Food and Chemical Toxicology 124 (2019) 385–399

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

Assessing the *in vivo* data on low/no-calorie sweeteners and the gut microbiota

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A B S T R A C T

Low/no-calorie sweeteners (LNCS) are continually under the spotlight in terms of their safety and benefits; in 2014 a study was published linking LNCS to an enhanced risk of glucose intolerance through modulation of the gut microbiota. In response, an in-depth review of the literature was undertaken to evaluate the major contributors to potential changes in the gut microbiota and their corresponding sequelae, and to determine if consuming LNCS (*e.g.,* acesulfame K, aspartame, cyclamate, neotame, saccharin, sucralose, steviol glycosides) contributes to changes in the microbiome based on the data reported in human and animal studies. A few rodent studies with saccharin have reported changes in the gut microbiome, but primarily at high doses that bear no relevance to human consumption. This and other studies suggesting an effect of LNCS on the gut microbiota were found to show no evidence of an actual adverse effect on human health. The sum of the data provides clear evidence that changes in the diet unrelated to LNCS consumption are likely the major determinants of change in gut microbiota numbers and phyla, confirming the viewpoint supported by all the major international food safety and health regulatory authorities that LNCS are safe at currently approved levels.

1. Introduction

The trillions of symbiotic microorganisms present in the human body, the majority of which are located within the gastrointestinal tract, are collectively referred to as the microbiota. The human gut microbiota is seeded at birth, develops intensely during the first 3 years of life, and continues to evolve and adapt throughout the lifetime of an individual (Koenig et al., 2011). The gene set of the gut microbiota (the gut microbiome) is estimated to be about 3 million genes, about 150 times larger than that of the human genome ((Qin et al., 2010). Over 1000 species have been identified in total, with around 160 being present in the gut of any one individual (Rajilic-Stojanovic and De Vos, 2014). The predominant phyla, which encompass about 90% of the bacteria, are *Firmicutes* and *Bacteroidetes*; other common phyla include *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia,* and *Fusobacteria*, together with a limited number of species (mostly methanogens) that fall into the domain Archaea (Eckburg et al., 2005). The relative proportions of these phyla and the species present can vary enormously between individuals and the exact contributions of a variety of factors to this diversity, as well as to the initial development and continuing evolution of the gut microbiota, including host genetics, environmental elements, and diet, remain to be elucidated (Tilg and Kaser, 2011; Ursell et al., 2012; Xu and Knight, 2015). Mounting evidence, however, indicates that diet, both habitual, and long-term and shorter-term dietary changes, appear to be the most significant factors influencing the overall composition of the gut microbiota and its functionality (Muegge et al., 2011; Wu et al., 2011; David et al., 2014; Graf et al., 2015).

In addition to its taxonomic diversity, the gut microbial community has an equally extensive metabolic repertoire including hydrolysis, reduction, dehydroxylation, deamination, and ring fission that complements the activity of mammalian enzymes in the liver and gut mucosa (Nicholson et al., 2012). The human gut microbiota makes an important contribution to the breakdown, absorption, and metabolism of key dietary components by contributing enzymes that are not encoded by the human genome. These enzymes contribute to the breakdown of polysaccharides, polyphenols, and the synthesis of vitamins. In turn, the microbiota composition and activities can be influenced by diet. Dietary components of particular importance in modulating the microbiota are those that are poorly digested in the stomach and small intestine, such as dietary fiber, and hence, reach the colon where they become substrates for microbial fermentation.

The impact of consuming sweeteners on the gut microbiota has recently received significant media attention as a result of a 2014 study

Abbreviations: acesulfame K, acesulfame potassium; ADI, acceptable daily intake; AUC, area under the curve; FDA, Food and Drug Administration; FISH, fluorescence *in situ* hybridization; HITChip, Human Intestinal Tract Chip; LNCS, low/no-calorie sweeteners; NAS, non-caloric artificial sweetener; NHDC, neohesperidin dihydrochalcone; NSP, non-starch polysaccharides; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PYY, peptide YY; rRNA, ribosomal RNA; RS, resistant starch; SUCRAM, saccharin + neohesperidin dihydrochalcone

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https://doi.org/10.1016/j.fct.2018.12.005
Received 17 August 2018; Received in revised form 3 December 2018; Accepted 6 December 2018
Available online 14 December 2018

reported by Suez et al. (2014). The authors of this study concluded that consumption of low/no-calorie sweeteners (LNCS) modulates the composition and function of the gut microbiota, leading to enhanced risk of glucose intolerance. Critical review of this paper, however, has revealed a number of limitations in experimental design and issues in both the analysis and reporting of the study data, bringing into question the conclusions drawn by the authors (Magnuson, 2015). The purpose of the present review is to (a) discuss known major contributors to gut microbiota fluctuations to give context to the outcomes of studies on LNCS, and (b) survey and assess the scientific literature relevant to the potential for LNCS to affect the gut microbiota, with particular regard to the potential for any adverse health outcome. This included studies on acesulfame potassium (acesulfame K), aspartame, cyclamate, neotame, saccharin, sucralose, and rebaudioside A.

2. The effects of dietary composition on the gut microbiota

2.1. Methods for studying the gut microbiota

The methodology for analyzing the composition of the gut microbiota has seen great advances over the last 10–20 years. Traditional approaches were based on cultivation of fecal organisms which were time consuming and insensitive and required fecal samples to be stored and processed in specialized anaerobic culture systems (Sankar et al., 2015). The advent of high-throughput DNA sequencing technologies has provided insights into gut microbial ecology without the need for cultivation and has revealed the complexity and diversity of the microbiota. It is estimated that only about 30% of the human gut microbiota can currently be cultured. A wide range of techniques are now available to explore the gut microbiota and these have been applied to studies of dietary effects and the relationship of microbiota composition in health and disease (Morgan and Huttenhower, 2012). It should be noted that differences in DNA extraction and recovery, sequencing technology, and polymerase chain reaction (PCR) amplification can affect the recovery of different groups of bacteria, and lead to inconsistencies between studies (Kennedy et al., 2014; Walker et al., 2014). Furthermore, each of the methods has inherent advantages and disadvantages, which have been reviewed by Sankar et al. (2015). The molecular approaches broadly divide into shotgun metagenomic sequencing and 16S ribosomal RNA (rRNA) methods. Whole community shotgun sequencing of all DNA fragments in a sample is a very powerful technique especially with the latest high throughput methods, which allows the production of large metagenomic datasets from a large number of individual samples such as those generated by the Human Microbiome Project and MetaHIT programs. The method can provide information on microbial diversity and metabolic function as well as the relationship to health and diseases such as inflammatory bowel disease, diabetes, and obesity (Sankar et al., 2015; Walker et al., 2015). The method does have limitations, including for example, that (a) some of the reads cannot be assigned a taxonomic identification or function where species are in low abundance, (b) gene prediction is dependent on the read length, and (c) insufficient reference databases currently exist.

Analyses based on PCR amplification of sequences from the 16S rRNA gene, which is present in all bacteria and archaea, can provide a more targeted approach to characterizing the gut microbial community. The 16S rRNA gene provides the basis for fluorescence *in situ* hybridization (FISH) and quantitative PCR and microarray methods such as the Human Intestinal Tract Chip (HITChip), which can detect less abundant, but nevertheless potentially important, microbial groups (Morgan and Huttenhower, 2012; Walker et al., 2015). Again, the methods do have limitations including biases in DNA recovery and PCR amplification that can have differential effects on bacterial groups, limitations in the reference databases, and relatively low resolution at species level than at higher taxonomic levels (Graf et al., 2015; Sankar et al., 2015).

2.2. Effect of different dietary components and dietary composition on microbiota

Two main mechanisms are proposed to underlie the modulation of microbiota composition by diet (Flint et al., 2015). Firstly, the diversity in composition of the microbiota is reflected in great metabolic diversity, with the various microbial types exhibiting different abilities to utilize substrates reaching the colon. Thus, the availability of particular substrates can favor growth of those genera or species capable of exploiting them. This mechanism is especially relevant to the effects of fermentable fibers, resistant starches, and oligosaccharides, which are poorly digested in the upper gastrointestinal tract and hence reach the large bowel and provide substrates for intestinal bacterial fermentation. A second mechanism by which diet may alter microbiota composition is *via* the modulation of physico-chemical conditions in the gut since microbial species differ in their tolerance of environmental factors such as pH and bile salts (Flint et al., 2015). Both mechanisms seem to play a role in determining the response of the microbiota to dietary composition.

There is evidence for the effects of both long-term and short-term diets on gut microbial composition and metabolism. The evidence for a role of long-term, habitual dietary intake is indirect and derived from observational studies of human population groups. For example, in a survey of adults from the United States, Wu et al. (2011) found associations between high levels of fecal *Prevotella* and consumption of dietary fiber, whereas high numbers of *Bacteroides* were associated with protein and fat intake. A positive association of *Prevotella* abundance with habitual dietary fiber intake was also noted by David et al. (2014) in a United States cohort. These data are consistent with a previous study comparing fecal microbiotas of rural African children with those from Italy (De Filippo et al., 2010). The former exhibited a greater abundance of *Prevotella* and the latter more *Bacteroides*, potentially reflecting the higher fiber intake of rural Africans and higher fat and protein intake of the western diet. There are a few observational studies of vegetarians and omnivores, and although these studies have indicated differences in microbiota composition, there is little consistency probably due in part to the studies being conducted in different countries as well as various limitations in study design (reviewed by Graf et al., 2015).

