Studying changes of the human gut microbiome in response to sweeteners using RapidAIM

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Abstract

The human gut microbiome is composed of millions of microbial genes, performing a variety of functions contributing to the host's homeostasis. The disturbance of normal gut microbiome composition and function is associated with diseases. Dietary components including food additives, i.e., sweeteners, play a pivotal role in shaping the human gut microbiome. Despite many studies pointing out the association between sweeteners consumption and health issues, the mechanisms are still unclear and whether sweeteners can directly change the gut microbiome remains largely unknown. In this study, we investigated the responses of the human gut microbiome to 20 common sweeteners, using an approach combining high-throughput in-vitro microbiome culturing and metaproteomics, which provided both taxonomic and functional profile. Sweeteners that belonged to sugar alcohols and glycosides were revealed to induce larger changes in the microbiome metaproteome, as compared with other non-caloric artificial sweeteners (NAS). Changes in taxa abundance were found to be associated with all tested sweeteners at genus level. Clustering analysis based on functional profiling categorized sweeteners into two major clusters, including one cluster comprising 6 sugar alcohols which induced greater functional responses including reduced transport and metabolism of lipid and amino acids, and promoted translation, ribosomal structure and biogenesis, as compared with the other cluster comprising NAS. Taxon-specific functional analyses showed that microbial enzymes from *Lachonospiraceae*, *Faecalibacterium*, *Eubacterium*, *Coprococcus* and *Roseburia hominis* were the major contributors to altered butyrate-producing pathways by sweeteners. This study provides a comprehensive profiling of sweeteners-induced gut microbiome changes, and may serve as a basis to understand sweeteners-relevant health issues from a microbiome point of view.

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List of Abbreviations

ADI	Acceptable daily intake
BMI	Body mass index
CAZymes	Carbohydrate- active enzymes
CD	Crohn's disease
CHO	Carbohydrate
COG	Cluster of orthologous groups
DGGE	Denaturing gradient gel electrophoresis
DTT	dithiothreitol
FISH	Fluorescence in situ hybridization
FOS	Fructooligosaccharide
GC	Gas chromatography
GO	gene ontology
HFD	High-fat diet
HMO	Human milk oligosaccharide
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IGC	Integrated gene catalog
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCA	Lowest common ancestor
LC-MS	Liquid chromatography-mass spectrometry
LFQ	Label-free quantification
LPS	Lipopolysaccharide
MBRAs	Minibioreactor arrays
NAS	Non-caloric artificial sweeteners
NOG	Non-supervised orthologous groups
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCA	Principal component analysis
qPCR	quantitative Polymerase Chain Reaction
RapidAIM	Rapid assay of individual microbiome
rRNA	ribosomal RNA
RS	Resistant starch
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulfate
SHIME	Simulators of the human intestinal microbial ecosystem
T1R	Taste 1 receptor
T2D	Type 2 diabetes
T2R	Taste 2 receptor
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
T-RFLP	Terminal restriction fragment length polymorphism

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1.0 Introduction

1.1 The human gut microbiome and association with human health

The human microbiome refers to the microorganism communities that inhabit the human body, comprising bacteria, archaea, virus, fungi, and other unicellular and multicellular eukaryotes[1-2]. It is present in different parts of the body including surface of skin, gastrointestinal tract, genitourinary tract and respiratory tract[3], with 500-1000 bacterial species estimated to be present in the whole body at any given time[4]. The estimated number of microbial cells in the human body is hundreds of trillions, with the gastrointestinal tract hosting the largest number of microbial cells[3] at 10-100 trillion[5]. The colon is the primary site in the gastrointestinal tract to host microorganisms[5], termed the gut microbiome. The human gut microbiome comprises more than 9.9 million genes, encoding over 170 million protein sequences[6-7]. The human gut microbiome contributes a variety of important functions to the host[8] (Figure 1.1), including immunomodulation[9], resisting pathogen overgrowth[10], modulation of host-cell proliferation[11] and vascularization[12], intestinal endocrine regulation[13], neurologic signaling[14], bone development[15], energy production[16], biosynthesizing a variety of metabolites including neurotransmitters[14], vitamins[17], short-chain fatty acids (SCFAs)[16], and the metabolism of different compounds including bile salts[18], drugs[19] and dietary components[20]. Factors including delivery mode[21], host genetics[22], gut habitat[23], diet[20], physical activity[24], psychological state[25], medication[26] and age[27] have been found to positively or negatively affect the gut microbiome, leading to health issues. Disease types associated with the gut microbiome include gastrointestinal (i.e., inflammatory bowel disease (IBD)[28]), metabolic (i.e., obesity[3], type 2 diabetes(T2D)[29], metabolic syndrome[3]), cardiovascular[30], psychiatric[25],

oncologic[31], autoimmune[32] and neurologic[33]. Causation relationship between dysbiosis and the development of many diseases have been established, including a study by Ridaura et al. showing lean and obese phenotypes were transferrable through fecal transplantation, and rescuing of obesity was achieved by invasion of selected taxa from "lean" to "obese" microbiome[34]. However, the mechanism by which the gut microbiome composition and function led to diseases, which is crucial to understand the relationship between the gut microbiome and human health, and the development of microbiome targeted therapies, remains largely unknown.

