 of the 'responders' were

sequenced 2 and 8 weeks, respectively, following cessation of saccharin exposure, and were found to fully return to their original configurations (data not shown). The extent and kinetics of such reversibility after longer exposure periods merits further exploration. Likewise, it will be interesting to determine which members of the microbiome are important in the "resistance" or "susceptibility" to NAS effects on host metabolism. Results of such human-based studies would enable to better understand the scope and relevance of personalized microbiome-driven NAS effects, with respect to human health and public policy. Overall, the inter-individual difference noted in the response to NAS serves as an example of the need for a personalized approach to diet, tailored to the individual's glycemic responses and microbiome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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