The susceptibility of the gut microbiota to shorter term dietary change has been explored in a number of human intervention studies, some of which have involved gross dietary changes such as animal-*versus* plant-based diets, while others have focused on changes in specific dietary components (*e.g.,* resistant starch). David et al. (2014) conducted a study in which 10 subjects consumed either an animal-based diet (meat, eggs, and cheese) or a plant-based diet (rich in legumes, grains fruits, and vegetables) for 5 days with a 6-day washout period. Fecal microbiota diversity and composition were assessed by 16S rRNA gene sequencing. In comparison to the subjects' baseline diets, the animal-based diet increased fat and protein intake by 2-fold and decreased fiber intake to virtually zero and was associated with significant changes in relative abundance of 22 bacterial taxonomic clusters. This diet increased the abundance of bile-tolerant microbes, notably species of *Alstipes, Bilophila, Bacteroides*, and decreased levels of *Roseburia, Eubacterium*, and *Ruminococcus* that ferment plant polysaccharides. The plant-based diet, which increased fiber intake nearly 3-fold to 25 g/1000 kcal and decreased protein and fat consumption, had less potent effects on the microbiota with only 3 taxonomic clusters being affected. Klinder et al. (2016) conducted a randomized controlled trial in which fruit and vegetable intake was increased by up to 6 portions a day and at this level of intake an increase in *Clostidium leptum-Ruminococcus bromii/flavefaciens* group was seen. Salonen et al. (2014) reported a cross-over study of 14 obese men fed 3 fully-controlled diets for 3 weeks each: a diet high in resistant starch (RS); a diet high in wheat bran, providing non-starch polysaccharides (NSP), but otherwise of similar macronutrient composition; and a high protein

weight loss diet. Microbiota analysis was by phylogenetic microarray (HITChip) and by quantitative PCR analysis. Despite the diet type explaining only 10% of the total variance (less than that between subjects) each one induced distinct changes. In particular, increases in *Ruminococcaceae* phylotypes (*Clostridium leptum*, *C. cellulosi*, *Oscillospira* spp.) and certain *Bacteroidetes* were evident during the RS diet period, whereas on the NSP diet, *Lachnospiraceae* phylotypes increased and members of *Ruminococcaceae* decreased. During the weight loss diet, bifidobacteria decreased. Importantly, the dietary responsiveness of each individual's microbiota varied considerably, suggesting that individuals can be divided into responders and non-responders based on certain features of their gut microbiota. In these subjects, a low response to dietary change was associated with microbiotas with high phylogenetic diversity, which may promote higher stability of the ecosystem. Diversity, however, may not be a universal predictor of dietary responsiveness, as a study of obese subjects from Belgium, Finland, and the United Kingdom identified the main predictors to be the abundance of certain *Firmicutes* phylotypes, particularly those in clostridial clusters IV, IX, and XIV, and not microbiota diversity (Korpela et al., 2014). One reason why certain gut microbes respond to dietary change in some individuals but not others may be related to baseline abundance. This has been demonstrated for bifidobacteria stimulation by prebiotics, which is more apparent when baseline numbers are low (Tuohy et al., 2001). Several studies have also investigated the impact of overall calorie reduction on gut microbial composition, primarily in populations of obese adults (reviewed by M.C. Dao et al., 2016). Changing between diets higher in calories/energy (*i.e.,* higher in carbohydrate and/or fat) to those with lower caloric content (*i.e.,* higher in protein and/or fiber) consistently results in modulation of the gut microbiota, including shifts in specific bacterial phyla and/or overall microbial diversity.

Whole grain products, including maize, wheat, barley, and rice, have been the focus of a number of human trials. In general, these studies revealed increases in bifidobacteria and the proportion of *Lactobacillus/Enterococcus* group (Carvalho-Wells et al., 2010; Costabile et al., 2012) with 1 study (Martínez et al., 2013) also showing an increase in *Firmicutes* (*Blautia* and *Roseburia*). Increases in bifidobacteria were also apparent in studies of phytochemical rich foods such as blueberries, red wine, and cocoa polyphenols (Tzounis et al., 2011; Vendrame et al., 2011; Queipo-Ortuño et al., 2012). The main bifidogenic food components are the non-digestible oligosaccharides, such as fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, and inulin, usually referred to as prebiotics. Numerous controlled dietary trials in young and old adults as well as children have demonstrated increases in numbers of bifidobacteria after prebiotic consumption (reviewed by Rastall and Gibson, 2015). In some studies numbers of other bacterial groups, such as lactobacilli, change, but bifidobacteria increases are the most consistent (Graf et al., 2015). Other non-digestible carbohydrates that have been investigated in human trials include polydextrose, soluble corn fiber, and resistant maltodextrin. Intake of polydextrose or corn fiber for 21 days increased the concentration of *Clostridiaceae* and *Eubacteriaceae* and also increased the abundance of *Faecalibacterium, Phascolarctobacterium*, and *Dialister* (Hooda et al., 2012). A second study of polydextrose given for 3 weeks reported increases in *Ruminococcus intestinalis* and in *Clostridium* clusters I, II, and IV (Costabile et al., 2012). It is important to note that dietary interventions can have a significant impact on microbiota functions, even without major changes in composition. In the study by David et al. (2014) on animal- and plant-based diets, the gene expression profile was strongly linked to diet type and was associated with changes in carbohydrate and protein fermentation, as well as vitamin synthesis. Their data suggested that gene expression differences were due to both regulatory and taxonomic shifts within the microbiota.

Overall, the dietary studies indicate that the composition and functionality of the microbiota are modified by dietary changes and that there can be important inter-individual differences in the response of gut microbiotas, thereby making it difficult to generalize about the influence of specific dietary components. As a consequence, it is clear that when conducting dietary intervention studies to assess the effects of various ingredients that are added to the diet in small amounts, such as LNCS, the habitual diet of the subjects should be well-characterized and the intervention diets should be carefully controlled.

3. Review of the scientific literature: low/no-calorie sweetener exposure and impact on the gut microbiota

To investigate whether an association exists between LNCS consumption and changes in the composition of the gut microbiota a literature search was conducted using ProQuest to identify publications studying sweeteners (“high-intensity” or “high-potency” or “intense” or “artificial” or “low-calorie" or “non-caloric" or “no-calorie” or “non-nutritive” sweetener) and the gut microbiota (gut or intestinal “microbiome” or “microbiota” or “microbes” or “microflora” or “microorganisms”). Specific sweetener names were also used in the search as follows: Aspartame or Nutrasweet; or Sucralose or Splenda; or Saccharin or SugarTwin or “Sweet’N Low”; or “Acesulfame potassium” or “Acesulfame K” or Ace-K; or Sunett or “Sweet One”; or Neotame or Advantame; or Stevia or “Steviol glycosides” or “Rebaudioside A” or “Stevioside” or PureVia or Truvia; or Mogrosides; or Nectresse or “Norbu Sweetener”; or Cyclamate. Applying first the sweetener search terms followed by the gut microbiota search terms, the following databases were queried on 18 May 2018 (with no date restrictions, for all languages, for all article types including both peer-reviewed and non-peer-reviewed [*e.g.,* research articles, reviews, conference abstracts, news articles, interviews, and book chapters]): AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The search generated 123 hits and all articles were screened for relevance based on the following inclusion criteria: (a) *in vivo* studies conducted in animals and humans (all *in vitro* studies were excluded); and (b) oral exposure to 1 or more LNCS, and (c) reported measurement of microbial populations in the gut. Published abstracts from presentations and/or conferences were matched with full articles, where applicable, and remaining abstracts were included in the search results so long as criteria (a), (b), and (c) listed above were met. Review articles and opinion pieces responding to recent publications in the field were excluded; however, where applicable, reference lists were reviewed for additional relevant publications. Following application of the defined screening criteria, 17 publications were identified as relevant primary research articles investigating the administration of LNCS to animals or humans and effects on the gut microbiota. The experimental details and outcomes of these studies, including study size, subjects, interventions, comparisons, outcomes related to the gut microbiota, and study design, are summarized and grouped by sweetener below. For publications that investigated more than one single LNCS, the results have been evaluated on a per sweetener basis and are reported individually in the respective subsections below. A summary of all identified studies, including study design, microbiome-related results, and potential confounding factors, is presented for comparison in Table 1.

3.1. Acesulfame potassium (acesulfame K)

Acesulfame K was administered to CD-1 mice (N = 5 per sex per group) *via* gavage for 4 weeks at a dose of 37.5 mg/kg/day, and the control group received only water (Bian et al., 2017a). Fecal samples were collected after 4 weeks for 16S rRNA gene sequencing and functional gene enrichment analysis. The authors reported that weight gain from baseline to the end of the study increased significantly in males compared to the respective control group, but not in females. No data

Table 1
Summary of studies evaluating low/no-calorie sweeteners and the gut microbiota.