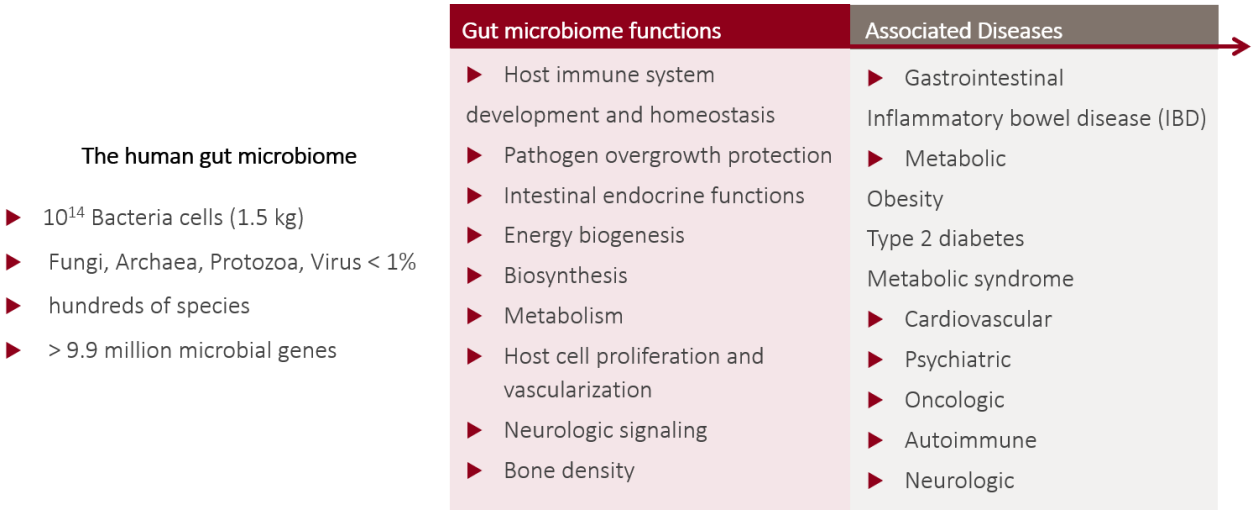


Figure 1. 1. Summary of gut microbiome function and associated diseases.

1.2 Dietary impact on the human gut microbiome

Dietary components, which include carbohydrates, proteins, fats, minerals, food additives and other compounds, have been shown to play major roles in shaping the composition and function of the gut microbiome, therefore the associated health consequences[20, 35]. Dietary modulation of the human gut microbiome occurs across the whole lifespan, with human milk oligosaccharides (HMOs) helping the establishment of the microbiome during infancy[36], whereas diet diversity[37],

quantity[38-39] and temporary diet[40] serves as determinants of the gut microbiome in later stages of life. Both direct and indirect mechanisms have been found through which dietary components change the gut microbiome. In addition, certain bacteria, fungi and viruses can be introduced and incorporated into local gut microbiome through passive transfer[40].

Indirect mechanisms shaping the gut microbiome include dietary components modulating the host's immune system and metabolism. For instance, a combination of vitamin D deficiency and high-fat diet (HFD) resulted in suppressed ileum immune system, leading to dysbiosis, endotoxemia, systemic inflammation, and phenotype including hepatic steatosis and insulin resistance[41]. These phenotypes could be rescued by supplementing alpha-defensins to complement the immune system[41].

Direct mechanisms involve dietary components interacting with microbial cells to directly promote or inhibit their growth and metabolic pathways, which in return affect the health of the host. Common examples include the gut microbiome metabolizing dietary components into metabolites which plays important roles in both the microbial communities and the host, exemplified by the following major food components.

The human genome encodes few lyases capable of degrading dietary polysaccharides. Instead, the bulk of the fermentation of polysaccharides is performed by the microbiome providing energy for degrader species, and metabolites such as SCFAs (acetate, propionate, formate and butyrate) important to the host and for microbial cross-feeding network[20]. Gene content encoding a fructan utilization locus in *Bacteroides spp.* was shown to be indicative of species competitiveness to use fructan as energy source[42], while the absence of polysaccharides in food saw *Bacteroides thetaiotaomicron* turn to the degradation of the host mucus glycans[43], gaining colonization advantages while leading to disrupted intestinal barrier function and enhanced pathogen

susceptibility[44]. SCFAs, especially butyrate, serve as energy source for the colonocytes, histone deacetylases inhibitors and G protein-coupled receptors ligands[45], positively contributing to host health by protecting against inflammation and carcinogenesis[46]. Dietary supplementation of fibre has been associated with beneficial health effects. Menni et al. showed that dietary fibre intake promoted gut microbiome diversity, and negatively correlated with long-term weight gain[47]. In T2D patients, dietary fiber interventions were found to increase the abundance and diversity of acetate and butyrate producing bacteria and to improve T2D[29]. In another study, colon acetate derived from fibre fermentation was found to enter the brain which led to appetite suppression[48].

Dietary fat, especially unsaturated fat, has been associated with metabolic endotoxemia, resulting from promoted lipopolysaccharide (LPS) producing bacteria[20]. In a mice study, HFD was shown to reduce gram-negative *Bacteroides*, gram-positive *Eubacterium rectale-Clostridium coccoides* and bifidobacterial, increase the plasma LPS level and induced endotoxemia. This was concomitant with weight gain in the whole body and several organs, insulin resistance[49], increased intestinal permeability and reduced tight junctions protein levels. Antibiotic treatment reduced the level of LPS and endotoxemia[50].

L-carnitine present at high levels in red meat, can be metabolized by the gut microbiome, producing trimethylamine (TMA), and further metabolized to proatherogenic trimethylamine-N-oxide (TMAO) by the liver. Increased plasma L-carnitine and TMAO levels have been associated with elevated risk of cardiovascular disease and increased incidence of major adverse cardiac events[30]. In humans, vegan diet has been shown to produce less TMAO from L-carnitine compared with omnivore diet, with the genus *prevotella* from the gut microbiome associated with an elevated amount of TMAO[30].

Food additives, i.e., food coloring, preservatives, emulsifiers, fortifying agents and sweeteners, are used for the purpose of preservation or enhancing food quality, and their presence in food have been increasing over the past decades[51]. Food additives are approved as safe to use based on toxicology studies on humans or experimental animals. For the majority of food additives, we do not know whether they induce changes in the gut microbiome first and then affect human health. Some more recent studies of the effects of food additives on the microbiome point to potential human health impacts. For instance, emulsifiers carboxymethylcellulose and polysorbate-80 have been shown to change the gut microbiome composition in mice, leading to low-grade inflammation and metabolic syndrome[52]. Moreover, those induced phenotypes are transferable to germ-free mice by transplanting the disrupted microbiome[52]. As well, maltodextrin, a food thickening agent, was shown to suppress intestinal defense in mice, facilitating Salmonella colonization[53]. These studies point to a need to systematically investigate the effects of food additives on the gut microbiome.

1.3 Sweeteners induce changes in the gut microbiome

Sweeteners are food additives used to increase the sweetness of food, while contributing a negligible amount of energy. They are commonly found in foods including soft drinks, snack foods, mints and gums, and as tabletop sweeteners. In the United States, 25.1% of children and 41.4% of adults were reported to consume sweeteners based on data collected from 2009 to 2012[54], and the popularity of sweeteners has been continuously increasing[55]. They have been recommended as replacements of sugar for better caloric and glycemic control, while preserving the sweetness[56]. Sweeteners can be categorized into non-caloric artificial sweeteners (NAS), which are of high-sweetness intensity and carry little calories on their own, and sugar alcohols (nutritive sweeteners) with comparable sweetness to sucrose, indigestible by human, thus contributing few calories[57].

Despite the proposed health benefits of sweeteners consumption, many studies have associated their intake with the development of diseases and metabolic syndromes, some of which they were initially used to prevent. Consumption of artificially sweetened beverages was positively associated with overweight and obesity over a period of 7-8 years[58]. Similarly, diet soda consumption was associated with increased body mass index (BMI) and percent fat gain in female teenagers[59], and increased left ventricular mass, an indicator of cardiovascular diseases[60]. Both sugar and artificially sweetened beverages were shown to be associated with increased risk of hypertension and T2D[61]. Despite the associations, causal relationship between sweeteners consumptions and diseases have not been fully established, which adds to the difficulty to describe the physiological effects of sweeteners to human[62].

Three major mechanisms through which sweeteners induce metabolic syndromes and diseases have been proposed. Sweeteners can be recognized by taste 1 receptor (T1R) and taste 2 receptor (T2R) located on the taste buds[63], which activate downstream pathways and result in increased intracellular calcium levels and release of neurotransmitters[57]. They could also bind to sweet taste receptors on enteroendocrine cells found in the gut, promoting release of glucose homeostasis relevant hormones, which might contribute to obesity and T2D[57]. Sweeteners consumption may also induce learned responses to sweetness which contribute to energy homeostasis[57]. In addition, sweeteners have been found to induce changes in the gut microbiome, which may in return affect human health.

Notable research on the impact of NAS on the gut microbiome include a study by Suez et al., where saccharin-induced glucose intolerance in healthy volunteers (responders) was found to be transferable to germ-free mice through fecal transplantation, compared with mice receiving fecal samples from human non-responders who did not develop saccharin-consumption induced glucose

intolerance[64]. Taxonomic and functional analysis of altered microbiomes revealed an increase of *Bacteroides fragilis* and *Weissella Cibaria*, and a decrease of *Candidatus Arthromitus*, which potentially contributed to disrupted glucose homeostasis in responders[64]. This study established the causation relationship between saccharin-consumption induced dysbiosis and host glucose intolerance. Sugar alcohols, on the other hand, while being low-digestible to the human body, could reach the colon intact and be fermented by the gut microbiome. They have been associated with gastrointestinal symptoms including flatulence, abdominal discomfort, and laxative effects[65], while there has been a limited number of studies investigating their impact on the gut microbiome.

Experimental studies that investigated gut microbiome responses to sweeteners are summarized in Table 1. Most of the studies have focused on the compositional changes, while techniques including metagenomics and metabolomics have been used by some to reveal functional changes in the microbiome. Moreover, there has been no systematic studies on sweeteners and no consistency of approaches used in smaller scale studies. Therefore, comparison between the limited studies on the effects of sweeteners on the microbiome is difficult.

Table 1. 1. Summary of experimental studies on gut microbiome responses to sweeteners

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Acesulfame K	Composition: Increased *Bacteroides*, *Anaerostipes* and *Sutterella* in male mice; Reduced *Lactobacillus*, *Clostridium*, an unassigned *Ruminococcaceae* genus and an unassigned *Oxalobacteraceae* genus, increased *Mucispirillum* in female mice; Function: Reduced genes in key energy metabolism pathways and polysaccharide hydrolysis and degradation in female mice; increased genes in carbohydrate absorption, metabolism and fermentation pathways in male mice; increased genes involved in LPS synthesis in both gender; gender-specific alteration of lactic acid, succinic acid, pyruvic acid, cholic acid, deoxycholic acid, 2-oleoylglycerol.	mice, in-vivo 16S rRNA sequencing, Gas Chromatography (GC)-MS metabolomics	37.5 mg/kg bw/d	28594855 Bian et al. 2017[66]
Acesulfame K	Did not observe significant change in microbiome composition	mice, in-vivo 16S rRNA sequencing, Denaturing gradient gel electrophoresis (DGGE)	15 mg/kg bw/d (ADI)	28587159 Uebanso et al. 2017[67]
Acesulfame K and sucralose	Increased *Lachnospiraceae* and *Ruminococcaceae*, reduced *Akkermansia muciniphila* in pups but not in mothers.	mice, in-vivo 16S rRNA sequencing	combined ACE and SUC at ADI and 2XADI (15 & 5 mg/kg bw/d)	31281295 Stichelen et al. 2019[68]
Aspartame	Increased *Clostridium leptum* in normal chow and HFD fed mice, increased total bacteria, *Enterobacteriaceae* and *Roseburia spp.* with HFD. Attenuated HFD-induced increase in *Clostridium cluster XI*.	rat, in-vivo 16S rRNA sequencing	60 mg/L in drinking water	25313461 Palmnas et al. 2014[69]

Table 1.1 (*Continued*)