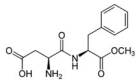
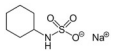
Low/no-calorie sweetener	Subjects	Dose and duration	Microbiome-associated changes[a]	Study confounders	Reference
Acesulfame potassium ADI: 15 mg/kg Sucrose equivalence: 200x 	Humans "consumers" (N = 7) "nonconsumers" (N = 24)	1.7–33.2 mg/d, based on daily food records over 4 days	Fecal bacterial diversity different between "consumers" & "nonconsumers"	Habitual diet not controlled	Frankenfeld et al. (2015)
	Mice, CD-1 Exposed (N = 5) Control (N = 5)	37.5 mg/kg/d, 4 weeks	• Males: ↑ in fecal *Bacteroides, Anaerostipes,* and *Sutterella* • Females: ↑ in fecal *Mucispirillum*; ↓ *Lactobacillus, Clostridium,* an unassigned *Ruminococcaceae* genus, and an unassigned *Oxalobacteraceae* genus	• Food consumption not reported • Dose in excess of ADI (2.5x) • Small sample size	Bian et al. (2017a)
	Mice, C57Bl/6J Exposed (N = 9) Control (N = 8)	15 mg/kg/d, 8 weeks	• No changes in fecal microbiota between groups	• None to report	Uebanso et al. (2017)
	Pregnant mice/dams and their offspring	Pregnant mice/dams consumed sucralose/acesulfame-K mixture in diet, dose and duration not reported	Assessment of 19-day old pups: • ↓ in fecal *Akkermansia muciniphila* • ↑ in fecal *Firmicutes*	• Acesulfame-K exposure (mg/kg) unknown • Food consumption not reported • Sample size unknown	Olivier-Van Stichelen et al. (2017)
Aspartame ADI: 40 mg/kg Sucrose equivalence: 200x 	Humans "consumers" (N = 7) "nonconsumers" (N = 24)	5.3–1112 mg/d, based on daily food records over 4 days	Bacterial diversity different between "consumers" & "nonconsumers"	Habitual diet not controlled	Frankenfeld et al. (2015)
	Rats, SD Normal rats (N = 10–12) Obese rats (N = 10–12)	Normal, 5 mg/kg/d; Obese, 7 mg/kg/d; 8 weeks	• Normal rats: ↑ fecal *C. leptum* • Obese rats: ↑ fecal total bacteria, *Bifidobacterium* spp., *Enterobacteriaceae*, *C. leptum*, and *Roseburia* spp.	Food, water consumption not equivalent between aspartame and control groups	Palmnäs et al. (2014)
	Mice, C57Bl/6 Exposed (N = 20) Control (N = 20)	1333 mg/kg/d, 11 weeks	Elevated glycemic response	• Dose in excess of ADI (~30x) • Food, water consumption not equivalent between groups • Unconventional grouping of data; invalid statistical analysis	Suez et al. (2014)
Cyclamate ADI: 0–11 mg/kg Sucrose equivalence: 30–50x 	Monkeys Exposed (*Macacairus,* N = 1) Control (2 *Macacarhesus*, 1 *Cynopithecus niger*)	250 mg/kg/d sodium cyclamate, 30 days	Compared 1 cyclohexylamine producer to controls not consuming cyclamate: • No change in total fecal bacteria and several microbial populations (*Bacteroidaceae*, catenabacteria, bifidobacteria, peptostreptococci, lactobacilli, streptococci, enterobacteria, *Clostridia, Veillonella,* staphylococci)	• Small sample size • Control animals were different species than test group	Matsui et al. (1976)
Saccharin ADI: 5 mg/kg Sucrose equivalence: 400x 	Rats, Charles River Exposed (N = 7) Control (N = 5)	10–14 g/kg/d, 10 days	• ↑ number of aerobes in cecum • ↓ cecal anaerobe:aerobe ratio	Dose in excess of ADI (~2000x)	Anderson and Kirkland (1980)
	Humans Exposed (N = 7)	5 mg/kg/d, 1 week	• Elevated glycemic responses in 4 subjects, classified as "responders" • "Responders" microbiome configurations clustered differently	• No control group • Habitual diet not controlled • Unconventional grouping of data	Suez et al. (2014)
	Mice, C57Bl/6 Exposed (N = 20) Control (N = 20)	3333 mg/kg/d, 11 weeks	• Elevated glycemic response • ↑ fecal abundance *Bacteroides, Clostridiales* • ↓ fecal *L. reuteri*, members of *Clostridiales*	• Dose in excess of ADI (~650x) • Food, water consumption not equivalent between groups • Unconventional grouping of data; invalid statistical analysis	Suez et al. (2014)
	Mice, Swiss-Webster Exposed (N = 16) Control (N = 16)	5 mg/kg/d, 5 weeks: fecal transplant → germ-free mice	• Elevated glycemic response transferred *via* feces to germ-free mice • Minor changes (± 1.2-fold) in fecal composition	• Glycemic responses in donor animals not consistent – unclear if all animals were donors, or if only high-response animals were donors	Suez et al. (2014)
	Piglets, Landrace X Large White Exposed (N = 8) Control (N = 8)	0.015% SUCRAM, 2 weeks	• ↑ in fecal *Lactobacillus* OTU4228 [authors reported NHDC component of SUCRAM responsible] • ↓ in fecal *Veillonellaceae* and *Ruminococcaceae*	• Food consumption not reported • Saccharin dose (mg/kg) unknown	Daly et al. (2014) Daly et al. (2016)

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Table 1 (*continued*)

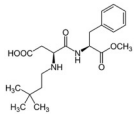
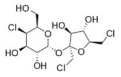
Low/no-calorie sweetener	Subjects	Dose and duration	Microbiome-associated changes[a]	Study confounders	Reference
	Mice, C57Bl/6J Exposed (N = 10) Control (N = 10)	~27–65 mg/kg/d, 6 months	• ↑ in fecal *Corynebacterium, Roseburia, and Turicibacter* • ↓ in fecal *Ruminococcus, Adlercreutzia*, and *Dorea*	• Food consumption not reported • Dose appears to be in excess of ADI (at minimum ~5x)	Bian et al. (2017b)
Neotame ADI: 0.3 mg/kg Sucrose equivalence: 7000 to 13000x 	Mice, CD-1 Exposed (N = 5) Control (N = 5)	0.75 mg/kg/d, 4 weeks	• ↑ in fecal *Bacteroidetes*, primarily genus *Bacteroides* and undefined genus in family S24-7 • ↓ in fecal Firmicutes, primarily members of *Lachnospiraceae* and *Ruminococcaceae families (e.g., Blautia, Dorea, Oscillospira, and Ruminococcus)*	• Dose in excess of ADI (2.5x)	Chi et al. (2018)
Sucralose ADI: 15 mg/kg Sucrose equivalence: 600x 	Mice, C57Bl/6 Exposed (N = 20) Control (N = 20)	1666 mg/kg/d, 11 weeks	Elevated glycemic response	• Dose in excess of ADI (~100x) • Food, water consumption not reported • Unconventional grouping of data; invalid statistical analysis equivalent between groups	Suez et al. (2014)
	Mice, SAMP1/YitFc (Crohn's disease-like ileitis) and AKR/J (ileitis-free) All groups (N = 6)	Splenda 1.08 mg/mL (SAMP only) or 3.5 mg/mL (SAMP & AKR) in drinking water, dose not reported, 6 weeks	• Low dose: ↑ in fecal *E. coli* • High dose: ↑ in *Proteobacteria*, including *Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Deltaproteobacteria*, and *Gammaproteobacteria* • ↓ in fecal lactobacilli and clostridia	• No control for sucralose alone • Sucralose dose (mg/kg) unknown • Food consumption not reported	Rodriguez-Palacios et al. (2018)
	Rats, SD Exposed (N = 10 per group) Control (N = 10)	Splenda sucralose equivalents 1.1, 3.3, 5.5, or 11 mg/kg/d, 12 weeks	• ↓ in fecal total anaerobes, bifidobacteria, lactobacilli, and *Bacteroides* (all doses) • ↓ *Clostridia* abundance & total aerobic bacteria (3 highest doses)	• No control for sucralose alone • Significant differences in body weight reported • Food consumption not reported • Bacterial counts not standardized: fecal weights reported on wet basis	Abou-Donia et al. (2008)
	Mice, C57BL/6J Exposed (N = 10) Control (N = 10)	~9–22 mg/kg/d, 6 months	• ↑ in fecal *Turicibacter, Roseburia, Akkermansia, Clostridiaceae, and Christensenellaceae* • ↓ in fecal *Ruminococcus, Streptococcus, Dehalobacterium*, and *Erysipelotrichaceae*	• Food consumption not reported • Unclear if exposure was below ADI for entirety of study	Bian et al. (2017c)
	Mice, C57Bl/6J Exposed (N = 9 per group) Control (N = 8)	~1.5 & 15 mg/kg/d, 8 weeks	• ↓ in fecal *Clostridium IVXa*, dose-dependent	• None to report	Uebanso et al. (2017)
	Mice, PYY knockout and wildtype	1% in drinking water, dose not reported, 1 week	• ↓ in fecal *Bacteroidetes* in wildtype; ↓ attenuated in PYY knockouts	• Dose (mg/kg) unknown • Food consumption not reported • Sample size unknown	Farzi et al. (2017)
	Pregnant mice/dams and their offspring	Pregnant mice/dams consumed sucralose/acesulfame-K mixture in diet, dose and duration not reported	Assessment of 19-day old pups: • ↓ in fecal *Akkermansia muciniphila* • ↑ in fecal *Firmicutes*	• Sucralose exposure (mg/kg) unknown • Food consumption not reported • Sample size unknown	Olivier-Van Stichelen et al. (2017)
Rebaudioside A ADI:4 mg/kg, steviol equivalents Sucrose equivalence: ~300x	Mice, SPF BALB/c Exposed (N = 5 per group) Control (N = 5)	5.5 or 139 mg/kg/d, 4 weeks	• ↑ diversity of fecal lactobacilli (high dose only)	• High dose in excess of ADI (~10x) • Food consumption not reported	Li et al. (2014)

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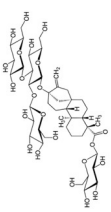
Table 1 (*continued*)

Low/no-calorie sweetener	Subjects	Dose and duration	Microbiome-associated changes[a]	Study confounders	Reference
					

ADI = acceptable daily intake; d = day; NHDC = neohesperidin dihydrochalcone; SUCRAM = saccharin + NHDC.

[a] Compared to respective control group, unless otherwise stated.

on food consumption monitoring, however, was provided so it is unclear if this group may have consumed more chow. The relative abundance of *Bacteroides, Anaerostipes*, and *Sutterella* significantly increased in male mice, whereas in female mice *Lactobacillus, Clostridium,* an unassigned *Ruminococcaceae* genus and an unassigned *Oxalobacteraceae* genus decreased, and *Mucispirillum* increased. Despite these findings in the gut microbiome, several limitations are discussed by the authors and include the use of a high dose of acesulfame K (2.5x the acceptable daily intake [ADI]), lack of food intake monitoring, and small sample size, and the authors indicate that ongoing studies have been designed to address these shortcomings.