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Aspartame	In the presence of dietary fibre, increased *Bifidobacterium*, *Clostridium coccoides*, reduced *Bacteroides/Prevotella* ratio increased acetic acid, propionic acid and caprylic acid, reduced valeric acid, iso-valeric acid, isobutyric acid	human, in-vitro 16S rRNA sequencing; GC with with flame ionisation detector	2 mg/mL	31853641 Gerasimidis et al. 2020[70]
Neotame	Composition: Reduced alpha-diversity, increased Bacteroidetes, with *Bacteroides* and an undefined genus in family S24-7 as main contributor, reduced Firmicutes, with over 12 genera altered including *Blautia*, *Dorea*, *Oscillospira* and *Ruminococcus*; Function: Enriched amino acid metabolism, LPS biosynthesis, antibiotics biosynthesis and folate biosynthesis pathways, reduced fatty acid metabolism, carbohydrate metabolism, lipid metabolism and ABC transporters. Reduced enzymes involved in butyrate producing pathways.	mice, in-vivo 16S rRNA sequencing	0.75 mg/kg bw/d (2.5 ADI)	29425148 Chi et al. 2018[71]
Saccharin	Fecal transplantation to germ-free mice showed increased *Bacteroides fragilis* (Bacteroidales) and *Weissella cibaria* (Lactobacillales) by 20-fold, and reduced *Candidatus Arthromitus* (Clostridiales).	human, in-vivo 16S rRNA sequencing	5 mg/kg bw/d (ADI)	25231862 Suez et al. 2014[64]
Saccharin	Composition: Increased *Bacteroides,* members of Clostridiales either being under-represented or over-represented. Reduced *Lactobacillus reuteri*; metagenomics showed increased *Bacteroides vulgatus* and decreased *Akkermansia muciniphila*; Function: Increased glycan degradation pathway, much of with attribute to five species including two from *Bacteroides*, as well as pathways in starch and sucrose metabolism, fructose and mannose metabolism, and folate, glycerolipid and fatty acid biosynthesis; reduced glucose transport pathways. Increased ascorbate and aldarate metabolism, LPS biosynthesis and bacterial chemotaxis with HFD	mice, in-vivo 16S rRNA sequencing, metagenomics	5 mg/kg bw/d (ADI)	25231862 Suez et al. 2014[64]

Table 1.1 (*Continued*)

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Saccharin	Composition: Increased Bacteroidetes, reduced Firmicutes; fecal transplantation to germ-free mice showed over-represented *Bacteroides* members, and under-represented Clostridiales members Function: Enriched glycan degradation pathways and sphingolipid metabolism pathways, reduced glucose transport pathways.	mice, in-vitro 16S rRNA sequencing, metagenomics	5 mg/mL	25231862 Suez et al. 2014[64]
Saccharin	Composition: Increased *Sporosarcina*, *Jeotgalicoccus*, *Akkermansia*, *Oscillospira* and *Corynebacterium* after three months, and *Corynebacterium*, *Roseburia* and *Turicibacter* after six months; reduced *Anaerostipes* and *Ruminococcus* after three months, and *Ruminococcus*, *Adlercreutzia* and *Dorea* after six months. Function: Increased pro-inflammatory mediators including LPS biosynthesis orthologs, flagellar assembly ortholog, fimbrial orthologs, bacterial toxin orthologs and multidrug resistance orthologs; Increased daidzein and decreased equol.	mice, in-vivo 16S rRNA sequencing, metabolomics	15 mg/kg bw/d (ADI)	28472674 Bian et al. 2017[72]
Saccharin	Increased Bacteroidetes and Proteobacteria, reduced Firmicutes.	mice, in-vivo 16S rRNA sequencing	5 mg/kg bw/d (ADI)	32316544 Sunderhauf et al. 2020[73]
Sucralose	Reduced relative *Clostridium cluster XIVa* abundance in a dose-dependent manner.	mice, in-vivo 16S rRNA sequencing, DGGE	1.5 mg/kg bw/d 15 mg/kg bw/d (ADI)	28587159 Uebanso et al. 2017[67]
Sucralose	Reduced total anaerobes, bifidobacteria, lactobacilli, *Bacteroides* at lowest dose. Reduced total aerobes at higher dose.	mice, in-vivo colony counting	1.1, 3.3, 5.5 and 11 mg/kg bw/d	18800291 Abou-Donia et al. 2008[74]

Table 1.1 (*Continued*)

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Sucralose	Composition: Increased *Turicibacter, Roseburia, Akkermansia*, one unclassified member in *Clostridiaceae* and in *Christensenellaceae*; reduced *Ruminococcus, Streptococcus, Dehalobacterium*, one unclassified member in *Erysipelotrichaceae* Function: Increased genes related to bacterial pro-inflammatory mediators (including LPS synthesis, flagella protein synthesis), fimbriae synthesis, toxic shock syndrome toxin-1 and shiga toxin subunits.	mice, in-vivo 16S rRNA sequencing	5 mg/kg bw/d (ADI)	28790923 Bian et al. 2017[75]
Sucralose	Increased Firmicutes and reduced Bacteroidetes with both normal chow and HFD, increased *Bifidobacterium* with normal chow	mice, in-vivo 16S rRNA sequencing	3.3 g/kg bw/d (normal chow) 1.5 g/kg bw/d (HFD)	29975731 Wang et al. 2018[76]
Sucralose	Increased members of Proteobacteria in both healthy mice and mice with ileitis,	mice, in-vivo 16S rRNA sequencing	3.5 mg/mL to 35 mg/mL	29554272 Rodriguez-Palacios et al. 2018[77]
Sucralose	Did not change microbiome composition at phylum level.	human, in-vivo 16S rRNA sequencing	11.25 mg/kg bw/d (75% ADI)	31258108 Thomson et al. 2019[78]
Stevioside	Increased *Lactobacillus*, Reduced *Ruminococcaceae*, *Lachnospiraceae.*	Broiler chicken, in-vivo 16S rRNA sequencing	11.9 mg.kg bw/d	32981085 Jiang et al. 2020[79]
Rebaudioside A	Reduced number of *E. coli* HB101 colonies by 83% but not *E.coli* K-12.	E. Coli strain, in-vitro colony counting	2.5% (w/v)	29975731 Wang et al. 2018[76]
Rebaudioside A	With HFD, higher *Akkermansia/Bacteroides* ratio in REB consuming mice than high-carbohydrate diet consuming mice.	mice, in-vivo 16S rRNA sequencing	194 mg/L in drinking water	32317687[80] Xi et al. 2020

Table 1.1 (*Continued*)

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Stevia extract and erythritol	Commercial stevia extract (SN Stevia, containing 10% Rebaudioside D and 90% erythritol) or erythritol alone did not change microbiome composition and alpha diversity.	human, in-vitro 16S rRNA sequencing	6.2 mg/kg bw/d	31869223 Mahalak et al. 2020[81]
Stevia extract and erythritol	SN Stevia, STE and REB did not affect the growth of selected strains	bacterial strains, in-vitro growth curve	SN Stevia 25-100 μg/mL STE and REB 12.5-50 μg/mL	31869223 Mahalak et al. 2020[81]
Cyclamate (Sodium)	Composition: reduced facultative anaerobe count; Function: Reduced β-glucuronidase and nitroreductase activities, increased β-glucosidase and nitrate reductase activities	rat, in-vitro colony counting, bacterial enzyme determination	75 mM (about 15 mg/mL)	2416656 Mallett et al. 1985[82]
Neohesperidine DC and Saccharin	Increase the abundance of *Lactobacillus*, almost entirely contributed by *Lactobacillus* 4228. Reduced Veillonellaceae and Ruminococcaceae family; neohesperidin DC increases expression of lactose, sucrose and fructose transporter in *Lactobacillus* in vitro, independent of its metabolism.	pig, in-vivo; Lactobacillus 4228, in-vitro 16S rRNA sequencing, qPCR	0.015% (w/w) mixed sweetener in food; 0.5 mM NDC in vitro (about 0.3 mg/mL)	24382146 26058469 Daly et al. 2014[83] & 2016[84]
Sorbitol	Increased butyrate concentration in the colon and cecum. Increased *Lactobacillus sp.* AD102 and *Lactobacillus reuteri* in feces, colon and cecum.	mice, in-vivo DEEG, quantitative Polymerase Chain Reaction (qPCR)	water with 10% (w/v) sorbitol	17825531 Sarmiento-Rubiano et al. 2007[85]
Mannitol	Significant increased butyrate level.	human, in-vitro ion-exclusion HPLC	5 mg/mL	27810878 Sato et al. 2017[86]
Isomalt	Significantly increased Atopobium group and Actinobacteria; reduce *Roseburia intestinalis* and *Bacteroides*; reduced beta-glucosidase activity.	human, in-vivo Fluorescence in situ hybridization (FISH) enzyme activity assay	5 g/d to 30 g/d	16441915 Gostner et al. 2007[87]

Table 1.1 (*Continued*)

Sweetener	Description of gut microbiome responses	Tested model and analysis technique	Dose tested	References with PMID
Maltitol	Significantly increased Bifidobacteria, *Bacteroide*, *Eubacterium rectale*, *Ruminococcus flavefaciens* and *Atopobium*.	human, in-vivo FISH	22.8, 34.2 and 45.6 g per day	20370946 Beards et al. 2010[88]
Lactitol	Reduced *Enterobacteriaceae.*	cat, in-vitro FISH	2 mg/mL	25367521 Pinna et al. 2014[89]
Lactitol	Reduced *Bacteroides*, *Clostridium*, coliforms and Eubacterium, increased *Bifidobacterium*, *Lactobacillus* and *Streptococcus*.	human, in-vivo Colony counting	20 g per day	9145445 Ballongue et al. 1997[90]
Lactitol	A combination of lactitol and *Lactobacillus acidophilus* NCFM increased Bifidobacteria and Lactobacilli level, rescued decline of Blautia coccoides–Eubacterium rectale bacterial group and Clostridium cluster XIVab.	human, in-vivo qPCR	10 g per day	21853265 Bjorklund et al. 2012[91]
Lactitol	Significantly increased *Bifidobacterium*.	human, in-vivo Colony counting	5 g and 50 g per day	17623227 Finney et al. 2007[92]
Xylitol	Reduced *Bacteroides* at 200 mg/kg bw/d with both normal chow and HFD, increased Firmicutes, *Prevotella*, *Firmicutes*/*Bacteroides* and *Prevotella*/*Bacteroides* ratio with HFD.	mice, in-vivo DEEG, qPCR, 16S rRNA sequencing	40 and 200 mg/kg bw/d	28708089 Uebanso et al. 2017[93]
Xylitol	Increased Gram-positive bacteria after consumption, reduced Gram-negative bacteria after 6-10 h.	human, in-vivo Gram-staining and colony counting	30 g	4076932 Salminen et al. 1985[94]
Xylitol	Increased butyrate level, increased *Anaerostipes hadrus*, *Anaerostipes caccae*, *Bacteroides plebeius, Bacteroides fragilis and Eubacterium contortum.* Xylitol was largely utilized by *A. caccae* to produce butyrate.	human, in-vitro DEEG, qPCR, ion-exclusion HPLC	5 mg/mL	27810878 Sato et al. 2017[86]
Xylitol	Reduced *Bacteroides*, increased *Bifidobacterium* and *Prevotella*.	mice, in-vivo Terminal restriction fragment length polymorphism (T-RFLP)	5% xylitol in diet	24336061 Tamura et al. 2013[95]

1.4 Studying the microbiome with in vitro culturing based approaches

Different in-vitro approaches have been applied to study the gut microbiome, microbiome-host interaction and microbiome responses to perturbations and treatments. Compared with in-vivo models, the advantages of in-vitro approaches include: 1. Having controllable microbial composition and culturing environment, which allows direct comparison between different groups, compared with unique and host-specific microbiomes in in-vivo models[96-97]; 2. Allowing study solely on the microbiome communities without host interference, and reveals the direct effect of treatment to the microbiome, compared with end-point measurement in in vivo models[97]; 3. Cost effective and easier ethical approval, thus more freedom in experimental design[97].

In-vitro models comprising both the microbiome and host cells can be used to study microbiome-host interaction by controlling changes in either the microbiome or the host. The human gut-on-a-chip model was described by Kim et al.[98], where commensal microbes were cultured with live human intestinal epithelial cells on a microfluidic device. The environment of the gut was further mimicked by physiologically relevant luminal flow and mechanical deformations to form intestinal villi[98]. By controlling mechanical deformations of epithelial cells to simulate IBD condition, four proinflammatory cytokines were found to be produced upon stimulation by immune cells and LPS, resulting from bacterial overgrowth. Being an effective tool to study the microbiome-host tool in vitro, this model still had disadvantages including long stabilization period (2 weeks), relatively difficult to build, and only selected known commensal microbes were cultured with this model.