Uebanso et al. conducted a study with male C57Bl/6J mice and exposed them to acesulfame K in the drinking water for 8 weeks (N = 9) (Uebanso et al., 2017). Control mice (N = 8) received distilled water, and the authors measured body weight and fluid intake and reported an average exposure of 12.9 mg/kg/day for the acesulfame K group over the course of the study. Liquid and energy consumption was monitored and reported to be equivalent between the control and acesulfame K groups. Fecal samples and cecal contents were obtained at the end of the study for 16S rRNA analysis, and the relative amounts of total bacteria, *Firmicutes, Bacteroidetes, Bacteroides, Clostridium IV* and *Clostridium IVXa* were equivalent between groups. Furthermore, the relative band intensities of different 16S rRNAs from the V2 to V3 region for the cecal contents as well as the feces were not changed following acesulfame K exposure. The authors concluded that acesulfame K did not significantly alter the gut microbiota in mice exposed to doses equivalent to the human ADI.

The effects of acesulfame K on the gut microbiota was investigated in a cross sectional clinical study, where participants completed a daily food record for 4 days and fecal samples were collected on the fifth day to assess gut microbial composition by 16S rRNA analysis (Frankenfeld et al., 2015). Of the 31 participants in the study, 7 reported consumption of acesulfame K over the 4-day period in amounts of 1.7–33.2 mg/day ("consumers"). Consumers that reported consuming both acesulfame K and aspartame were analyzed separately. The median percent abundance of bacteria at the order and class level was compared between consumers and non-consumers of acesulfame K and no significant differences were found. The median *Bacteroidetes*:*Firmicutes* ratio remained constant between the 2 groups, whereas statistically significant differences in bacterial diversity evaluated with UniFrac analysis were noted across consumers and non-consumers. Given that habitual diet was not controlled for it is probable that the difference between the 2 groups was related to diet alone and as such it is not possible to come to any conclusion regarding the impact of acesulfame K on the gut microbiota.

3.2. Aspartame

Aspartame's effects on the gut microbiota were also investigated in the cross sectional clinical study described above for acesulfame K (Frankenfeld et al., 2015). Of the 31 participants in the study, 7 were found to consume 5.3–1112 mg/day of aspartame over the 4-day study period ("consumers"). These were 7 different individuals from those described in Section 3.1 that consumed acesulfame K, and co-consumers of both sweeteners, which were analyzed separately. Similar to what was reported for acesulfame K, no significant differences between consumers and non-consumers were found for the median percent abundance of bacteria at the order and class level, and the median *Bacteroidetes*:*Firmicutes* ratio did not change between the 2 groups. Statistically significant differences in bacterial diversity were noted across aspartame consumers and non-consumers, though as outlined above, the significance of this finding can be called in to question as habitual diet was not controlled for between the 2 groups.

The effect of aspartame consumption on the composition of the gut microbiota of male Sprague-Dawley rats (N = 10 to 12 per group), with and without diet-induced obesity, was assessed over 8 weeks (Palmnäs

et al., 2014). Aspartame was administered in the drinking water at a dose of 5 mg/kg/day to the rats with diet-induced obesity and 7 mg/kg/day to those without diet-induced obesity. Control animals consumed only water. Fecal samples were collected at the end of the study and evaluated by 16S rRNA analysis. The only significant change observed in the normal weight rats fed aspartame was an increase in the levels of *Clostridium leptum* compared to the normal weight control group. More changes in the composition of the gut microbiota of the obese rats fed aspartame compared to the obese control group were reported, including increased total bacteria, *Bifidobacterium* spp., *Enterobacteriaceae*, *C. leptum*, and *Roseburia* spp. Unfortunately, fecal samples were not analyzed prior to dosing so it is unclear if these differences were due to the administration of the sweetener or were inherent differences in the gut microbiota between obese and normal weight animals. The study would have been more informative if it had measured the microbiota changes over the 8-week intervention period between treatment groups. In addition, large differences were noted with respect to food and water consumption by both normal weight and obese rats fed aspartame compared to their respective control groups; all aspartame-exposed rats ate less food, therefore consuming fewer kcal per day, and drank more water compared to their respective control groups. As discussed in Section 2, differences in food consumption and caloric intake influence the composition of the gut microbiota, and therefore without the inclusion of an isocaloric control group, the reported changes in gut microbial composition cannot be attributed to aspartame due to the limitations in study design.

A study published by Suez et al. (2014) investigated the impact of low-calorie sweetener exposure first on glucose metabolism and then extended some studies to include analysis of the intestinal microbiota. Commercial aspartame (Sweet'n Low Gold, 4% aspartame) was administered to C57Bl/6 male mice (N = 20) in the drinking water for 11 weeks, providing an approximate daily dose of 1333 mg/kg aspartame (approximately 30 times higher than the ADI of 40 mg/kg/day), as estimated from the liquid intake data reported in the supplemental data. Saccharin and sucralose were also investigated and the results for these compounds are discussed in their respective Sections 3.4 and 3.5 below. Control groups (N = 20 per group) consumed water, sucrose (33 g/kg/day), or glucose (50 g/kg/day). To detect changes in glucose metabolism, an oral glucose tolerance test (OGTT) was administered at the end of the study and glycemic response was calculated as the area under the 2-h blood glucose response curve. No baseline OGTT results were reported. The statistical analysis grouped all data for the low-calorie sweeteners together (aspartame, saccharin, and sucralose) and compared this group to all control data (water, sucrose, and glucose); glycemic response was reported to be significantly higher in the low-calorie sweetener group ($P < 0.001$). Scientific justification for this mass grouping of data was not provided by the authors, yet, based on this statistical assessment they concluded that "artificial sweeteners induce glucose intolerance". Furthermore, the reported median and range of the aspartame data set was not visually different from that for the water, sucrose, and glucose control data sets, suggesting that aspartame itself had no effect on glycemic responses. The authors asserted that aspartame affected the gut microbiota, based on a reported increase in glucose response to a single OGTT in mice receiving a commercial formulation of aspartame in the drinking water for 11 weeks, which was not seen when the mice were treated with antibiotics. However, the study had no actual measure of microbiota in mice administered aspartame alone. Furthermore, the lack of change in the post-prandial response to the OGTT when aspartame-treated mice were given antibiotics is insufficient evidence to conclude that there was an effect on the gut microbiota.

3.3. Cyclamate

A number of studies show that cyclamate may be converted by the gut microbiota to cyclohexylamine, which is absorbed and excreted in urine. One study also assessed the effect of cyclamate ingestion on the distribution of fecal microbiota *in vivo*. In this study, a *Macaca irus* monkey was orally administered 250 mg/kg/day sodium cyclamate for 30 days (Matsui et al., 1976). Urine samples from this monkey confirmed a significant conversion of the ingested cyclamate to cyclohexylamine. The control group consisted of 3 monkeys (2 *M. rhesus*, 1 *Cynopithecus niger*) that did not consume sodium cyclamate. Fecal samples were collected 3 times from all monkeys at 10-day intervals. Total fecal bacteria as well as the levels of individual microbial populations in the feces (*Bacteroidaceae*, catenabacteria, bifidobacteria, peptostreptococci, lactobacilli, streptococci, enterobacteria, *Clostridia*, *Veillonella*, staphylococci) were reported to be equivalent between the control monkeys and the sodium cyclamate metabolizing monkey at all 3 time points.

3.4. Neotame

Neotame was administered to male CD-1 mice (N = 5 per group) *via* gavage for 4 weeks at a dose of 0.75 mg/kg/day, and the control group received only water (Chi et al., 2018). Fecal samples were collected for 16S rRNA gene sequencing and functional gene enrichment analysis before and after 4 weeks of neotame exposure. Body weight did not significantly differ between the 2 groups throughout the study and the authors reported that "no difference of eating behavior or other behaviors were observed between two groups". The relative fecal abundance of *Bacteroidetes* significantly increased in the neotame exposed group, which at the genus level was primarily due to elevated *Bacteroides* and an undefined genus in family S24-7. The relative fecal abundance of *Firmicutes* was significantly decreased following neotame exposure, with over 12 genera altered, most notably multiple components of the *Lachnospiraceae* and *Ruminococcaceae* families, such as *Blautia*, *Dorea*, *Oscillospira*, and *Ruminococcus*. Despite these changes in the gut microbiome following neotame exposure, some limitations are discussed by the authors including the use of a neotame dose 2.5x the human ADI and the small sample size, and the authors indicate that long-term studies in humans should be conducted.

3.5. Saccharin

A few studies were identified reporting effects of saccharin on the gut microbiota. An early study (Anderson and Kirkland, 1980) was conducted in male rats provided chow containing no saccharin (control; N = 5) or 7.5% sodium saccharin (N = 7) for 10 days. Final average saccharin intake was 10–14 g/kg/day. At the end of the 10-day study, the cecum was collected from each rat and weighed, and microbial contents were analyzed by culturing the aerobic and anaerobic bacteria. The cecal populations of the saccharin-fed rats contained increased numbers of aerobes and equivalent numbers of anaerobes compared to the control group, leading to a downward shift in the anaerobe/aerobe ratio. Furthermore, saccharin consumption was reported to prevent the growth of saccharin-sensitive anaerobes in the gut. Given that the average daily saccharin intake was approximately 2000 times higher than the 5 mg/kg/day ADI set by regulatory authorities for saccharin, the human dietary relevance of this study is evidently limited.