Stool samples have been used in in-vitro culture models to serve as a representation of the gut microbiome in both continuous culturing and batch culturing. Simulators of the human intestinal microbial ecosystem (SHIME)[99] is a dynamic and multicompartment gastrointestinal tract simulator based on continuous culturing. The model includes five interlinked vessels representing

the stomach, small intestine, and ascending, transverse and descending colon. Culture feed composed of essential nutrients and pancreatic juice are constantly flowing into the system allowing the stabilization of the cultured fecal microbiome, usually including an initial stabilization period of two to three weeks to reach compositional and functional stable[99]. This model was able to reproduce region specific microbial communities including saccharolytic microbes residing in proximal regions and mucin-degrading microbes residing in distal regions[99]. A twin-vessel based single-stage chemostat distal gut model was described by McDonald et al. including culturing of fecal inoculum from healthy donors with a maximal stabilization period of 36 days[96]. This model was able to show distinct microbial compositions between individual microbiomes in both the steady state chemostat microbiome communities and the initial fecal inoculum[96]. A higher throughput continuous-flow culture model, termed minibioreactor arrays (MBRAs) was described by Auchtung et al., which required less culturing volume, shorter stabilization period, and up to 48 reactors were able to run in one single anaerobic chamber[100]. Despite continuous-flow culture of fecal samples allowing studying the gut microbiome under simulated gut environment with controlled conditions for extended periods, it requires a stabilization period during which the microbial compositional changes, resulting in an inaccurate representation of the seed microbiome. For instance, increased Bacteroidetes/Firmicutes ratio, enriched *Clostridium* cluster IX and less represented *Clostridium* cluster XIVa have been observed in the studies described above[96, 99]. Different batch culturing methods, on the other hand, require culturing of stool samples with culture media without a stabilization period, allowing reduced culturing period commonly in 24 hours to 72 hours, and serve as efficient approaches to study microbiome responses to treatments[101-102]. Kim et al. investigated different stool culture medium and showed a low-concentration carbohydrate medium was able to maintain stool bacteria

population that were metabolically active, at an inoculation ratio of 3% and 1% supplementary fecal supernatant, the culturing condition was thus suitable for culturing microbiome with xenobiotics[103]. However, dependencies of batch culturing results on the culture medium have been reported, with inconsistent microbial responses under different culture conditions. Long et al. investigated gut microbiome responses to the same prebiotic formula with batch fermentation under both oligotrophic and eutrophic culture medium, where the major microbial responders were shown to be different between two conditions, with opposite responses observed for some operational taxonomic units (OTUs)[102].

High throughput screening based culturing approaches provides another effective way to study the impact of treatments on the gut microbiome on a large scale. Study by Maier et al.[104] included screening of 1,197 compounds against 40 representative human gut bacterial isolates, and optical density of each culture was measured over time as a proxy for bacterial growth. 24% of human-targeted drugs of all therapeutic classes, in addition to antibacterial drugs, were found to inhibit the growth of at least one tested strain, which indicated the potential risks of antibiotic resistance caused by non-antibiotics[104]. This study is an example of comprehensive drug antimicrobial effect investigation based on induced strain level growth change[104]. However, microbiome responses to treatments involve contribution from members of the whole community, which was not represented in this study, and biomass of individual taxa is not indicative of associated functional changes.

Rapid Assay of Individual Microbiome (RapidAIM) has been developed as an assay to study the responses of the gut microbiome upon drug treatment in a high throughput manner[105]. It involves an in-vitro stool sample culturing model which has been shown to preserve the compositional, functional and taxon-specific functional profile of the gut microbiome before and after culturing,

while increasing the viable bacteria counts by four-fold[101]. Microbiome responses to metformin in this model was shown to have a high degree of correlation with in-vivo study[101]. This model was designed as a 96 deep-well plates based approach, which allows large scale compound screening to study microbiome-compound interaction. Study with this model on gut microbiome responses to 43 drugs revealed antibacterial-like activities were associated with 35 non-antibiotic drugs[105]. Metaproteomics analysis revealed compound specific functional responses, and taxon-specific functional responses. The results revealed that many bacterial function shifts occurred without changes in taxa abundance[105]. RapidAIM has also been used to study the impact of berberine, a common non-prescription drug in Asia for diarrhea treatment, and berberine structural analogs on the gut microbiome, which revealed major taxonomic contributors of berberine and analogs induced functional changes[106]. Induced changes in butyrate producing pathways were shown to be indicative of butyrate concentration[106]. Using this approach, significantly altered microbial functions induced by different types of resistant starches (RS) have been identified, as well as associated taxa[107]. Functionally independent and co-regulated microbes were further revealed through network analysis[107]. Overall, RapidAIM could serve as a robust and reproducible approach to study the gut microbiome responses to treatments in a systematic manner.

1.5 Studying the microbiome with metaproteomics

Different meta-omic approaches have been used to investigate the composition and functional activities of the microbiome. 16S ribosomal RNA (rRNA) sequencing has been the most common technique to study microbiome compositions by associating 16S rRNA genes with OTUs[108], and has been applied in most data generation during the first stage of the Human Microbiome Project[5]. Metagenomics, which is based on whole-genome shotgun sequencing, can identify taxa more accurately at a lower level, and functional analysis of identified genes could also provide

information about microbiome functional activities[109-110]. Metatranscriptomics, based on sequencing microbiome mRNAs, provides information about genes that are actively expressed, a more accurate representation of microbiome functions[111]. However, problems with microbiome functional analysis with the methods above include levels of gene and mRNA being not sufficient to predict levels of proteins[112-113], which are directly associated with microbial function.

Metaproteomic approaches, which directly measure the levels of microbiome and host proteins simultaneously, can provide information about microbiome function and metabolic activities at a much deeper level, compared with other omic approaches[114]. Liquid chromatography-mass spectrometry (LC-MS) based metaproteomic studies generate mass spectral data of extracted peptides. Database searching is done against specialized microbiome databases, generated from pseudo-metagenomes of specific microbiomes, i.e., a catalog including close-to-complete sets of genes for most human gut microbes[6]. Optimized database-searching strategy have been used to deal with the large computational workload and low sensitivity resulting from the large size of those microbiome databases[115], i.e., generating target-limited databases during initial database search for more accurate subsequent identification and quantification[116]. Taxonomic assignment of metaproteomic data is usually performed at the peptide level using the lowest common ancestor (LCA) algorithm, while protein-centric and peptide centric functional annotation are both used, despite generating distinct result[115]. Tools including Unipept[117] or MetaGOmics[118] allow peptide-centric taxonomic and functional analysis, while tools including MEGAN[119] and MetaProteomeAnalyzer[120] provide protein-centric analysis.

Metaproteomics approaches have been used to study the gut microbiome of individuals with different diseases, which revealed altered microbiome taxonomic and functional profile, as well as host-microbe interactions compared with healthy individuals. In inflammatory bowel diseases,

metaproteomics identified microbiome and host derived protein biomarkers, as well as their associated functions[121]. Microbial pathways associated with oxidative stress, as well as host defence proteins present in extracellular vesicles were increased in IBD patients[28]. Metaproteomics also revealed altered microbial metabolic capacities in 14 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in patients with liver cirrhosis, with positive correlation between pathway coverage and disease severity, indicative of the role of the gut microbiome in the development of liver cirrhosis[122]. Changes in taxon specific metabolic pathways have been found by metaproteomics in HIV patients undergoing antiretroviral therapy, with *Succinivibrionaceae* exclusively expressing a protein transporter domain, potentially promoting immune recovery[123]. Metaproteomic approaches have also been used in in-vitro culturing models including RapidAIM to investigate microbial responses to different treatments[105-107, 124], which revealed rich information of compositional, functional and taxon-specific functional changes in the gut microbiome upon treatment.

1.6 Hypothesis and objective

I hypothesize that common sweeteners can modulate the human gut microbiome taxonomy and function, potentially affecting human health.

Objective: To profile the direct effect of common sweeteners on the human microbiome, and in particular, taxonomic and functional changes.

2.0 Materials and Methods

2.1 Sweeteners and the determination of concentrations

The concentration of sweeteners used in the assay was determined based on their consumption level in the general public, the acceptable daily intake (ADI), and the proportion of consumed sweeteners that could reach the colon (Supplementary Figure S2).

For sweeteners with defined ADI, the concentration used would represent a consumption level 100% of the ADI (cADI), calculated from the following equation:

$$\frac{\text{ADI (mg kg}^{-1}\text{ day}^{-1}) \times \text{average volunteers body weight 70.3 kg}}{(\text{weight of culture system 1 g})^{-1} \times \text{weight of colon content 200 g}} \times 1\ \text{ml}^{-1} \times \text{MW}^{-1}$$

$$\times \text{proportion that reaches the colon (\%)}$$

Where it was assumed that the proportion of consumed sweetener that reached the colon was distributed evenly in 200 g of colon content[105], the amount of consumed sweetener was based on the averaged weight of recruited volunteers at 70.3 kg. In the case of aspartame, salt of aspartame-acesulfame and thaumatin, the sweeteners were completely metabolized before reaching the colon [125-126], resulting in cADI of 0. They were tested at a standardized concentration of 2 mg/mL in an assumed circumstance where they reached the colon, despite not occurring naturally. Thaumatin was also tested at cADI (without considering the proportion reaching the colon), assuming all consumed thaumatin at this level reached the colon. Since the cADI of advantame exceeded its solubility and the ADI would represent a consumption level much higher than that in the general public[127], advantame was tested at 2 mg/mL, about 1/5 of cADI. Acesulfame K, saccharin and

neotame had cADI much lower than 2 mg/mL, they were tested both at cADI and 2 mg/mL for comparison between sweeteners.

For sweeteners without defined ADI, in the case of all sugar alcohols, the concentration used was standardized to 2 mg/mL, which would represent consumption levels lower than those in the general public[128-130].

For the full list of sweeteners names, abbreviations, suppliers and concentrations in culture medium, see Supplementary Table S1. One day before culturing, 20X stock solutions of all compounds were prepared with sterile phosphate-buffered saline (PBS, cat# 311-010-CL, Wisent inc., Canada). The stock solutions were filtered with 0.22 μm filter (CELLTREAT, USA) to remove potential bacterial contaminants from sweeteners solid, with exceptions of advantame, neotame, neohesperidin dihydrochalcone, aspartame, salt of aspartame-acesulfame, thaumatin and FOS due to incomplete dissolution. Stock solutions were stored at -20ºC until the culturing experiment.

2.2 Human stool sample collection and culturing

Stool sample collection protocol used in this study was approved by the Ottawa Health Science Network Research Ethics Board at the Ottawa Hospital (# 20160585-01 H). Stool samples from 5 healthy volunteers (age from 23 to 48 y, 3 males and 2 females) were included in this study. Exclusion criteria included diagnosis of irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) or diabetes; antibiotic use or gastroenteritis episode in the last 3 months; use of pro-/pre-biotic, laxative, or antidiarrheal drugs in the last month; or pregnancy. Volunteers included in this study were also self-accessed as non sweetener consumers. On the date of sample collection, each volunteer was given a collection kit on-site, including a dry plastic container which stool

sample was voided into. The sample container was then immediately transferred into an anaerobic workstation (5% H_2, 5% CO_2, and 90% N_2 at 37°C). A 20% w/v slurry of stool sample was made by mixing with sterile pre-reduced PBS containing 10% v/v glycerol and 1 g/L L-cysteine (freshly added), before homogenized with a vortex mixer and filtered through gauze. The filtered slurry aliquots were stored at -80ºC until future use.

In this study, a previously established microbiome in vitro culturing protocol was followed with a few modifications adapted to frozen fecal samples[105]. Briefly, the frozen fecal samples were taken out from -80ºC freezer and thawed at 37ºC immediately using water bath, before transferred into the anaerobic workstation, homogenized with vortex, and inoculated into 96 deep-well plates prepared shortly before containing optimized microbiome culture medium and sweeteners to be studied. In each well, 100 µl 20% w/v slurry of stool sample was mixed with 900 µl medium and 50 µl sweetener stock solution, to make a total volume of 1 mL and 2% w/v inoculation ratio. The components in the culture medium was: 0.45 g L^{-1} monopotassium phosphate, 0.45 g L^{-1} dipotassium phosphate, 0.9 g L^{-1} sodium chloride, 0.09 g L^{-1} magnesium sulphate heptahydrate, 0.09 g L^{-1} calcium chloride, 2 g L^{-1} peptone water, 2 g L^{-1} yeast extract, 4 g L^{-1} sodium bicarbonate, 0.25 g L^{-1} sodium cholate, 0.25 g L^{-1} sodium chenodeoxycholate, 2 mL L^{-1} Tween 80, 4 mg L^{-1} hemin, 10 mg L^{-1} vitamin K1, 4 g L^{-1} porcine gastric mucin, 0.5 g L^{-1} L-cysteine, as described previously[101]. The medium was prepared sterile and adjusted to a pH of 7.55 using 1 M hydrochloric acid. It was pre-reduced overnight in the anaerobic workstation before the culturing experiment. After the plates were set, they were covered with sterile silicone mats, which contained holes for each well pierced with a sterile syringe needle, allowing gas exchange. The plates were shaken at 500 rpm on shakers (MS3, IKA, Germany) in the anaerobic workstation for 24 hours.