Suez et al. (2014) reported a small clinical investigation in 7 healthy adults who did not normally consume low-calorie sweeteners. Subjects consumed 5 mg/kg/day of saccharin in 3 divided daily doses of approximately 120 mg/dose for 1 week. No control group was included in the study and only single-day baseline measurements were obtained. Fecal samples were collected, glucose tolerance tests were conducted daily, and the microbiota was analyzed by 16S rRNA analysis. The data were statistically assessed by creating data groups based on scientific outcomes, as opposed to being based on study design, which is particularly difficult to understand with such a small sample size. Specifically, the measured glycemic responses were used to classify the subjects as either "responders" (4 subjects that had elevated glycemic

responses at Day 5 and after) or "non-responders" (3 subjects that showed no change in glycemic response). The authors reported that the microbiome configurations of the responders were found to cluster differently from the non-responders both before and after saccharin consumption. Pronounced compositional changes were observed in the responder's microbiomes over the study week, whereas no changes were reported in the non-responders. Despite these measured differences between the microbiomes of the 2 groups, the arbitrary grouping criteria and lack of control group bring into question the biological significance of these reported findings. The lack of controlling for habitual diet may be a driving factor behind the reported gut microbial changes, which is supported by the fact that the composition of the gut microbiota of the responder and non-responder groups was already different prior to saccharin consumption (see Day 1, Fig. 1). Since the responder and non-responder populations were not comparable on Day 1 of the analysis, it therefore would have been more appropriate to compare each subject back to their own multi-day baseline to detect any saccharin-associated changes.

Suez et al. (2014) also evaluated effects of saccharin in mice. The commercial saccharin preparation, Sucrazit (5% saccharin, 95% glucose), was administered in the drinking water to C57Bl/6 male mice (N = 20 per group) for 11 weeks at a saccharin dose of approximately 3333 mg/kg/day (about 650 times higher than the ADI of 5 mg/kg/day). At the end of the study, an OGTT was conducted in all mice and fecal samples were collected for 16S rRNA analysis from 5 out of 20 mice in the saccharin group as well as each of the control groups. While glucose was reported to be statistically significantly increased in the treated mice (Suez et al., 2015), and this difference was not found when mice were treated with antibiotics, as noted above, this is not a measure of an effect on gut microbiota and therefore any cause and effect relationships can only be speculative. Moreover, based on fluid intake data reported, albeit limited to a subset of the mice studied, it appears that antibiotic treatment may have been greater in the saccharin *versus* control mice, which makes data interpretation more problematic. Additionally, from the data reported, it appears that the blood glucose area under the curve (AUC) following the OGTT was increased in only 3 of the 20 mice studied, and no baseline OGTT was available for comparison. The majority of data points (~17 out of 20), though, were found to fall within the range of control measurements. No direct statistical analysis was provided for saccharin treatment alone and as such it is unclear if there is truly a significant difference between the saccharin-treated animals and the controls. The reason for this postulated altered glycemic response in this limited number of animals is unclear, as in addition to being exposed to saccharin, chow consumption was reported to be unequal between the groups. Although food intake was measured for only a subset of mice (4 out of 20) over a portion of the study (3 days at the end of the study), instead of for all mice throughout the entire study, such differences in food intake alter the consumption levels of fiber, protein, fat, and carbohydrates, which directly influence blood glucose measurements. These differences in chow consumption could also have an effect on the composition of gut microbiota.

With regard to the fecal analyses conducted, the authors reported treatment-related alterations in the abundance of numerous operational taxonomic units (± 1.2-fold), including increases in the relative abundance of the *Bacteroides* genus and the *Clostridiales* order, and decreases in *Lactobacillus reuteri*, as well as other members of the *Clostridiales* order. It is difficult to interpret the results of these findings, for a variety of reasons. First, the treated mice received water with a commercial saccharin formulation, not pure saccharin. This means there was 11 weeks of exposure to a nutritive sweetener (carrier substance for the commercial saccharin formulation) in the drinking water of the treated mice, where control mice received only water. Second, the study reported between-group differences in both water and solid food consumption that, irrespective of caloric considerations, can introduce physiologic factors that could influence gut microbiota populations. Third, the data on gut microbiota changes are from only a subset of the population, about which there is no specific information on food or water intake. In all, it is not possible to know whether between-group differences observed in the gut microbiota measures are reflective of changes in diet and/or nutrient and/or fluid intake or changes related to the presence of saccharin. Finally, the saccharin dose delivered was far above expected human intakes.

In this same publication, Suez et al. (2014) did report other investigations in mice treated with pure saccharin. Saccharin was administered for 5 weeks in the drinking water at a concentration of 0.1 mg/mL (N = 20), leading to a daily exposure of about 5 mg/kg. In contrast to the previous study, both liquid and chow consumption were reported to be equivalent between the 2 groups; however, the data again represented only a subset of the population (8 out of 20 mice per group). This snapshot of food and water consumption may or may not be representative of average consumption in all animals over the entire length of the study. OGTTs were conducted and fecal samples were collected at the end of the 5-week study. Glycemic response was significantly higher in the saccharin-exposed mice, albeit with high variability in the range of responses of the individual animals; about half (N = 10) were within the range of control responses (AUC ≈ 18 to 44 × 10^3) whereas the other half were above of this range (AUC ≈ 45 to 56 × 10^3). The fecal samples were transplanted into germ free mice (N = 16 per group) *via* gavage, but no information was provided as to if the fecal samples were collected from all 20 animals and then pooled, or if only select animals were chosen as donors. The fecal-recipient mice were provided with standard rodent chow and water for 6 days, at which point OGTTs were conducted and fecal samples were collected for 16s rRNA analysis. The investigators report an "impaired glucose tolerance" in the germ-free mice implanted with fecal samples from saccharin-treated *versus* control mice. It is difficult to determine, however, if there is actually a treatment-related effect. The study found statistically significant increases in the post-OGTT glucose level at 30, 60, and 90 min; however, (a) baseline blood glucose level was statistically significantly higher in the saccharin-fecal recipients *versus* control-fecal recipients and the later observed differences were small, which may mean that the later differences were wholly unrelated to treatment; (b) total glucose AUC was not reported and may not be significantly different between the 2 groups; (c) peak blood glucose levels were not different for the 2 groups of mice; and (d) there was no baseline OGTT for comparison to understand potential intra-group/intra-individual variability with glucose response to an OGTT. As a result, it is difficult to conclude from this single OGTT result that gut microbiota from saccharin-treated mice affected a transference of

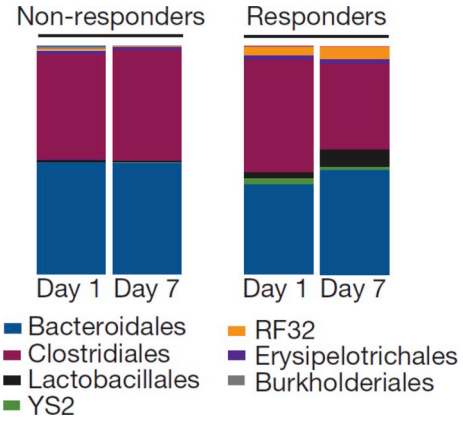


Fig. 1. Order-level relative abundance of taxa samples reproduced from Suez et al. (2014), Figure 4f. Adapted by permission from Macmillan Publishers Ltd: Suez et al. (2014). A clinical investigation was conducted in 7 subjects consuming 5 mg/kg/day of saccharin for 7 days. Subjects were classified as non-responders or responders, based on having no change or elevated glycemic responses, respectively, following saccharin consumption. Fecal 16S rRNA was measured on Day 1 (baseline) and on Day 7.

"impaired glucose tolerance" to germ-free mice. In the evaluations of actual changes in gut microbiota, the abundance of only a few operational taxonomic units were reported when the saccharin-recipient and control-recipient fecal microbiota were compared (± 1.2-fold). A few increases in relative abundance were observed in the *Bacteroides* genus and the *Lactobacillus reuteri* species, and decreases were mainly observed in a few members of the *Clostridiales* order and *Ruminococcaceae* family. These changes are not clearly known to be ones that can elicit a change in the ability of mice, or humans, to appropriately process a bolus load of glucose.

For an additional 4 weeks, 10 mice per group were given *ad libitum* access to food and water with 0 or 5 mg/kg/day pure saccharin, and with or without antibiotic (Gram-negative-targeting antibiotics ciprofloxacin [13 mg/kg/day] and metronidazole [67 mg/kg/day]). The results showed a similar response to the OGTT for the saccharin and control-treated mice when exposed to an antibiotic, from which the authors concluded that changes in gut microbiota were causative in the increased blood glucose levels seen when the mice received water with saccharin *versus* plain water. As discussed above, a potential difference in baseline fasting levels is not considered in this conclusion and intra-individual differences may account for the responses. In addition, it is unclear if antibiotic exposure was equivalent in the different treatment groups and there is also no information regarding how animals were selected to continue with the antibiotic-arm of the study. With the individual differences reported in glycemic response, this should have been reported.

Bian et al. (2017b) recently conducted a study in mice over a 6-month period, where C57BL/6J male mice were exposed to saccharin in the drinking water (0.3 mg/mL) or consumed just water (N = 10 per group). Water consumption was monitored and the authors stated that the exposure to saccharin was equivalent to the ADI of 5 mg/kg; however, the consumption data was not published. In the absence of the liquid consumption data, based on the average weight of the mice at the beginning and end of the study (23–33 g) and the usual daily water consumption of a mouse (~ 3–5 mL), it appears that daily exposure was more likely between 27 and 65 mg/kg (at least ~ 5x the ADI). Fecal samples were collected at baseline, 3, and 6 months for 16S rRNA analysis and the authors reported that 11 genera were significantly altered following saccharin exposure (*i.e., Sporosarcina, Jeotgalicoccus, Akkermansia, Oscillospira,* and *Corynebacterium* increased at 3 months; *Corynebacterium, Roseburia, and Turicibacter* increased at 6 months; *Anaerostipes* and *Ruminococcus* decreased at 3 months; *Ruminococcus, Adlercreutzia, and Dorea* decreased at 6 months). Given the suspected high exposure to saccharin, and the lack of food consumption data, the significance of these findings is unclear.

Two studies have investigated the impact of adding SUCRAM (saccharin + neohesperidin dihydrochalcone [NHDC]) to piglet feed. In the first study, feed containing 0.015% SUCRAM was fed to male and female suckling Landrace X Large White piglets for 2 weeks (N = 8, mg/kg/day dose not reported) (Daly et al., 2014). Control piglets (N = 8) consumed regular feed. Cecal and rectal contents were collected at the end of the study and the microbiota was evaluated by 16S rRNA analysis. A significant increase (2.5-fold) in the relative population size of *Lactobacillus* in the cecum was reported in the piglets fed SUCRAM compared to control, and 1 *Lactobacillus* phylotype, OTU4228, was found to be predominantly responsible for this elevation. In the second study, the same experimental conditions were employed, with the addition of treated and control groups containing male and female Gloucester Old spot piglets (Daly et al., 2016). Similar to the findings of the first study, significant increases in the population abundance of the *Lactobacillaceae* family was measured in the cecal contents of both piglet strains fed SUCRAM, as well as significantly lower abundances of the *Veillonellaceae* and *Ruminococcaceae* families. The authors noted that based on the control groups used in their studies, it was unclear if the observed changes were due to saccharin or NHDC. To address this, they conducted *in vitro* tests to gain a better understanding of the mechanism of *Lactobacillaceae* enhancement following SUCRAM consumption. *Lactobacillus* phylotype 4228 was isolated from the intestine of the piglets, and the growth characteristics of this strain were observed in the presence of saccharin or NHDC, independently. The presence of saccharin had no effect on the growth of *Lactobacillus* 4228 *in vitro*, whereas NHDC present in the media at 0.5 mM significantly decreased the lag phase of *Lactobacillus* 4228 growth when switching from glucose-containing medium to lactose, sucrose, or fructose. Based on these data, the authors concluded that it appears that the NHDC component of SUCRAM, not saccharin, is responsible for the changes in *Lactobacillaceae* prevalence *in vivo*. Further studies have also been recently conducted to characterize the mucosa-associated microbiota along the entire length of the piglet intestinal tract and the effects of SUCRAM exposure (Kelly et al., 2017).

3.6. Sucralose

The study published by Suez et al. (2014), also asserted that sucralose affected the gut microbiome, based on a reported increase in glucose response to a single OGTT in mice receiving a commercial formulation of sucralose (Sucralite, 5% sucralose) in the drinking water for 11 weeks (approximately 1666 mg/kg sucralose per day), because a similar effect was not seen when the mice were treated with antibiotics. The lack of change in the post-prandial response to the OGTT when sucralose-treated mice were given antibiotics is insufficient evidence to conclude that there was an effect on the gut microbiota. There was also no actual measure of microbiota in the mice that consumed sucralose, so the study presents no evidence of an effect of sucralose on the microbiota.

In an article investigating artificial sweeteners and Crohn's disease, mice with Crohn's disease-like ileitis SAMP1/YitFc (SAMP), were administered Splenda® (sucralose:maltodextrin, 1:99, w/w) in the drinking water at concentrations of 0 or 1.08 mg/mL for 6 weeks (N = 6 per group) (Rodriguez-Palacios et al., 2018). Body weight was monitored and fecal samples were collected at the end of the study and analyzed by microbiological culturing. Culture data reported elevated levels of *E. coli* in the feces of the Splenda®-exposed mice and no changes in lactobacilli, total bacteria, or anaerobic clostridial species between the Splenda® and control SAMP groups. In the second study, SAMP mice and parental ileitis-free AKR/J (AKR) control mice were administered Splenda® in the drinking water at concentrations of 0 or 3.5 mg/mL for 6 weeks (N = 6 per group). Fecal samples were collected at the end of the study and analyzed by 16S rRNA analysis. Splenda® exposure in both SAMP and AKR mice significantly elevated the levels of the *Proteobacteria* phylum by increasing the *Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Deltaproteobacteria*, and *Gammaproteobacteria* classes. Splenda® reduced the levels of other phyla, such as lactobacilli and clostridia, with no overall impact on *Bacteroidetes* or *Firmicutes*. Based on histological analyses, the authors noted that Splenda® did not increase the severity of ileitis in the SAMP mice. Although no details were provided on monitoring food or water consumption, nor the daily exposure to Splenda® on a body weight basis, the authors did indicate that the 3.5 mg/mL concentration was equivalent to the maximum United States Food and Drug Administration (FDA)-approved dose, suggesting the doses in both studies were no greater than 5 mg/kg bw/day. Whether or not the microbial changes reported in the mice with Crohn's disease-like ileitis and the ileitis-free mice are due to sucralose itself cannot be determined as no controls were included in this study to separate out the components of Splenda®, which is primarily composed of the bulking agent maltodextrin. In addition to a lack of control for ingredients other than sucralose in the test material, it is also not clear if food consumption was equivalent between groups, which could affect measures of gut microbiota species.

Uebanso et al. conducted a study with male C57Bl/6J mice (N = 8 per group) and exposed them to low-dose (1.5 mg/kg/day) or high-dose (15 mg/kg/day) sucralose in the drinking water for 8 weeks (Uebanso

et al., 2017). Control mice received distilled water, and the authors measured body weight and fluid intake and reported an average exposure to sucralose of 1.4 mg/kg/day for the low-dose and 14.2 mg/kg/day for the high-dose groups. Liquid and energy consumption was monitored and reported to be equivalent between the control and sucralose groups. Fecal samples were obtained at the end of the study for 16S rRNA analysis, and the relative amounts of total bacteria, *Firmicutes, and Bacteroidetes, Bacteroides,* and *Clostridium IV,* were equivalent between all groups. The relative amount of fecal *Clostridium IVXa*, however, was reported to decrease significantly in the sucralose groups in a dose-dependent manner. Other analyses were conducted, to see if other sucralose-dependent changes were present in the gut microbiome, but the relative band intensities of different 16S rRNAs from the V2 and V3 region, and principal component analysis and hierarchical clustering did not differ between the groups.

Bian et al. (2017c) conducted a study in mice over a 6-month period, where C57BL/6J male mice were exposed to sucralose in the drinking water (0.1 mg/mL) or consumed just water (N = 10 per group). Water consumption and body weight were monitored. The authors stated that the calculated exposure to sucralose was within the U.S. FDA ADI of 5 mg/kg; however, these data were not provided. In the absence of the liquid consumption and body weight data, assuming an average body weight range of 23–33 g (as reported in the Bain et al., (2017a, b, c) study with saccharin that used the same strain, sex, and age of mice) and usual daily water consumption of ~3–5 mL, it appears that daily exposure was more likely between 9 and 22 mg/kg (at minimum ~2x the FDA ADI). Fecal samples were collected at baseline, 3, and 6 months for 16S rRNA analysis and the authors reported that 14 genera were significantly altered following sucralose exposure (*i.e., Ruminococcus* increased at 3 months; *Turicibacter, Roseburia, Akkermansia, Clostridiaceae,* and *Christensenellaceae* increased at 6 months; *Anaerostipes, Ruminococcus, Staphylococcus, Peptostreptococcaceae,* and *Bacillales* decreased at 3 months; *Ruminococcus, Streptococcus, Dehalobacterium,* and *Erysipelotrichaceae* decreased at 6 months). Food consumption was not monitored, so it is unclear if dietary intake was standardized between the groups, and the sucralose exposure level was not clearly defined. The significance of the findings reported in this study regarding the gut microbiota, therefore, are unclear.

In a 12-week study in male Sprague-Dawley rats (N = 10 per group), Splenda® (1.10% sucralose, 1.08% glucose, 4.23% moisture, and 93.59% maltodextrin) was dissolved in the drinking water at the following doses: 0, 100, 300, 500, or 1000 mg/kg/day (equivalent to 1.1, 3.3, 5.5, 11 mg/kg/day sucralose) (Abou-Donia et al., 2008). Fecal samples were collected weekly, fecal weights were recorded on a wet basis, and bacteriological analysis was conducted by subculturing fecal bacteria. Following 12 weeks of Splenda® exposure, the numbers of total anaerobes, bifidobacteria, lactobacilli, and *Bacteroides* significantly decreased in all Splenda® groups compared to control. At the 3 highest Splenda® doses, the abundance of *Clostridia* and total aerobic bacteria also decreased significantly. The comparison of the bacterial count data between groups, however, appears to be limited in value as it was not indicated that the counts were standardized appropriately based on water intake. Water consumption has a marked effect on stool formation and moisture content in the feces, and since the fecal weights were reported on a wet basis, this should have been accounted for when evaluating the bacterial counts. Moreover, significant differences in body weight were reported between the Splenda® and control groups but no data reporting food intake, energy consumption, or water intake were presented. Similar to the study with Splenda® in mice described above, no control groups were included to separate out the effects of the individual components of Splenda®, particularly the maltodextrin. The relevance of these reported changes in the microbial composition of the gut following Splenda® ingestion, therefore, is questionable. Indeed, an Expert Panel that thoroughly evaluated this study concluded that it was deficient in several critical areas and that its results cannot be interpreted as evidence that either Splenda®, or sucralose, produced adverse effects in the rats studied, including effects on the gastrointestinal microbiota (Brusick et al., 2009).

Two recently published conference abstracts that reported measures of the microbiome in animals exposed to sucralose met the defined search criteria and therefore were included in the search results. In the first study, differences in intestinal microbiota composition in gut hormone peptide YY (PYY) knockout and wildtype male mice were investigated under basal conditions and following exposure to sucralose (1%) in drinking water for 1 week (Farzi et al., 2017). At the phylum level, baseline fecal microbial composition was reported to be similar, with decreased *Bacteroidetes* in the wildtype mice following exposure to sucralose that was attenuated in the PYY-knockouts. In the second study, the impact of pre- and post-natal exposure to sucralose combined with acesulfame-K was investigated (Olivier-Van Stichelen et al., 2017). Pregnant mice consumed sucralose/acesulfame-K mixed in the diet (no dose reported) and continued to consume this same diet after pups were born. The authors presumed that pups were exposed to the sweeteners through placental circulation and breastmilk, but this was not confirmed experimentally nor was the length of exposure reported. The mucin-feeding bacteria *Akkermansia muciniphila* was noted to "disappear" in 19-day old pups exposed to the sweeteners compared to control, along with an increase in *Firmicutes*. Additional study design details, such as LNCS exposure on a mg/kg basis, and food/water consumption data, are required in order to comment on the direct relevance of these microbiome findings to LNCS consumption.

3.7. Rebaudioside A

The impact of rebaudioside A ingestion on the gut microbiota has been investigated in 1 study, where SPF BALB/c mice were orally administered rebaudioside A for a total of 4 weeks at a low dose of 5.5 mg/kg/day (N = 5) or a high dose of 139 mg/kg/day (N = 5), and distilled water was used as control (N = 5) (Li et al., 2014). Total viable cell counts were obtained in the feces over the 4-week study period, and *Enterobacteriaceae* and lactobacilli were measured at the end of the study by denaturing gradient gel electrophoresis. Rebaudioside A consumption, at both the low and high doses, was not associated with any significant changes in the total number of anaerobic bacteria, enterococci, enterobacteria, or lactobacilli and also had no effect on *Enterobacteriaceae* distribution. A significant increase was found in the diversity of lactobacilli species present in the feces, yet, given that this dose (46 mg/kg/day steviol equivalents) was more than 10-fold higher that the ADI of 4 mg/kg/day steviol equivalents, the human dietary relevance of this change is limited. Overall, the results at the lower dose of 1.8 mg/kg/day steviol equivalents indicate that the gut microbiota of mice was not affected by rebaudioside A consumption.

3.8. Undefined low/no-calorie sweeteners

Suez et al. (2014) conducted a cohort study in 381 non-diabetic individuals (44% males, 56% non-pregnant females, age 43.3 ± 13.2) that were participating in an ongoing clinical nutrition study. Parameters assessed during the clinical nutrition study included body mass index, body circumference, fasting glucose levels, complete blood counts, and general chemistry parameters. Subjects completed validated long-term food frequency questionnaires that included specific questions regarding artificial sweeteners and the authors indicated that they were able to quantify long-term non-caloric artificial sweetener (NAS) consumption directly from these answers. Based on this, subjects were classified as either 'high-consumers' (N = 40) or 'non-consumers' (N = 236), yet, no details were provided on the exact criteria used to establish this grouping nor was the exclusion of 105 'low-consumer' subjects explained. No information was provided on the types or amounts of NAS consumed by the 40 subjects identified as high-consumers and diet was not controlled for. The Spearman correlation test was used to conduct a non-parametric rank comparison of glycosylated

hemoglobin (HbA1C %) levels between the high- and non-consumers and was reported to be significantly higher in the high-consumer group. A brief look at the data (Fig. 2) indicates that some of the non-consumers had higher HbA1C values than the high-consumers and that all values for the high-consumers fell within the range of the non-consumers, suggesting that the difference may simply be due to the much lower sample size of high-consumers. Additionally, no explanation was provided to justify the use of a non-parametric test despite all the measurements appearing to be parametric. The authors proceeded to randomly select 172 individuals out of this cohort (N = 276) and characterize their fecal bacterial 16S rRNA, but no further details were provided on collection of the fecal samples. A parametric Pearson correlation test was conducted to identify correlations between taxonomic entities within the fecal microbiota and NAS consumption. The authors identified several significant positive correlations between the *Enterobacteriaceae* family, the *Deltaproteobacteria* class, as well as the *Actinobacteria* phylum, and NAS consumption, yet, in the absence of information on habitual diet and amount or type of sweetener consumed, it is not possible to draw any conclusions regarding the impact of NAS on the gut microbiota.

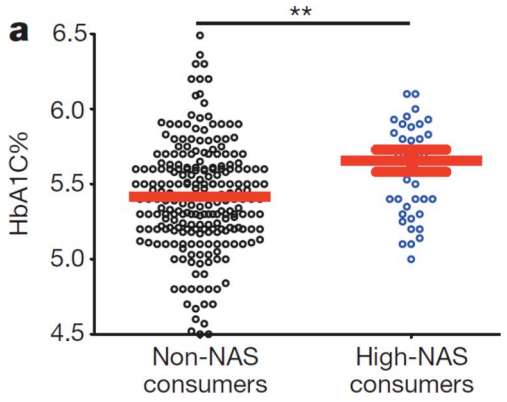


Fig. 2. Glycosylated hemoglobin (HbA1C %) levels reproduced from Suez et al. (2014), Figure 4a. Adapted by permission from Macmillan Publishers Ltd: Suez et al. (2014). HbA1C% levels of non-consumers of non-caloric artificial sweeteners (NAS) (N = 236) compared with high-consumers (N = 40) reported in a clinical nutrition study of non-diabetic individuals (N = 381).

4. Common critical issues when measuring the gut microbiome

Following review of the studies of LNCS that included evaluations of gut microbiota, certain critical issues were found to be common among the whole. The most common problem was the lack of proper control groups, particularly isocaloric controls. As outlined, dietary factors are key determinants in gut microbial composition, and as discussed in Section 2, differences in the types of food consumed (*i.e.,* plant-*versus* animal-based) and overall caloric intake lead to distinctly diverse gut microbiomes (David et al., 2014). To obtain meaningful outcomes in clinical studies aimed at measuring the response of the gut microbiota to low-level ingredient exposure, it is critical that background dietary consumption also be strictly controlled. Of the 3 clinical investigations assessed, none were designed to account for habitual dietary consumption (Suez et al., 2014; Frankenfeld et al., 2015). As such, any reported changes in the gut microbiota in these clinical studies could very likely be due to dietary differences between LNCS-exposed and non-exposed groups, and not necessarily the LNCS itself. It is much easier to control dietary composition in animal studies through the provision of standardized animal chow, and to effectively achieve this, food consumption must be monitored throughout the study, particularly if LNCS are mixed within the food, as caloric intake independently affects the composition of the gut microbiota. In the aspartame study in rats conducted by Palmnäs et al. (2014), for example, food intake was appropriately monitored and the authors did find that food consumption in the aspartame groups was significantly less than the controls. The authors, however, did not discuss these results in the context of possible effects on the gut microbiota and as such, the changes that were reported in microbial composition are likely to be related to these differences in caloric intake. Similar discrepancies in chow and liquid intake were reported in the 11-week mouse study by Suez et al. (2014), where addition of high concentrations of the commercial sweeteners aspartame, saccharin, and sucralose to the drinking water led to large variances in consumption between the sweetener and control groups. Despite the fact that these differences likely introduced significant confounding effects into measures of the gut microbiota, they were not discussed or factored into any conclusions drawn by the authors.

Another critical study design deficiency noted in a number of the animal studies was the use of LNCS doses that were greater than the currently established ADIs. In 2 studies in particular, doses in excess of 500 times higher than the ADI were utilized. Saccharin was administered to rats at doses of at least 10000 mg/kg/day in the study by Anderson and Kirkland (1980), which is 2000 times higher than the ADI of 5 mg/kg/day for saccharin. Similarly, Suez et al. (2014) fed mice doses of saccharin that were about 600 times higher than the ADI, equivalent to approximately 3333 mg/kg/day in the drinking water. It is well-documented to date that saccharin administered at high doses to rats does alter the gut microbiota, and the study by Anderson and Kirkland (1980), in fact, was one of the first publications to report these findings. In addition to the increased total number of aerobic microbes present in the cecal contents reported by Anderson and Kirkland (1980), a number of other gut-associated changes have been reported in rats fed high doses of saccharin, including marked enlargement of the cecum, increased stool sizes, as well as significant changes in the metabolism of amino acids by the gut microbiota leading to increased urinary excretion of the microbial metabolites indican and ρ-cresol and decreased excretion of phenol (Anderson and Kirkland, 1980; Lawrie et al., 1985; Lawrie and Renwick, 1987; Sims and Renwick, 1983). The fact that Suez et al. (2014) reported that high doses of saccharin in mice induced changes in the composition of the gut microbiota is not novel given the already well-established association in rats. Prior to the establishment of the saccharin ADI, some clinical investigations were conducted at doses higher than 5 mg/kg/day to confirm its safe consumption and to ensure that the gut-associated changes in amino acid metabolism observed in rats were not present in humans. For instance, 333 mg of saccharin was administered to 15 non-saccharin consuming volunteers (3 females, 12 males, average age 27.5 years) 3 times per day, equivalent to a daily dose of 14 mg/kg/day, for a period of 4 weeks. Subjects were instructed to consume their regular diet during the study and urine samples were collected before, during, and after saccharin exposure and analyzed for indican, ρ-cresol, and phenol content. Contrary to what was observed in rats, average urinary levels of these 3 microbial metabolites were unchanged following chronic saccharin consumption compared to baseline control (Lawrie and Renwick, 1987; Roberts and Renwick, 1985). This disparity between the effects reported at high doses in rats and the lack of changes reported in humans highlights the importance of utilizing biologically relevant doses in these types of investigations. The changes in the rodent gut microbiota in response to high doses of saccharin reported by Suez et al. (2014) and Anderson and Kirkland (1980) represent biochemical and physiological changes and cannot be extrapolated to humans who are exposed to profoundly lower levels in the diet. To date, dietary saccharin is associated with a safe toxicity profile in humans, and even the earliest clinical studies that were conducted at much higher doses in diabetic patients (4.8 g/day of saccharin for 5 months) were not associated with any adverse effects (JECFA, 1982).

A few of the animal studies, including the investigation of Splenda® consumption in SAMP and AKR mice (Rodriguez-Palacios et al., 2018), and the piglet studies with SUCRAM (Suez et al., 2015), did not provide sufficient information to convert LNCS consumption to exposures based

on body weight (*i.e.,* body weight of animals, food consumption by weight, water consumption by volume). In the absence of this information, it is not possible to comment on the relevance to humans in a dietary context, highlighting another common critical issue in the conduct of these studies. Of the animal studies that did employ LNCS doses that were equal to or less than the respective ADIs, the majority of studies reported no changes in the gut microbiota that could be definitively linked to sweetener consumption based on the study details provided. For instance, administration of rebaudioside A to mice at a dose of 1.8 mg/kg/day steviol equivalents, which is less than the ADI of 4 mg/kg/day steviol equivalents, was not associated with any significant changes in numbers of anaerobic bacteria nor alterations in the composition of the gut microbiota (Li et al., 2014) and likewise, acesulfame K exposure of 12.9 mg/kg/day for 8 weeks in mice had no effect on the fecal microbiome (Uebanso et al., 2017). Although Palmnäs et al. (2014) administered aspartame to rats at doses lower than the ADI of 40 mg/kg/day (normal weight rats, 5 mg/kg/day; diet-induced obese rats, 7 mg/kg/day), the findings from this study were inconclusive as significant differences in water and food consumption between sweetener and control groups were identified as confounding factors. Splenda® was administered to rats by Abou-Donia et al. (2008) at 4 relevant sucralose doses that were all below the ADI of 5–15 mg/kg/day (1.1, 3.3, 5.5, and 11 mg/kg/day), yet, the findings from this study were limited due to the authors failure to standardize the fecal bacterial count data or control for the bulking agent maltodextrin. In all, there are only 3 studies in which a LNCS has been directly tested *in vivo* at doses at or below the ADI that report a change in gut microbiota (Suez et al., 2014; Bian et al., 2017c; Uebanso et al., 2017), all of which were studies in mice, a species in which the level of reported changes in gut microbiota may also have no relevance to human health.

As with all studies that aim to associate low level ingredient exposure with a biological change or health outcome, utilizing well-designed clinical investigations or relevant animal or *in vitro* models that accurately reflect the appropriate human biological system is critical. Methods for studying the human gut microbiome have evolved significantly over the last decade and the establishment of rodent fecal-transplant models has provided a suitable animal model with human biological relevance to this field of study. The distal gut microbiota of mice, for instance, is comprised of the same bacterial phyla as humans, however, most of the bacterial genera and species present in mice do not exist in the human gut (Turnbaugh et al., 2009). Germ-free mice that are transplanted with human fecal microbiota, on the contrary, establish a bacterial gut community that is reflective of humans, and it is these types of animal models that have proven to be most informative when studying the gut microbiome. Associations between obesity and composition of the gut microbiota have been heavily investigated using these models, and for example, it has been demonstrated that obese or lean phenotypes can be transferred to germ-free mice through fecal transplants from human individuals harboring these distinct phenotypes (Xu and Knight, 2015). Only 1 publication was identified in our literature search that utilized fecal transplant models (Suez et al., 2014). The investigators in this case reported that impaired glucose tolerance was transferable to germ-free mice that were the recipient of feces obtained from saccharin-exposed mice (5 mg/kg/day). As discussed in Section 3.4, however, the evidence of an “impaired glucose tolerance” is questionable, and moreover, saccharin has been found to have no effect on glucose control in humans (Ambrus et al., 1976).

5. Molecular and metabolic considerations

Both the chemical structures (Table 1) and *in vivo* metabolism of approved LNCS support that they have no effect on the gut microbiota, either as individual sweeteners or as a class. Aspartame for instance, is a methyl ester of the dipeptide composed of the amino acids L-aspartic acid and L-phenylalanine, that when ingested, is rapidly hydrolyzed by gut esterases and peptidases to its amino acid components and methanol. These metabolites are absorbed in the small intestine, and neither aspartame nor its metabolites ever reach the colon for direct interaction with the microbiota there (Magnuson et al., 2007; EFSA, 2013). Since the vast majority of gut microbiota resides in the large intestine, potential effects of aspartame on gut microbiota are extremely limited. Numerous studies also show no effect of aspartame on the gastrointestinal tract (Bianchi et al., 1980), indicating no deleterious effects on the gut microbiota.

Metabolic studies in mice, rats, and humans with sucralose have shown that this sweetener is largely unabsorbed, but not digested in the gut, thus clearly demonstrating that it is not a substrate for gut microbiota. Research shows that there is no change in the metabolic profile after prolonged exposure (> 1 year), indicating no microbial metabolic adaptation, even with very high doses of orally consumed sucralose (Roberts et al., 2000; Sims et al., 2000). Similarly, neither saccharin nor acesulfame K, the potassium salt of 6-methyl-1,2,3-oxathiazine-4(3*H*)-one 2,2-dioxide, undergo gastrointestinal metabolism. In contrast to sucralose, both of these latter sweeteners are rapidly absorbed and excreted unchanged in the urine (McChesney and Golberg, 1973; Byard et al., 1974; Volz et al., 1991). Early research on each of these sweeteners, conducted in the assessment of their safety prior to FDA approval of their use, also supports no adverse effect on gut health or function, as evidenced by regulatory approvals worldwide (JECFA, 1984; JECFA, 1991; JECFA, 1999). Therefore, there appears to be no mechanism by which these LNCS can impact the microbiota in such a way as to impact health.

The metabolic pathway for steviol glycosides that can be extracted from the leaves of the *Stevia rebaudiana* plant directly involves the hydrolyzing action of the intestinal microbiota. The molecular structure of all steviol glycosides are similar, composed of a core steviol backbone that is conjugated to different numbers and types of sugar moieties, and these molecules, including stevioside and rebaudioside A, pass unabsorbed through the upper portion of the gastrointestinal tract and enter the colon intact (Koyama et al., 2003; Geuns et al., 2007). Once reaching the colon, the sugar moieties attached to the steviol backbone are sequentially removed by the gut microbiota, primarily of the *Bacteroidaceae* family (Gardana et al., 2003; Renwick and Tarka, 2008), and thus represent an energy source to these microbiota. Any energy contribution, however, is inconsequential, as total daily intake is very low (Renwick, 2008), and thus, would not be expected to have any meaningful effect on microbiota species involved in this metabolism. All steviol glycosides yield the common backbone, steviol, following removal of the conjugated sugars. Steviol is, itself, not a substrate for the intestinal microbiota, and is absorbed from the colon virtually entirely and intact. Following absorption, it is conjugated with glucuronic acid, and primarily excreted in humans as steviol glucuronide *via* the urine (Geuns et al., 2006; Wheeler et al., 2008). Furthermore, while gut microbiota actively act upon steviol glycosides, recent research (Kelly et al., 2017) showed that steviol glycosides, at levels comparable to the ADI, had no impact on the gut microbiome. Cyclamate is also known to be able to be metabolized by the gut microflora to cyclohexylamine, albeit in a small percentage of the population (Renwick, 1986); however, exposure to cyclamate does not appear to adversely affect microbial distribution in the gastrointestinal tract (Matsui et al., 1976).

In general, all LNCS also have a high sweetening potency, which means that dietary exposure will always be low - in milligram amounts - and well below the levels known to be needed by other dietary constituents to elicit a significant impact on the gut microbiome.

6. Conclusions

A review of the literature shows that the gut microbiota is able to be impacted by many dietary factors and is likely changing on a day-to-day basis. The science of how changes to the gut microbiota affect human health is also still developing and not perfectly understood. The data provide clear evidence that the major determinants of changes in

microbiota numbers and phyla result from fairly large changes in intakes of different types of nutrients. The changes to the microbiota most likely result from differing outcomes of metabolic processing of these nutrients, resulting in different intra-colonic metabolic by-products, possible changes in pH and osmotic character, and altered numbers and proportion of phyla.

In contrast, studies of LNCS establish no clear evidence of any adverse effect on the gut microbiota at doses relevant to human use. Results of metabolism and safety studies show no evidence of a likely mechanism for a clinically relevant effect on gut microbiota. The reports of correlated effects primarily stem from studies where doses employed are beyond the possible expected intakes of humans, where there existed significant study design issues that make conclusions of effects questionable, or where data were incorrectly evaluated/interpreted. A class-effect of LNCS on gut microbiota is similarly unsupported by the data and no adverse health effects mediated by alterations of the gut microbiota can be assumed from the published studies on this subject to date. Investigating how changes in the gut microbiome affect human health is not an inconsequential process, and therefore, studies evaluating the impact of food additive/ingredient consumption on the microbiota need to be conducted in relevant animal models or clinical populations at relevant exposure levels and be carefully controlled to account for the presence of numerous confounding factors, including the habitual or background diet. The safety databases that have been developed over decades for acesulfame K, aspartame, saccharin, sucralose, and steviol glycosides, which are structurally unrelated, indicate that these low or no-calorie sweeteners as a group, or individually, pose no safety concerns at their currently approved levels.

Statement of authors' contributions to the manuscript

Alexandra R. Lobach (A.R.L.) and Ashley Roberts (A.R.) were responsible for the design of the review. A.R.L., A.R., and Ian R. Rowland (I.R.R.) wrote the manuscript. A.R. had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest and funding disclosure

Intertek Scientific & Regulatory Consultancy (A.R.L. and A.R.) received financial support from the Calorie Control Council to assist in the preparation of the manuscript. The Calorie Control Council did not contribute to the origination, planning, implementation, or interpretation of this work. The Calorie Control Council did review the content of the complete manuscript; however, A.R. maintained responsibility for the final content. I.R.R. has no conflicts of interest to declare.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.005

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