2.3 Metaproteomic sample preparation

After culturing, the plates were taken out from the chamber and pelleted immediately by centrifugation at 2272 g/ 4ºC for 45 min. The supernatants were transferred into new plates, and the pH of supernatants were measured with a pH meter (Mettler Toledo, USA). The bacterial pellets were resuspended with 1 mL ice-cold PBS, and pelleted again by centrifugation at 2272 g/ 4ºC for 45 min. The bacterial pellets were resuspended with 1 ml ice-cold PBS and centrifuged at 300g / 4ºC for 5 min to remove large non-bacterial debris. The supernatants were transferred into new plates, and pelleted by centrifuged at 2272 g/ 4ºC for 45 min. The supernatants were removed, with bacterial pellets stored at -80ºC until LC-MS sample preparation.

The bacterial pellets were resuspended in a bacterial lysis buffer containing 4% w/v sodium dodecyl sulfate (SDS), 8 M urea, 50 mM tris-HCl and pH of 8.0. For each 10 mL lysis buffer, it also contained one dissolved PhosSTOP^{TM} tablet (MilliporeSigma) and one dissolved cOmplete mini^{TM} tablet (MilliporeSigma). The resuspensions were transferred into microplates and sealed with caps for better sonication. Sonication lysis of bacterial cells were performed using water bath sonicator (Qsonica, USA) at 8ºC, 50% amplitude, for a total lysis time of 10 min with a 10 s on 10 s off working cycle to prevent overheating of samples. After lysis was completed, the lysed mixtures were transferred into another set of tubes and centrifuged at 16000 g/ 8ºC for 10 min to remove cell debris. The concentration of supernatants containing bacterial protein was measured with DC assay (Bio-Rad Laboratories, USA), with each sample measured in triplicates. The concentration measured here was used to calculate the total biomass of the microbiome after culturing.

Proteins from each sample were precipitated by mixing lysis supernatant with a protein precipitation buffer containing 50% v/v acetone, 50% v/v ethanol, and 0.1% v/v acetic acid, with a volume ratio of 1:5. The precipitation buffer was stored at -20ºC for more than 24 hours before using. The precipitating proteins were kept at -20ºC for more than 24 hours.

The precipitated proteins were collected by centrifugation at 16000g/ 4ºC for 25 min, supernatants were then removed. The pelleted protein was washed with acetone three times to remove the remaining protease inhibitor and SDS, by repeating the following procedure three times: 1 ml -20ºC acetone was added, the protein was resuspended with the help of water bath sonication (Qsonica, USA) and pelleted by centrifugation at 16000g/ 4ºC for 25 min, with supernatants removed.

Protein pellets collected after the final acetone wash step were dissolved in a buffer containing 6 M urea and 50 mM ammonium bicarbonate. The protein concentrations of these solutions were measured with DC assay (Bio-Rad Laboratories, USA), with each sample measured in triplicates. For each sample, a volume which would give 50 µg protein was calculated, a protein solution with a concentration of 1 mg/mL was then prepared for each sample by transferring the determined amount of protein and topping up to a total volume of 50 µL. Protein reduction was performed by adding 2 µL 250 mM dithiothreitol (DTT) to make a final DTT concentration of 10 mM. Samples were shaken at 850 rpm/56 ºC for 30 min. Protein alkylation was then performed by adding 2 µL 500 mM iodoacetamide (IAA) to make a final IAA concentration of 20 mM. Samples were kept at dark for 40 min at room temperature. A 10-fold dilution of the solutions was made by adding 450 µL 50 mM ABC, to make the concentration of urea to approximately 0.6 M. Amount of trypsin (Worthington Biochemical Corp., Lakewood, NJ) required for protein digestion of each sample was based on a mass ratio of trypsin : protein = 1:50. 4 µL freshly prepared trypsin water solution

with a concentration of 0.25 μg/μL was added into each sample. Trypsin digestion was performed at 850 rpm/37 ºC on shakers (Eppendorf, Germany) for 19 hours.

Trypsin digestion was stopped by adding 50 μL 10% v/v formic acid to make a final pH of 2 to 3. Desalting was performed on in-house made 96-channel filter tip plates packed with 5 mg 10-μm C18 resin (Dr. Maisch GmbH, Ammerbuch, Germany). The resin was firstly activated by adding acetonitrile to the filter tips and spin down for three times, then balanced by adding 0.1% v/v formic acid and spin down for two times. Tryptic peptides were loaded onto the resin by adding to the filter tips and spin down the liquid, and repeated until all samples were loaded. The resin was washed by adding 0.1% v/v formic acid and spin down for two times. Peptides were eluted from the resin with an elution buffer containing 80% v/v acetonitrile and 0.1% v/v formic acid. 130 μL elution buffer was added into the filter tips, spined down, and repeated for another time resulting in 260 μL eluted peptide collected for each sample. The samples were then freeze-dried and stored at -20ºC before high-performance liquid chromatography (HPLC)-MS analysis.

2.4 HPLC-MS analysis

Each dry peptide sample was re-dissolved using 200 μL 0.1% v/v formic acid to make the final peptide concentration approximately 0.25 μg/μL. Thorough vortexing were required for complete dissolution and the samples were centrifuged at 16000 g/room temperature for 5 min to remove potential debris before transferred onto the loading plate of an Agilent 1100 Capillary LC system (Agilent Technologies, San Jose, CA). 4 μL samples which corresponded to 1 μg of peptide were loaded. Column used for peptide separation was of 75 μm inner diameter and 50 cm long, packed with reverse phase C18 resin (1.9μm/120 Å ReproSil-Pur C18 resin, Dr. Maisch GmbH, Ammerbuch, Germany). A 90-min gradient was used involving acetonitrile changing from 5% to

30% v/v at a flow rate of 200 nL/min. Solvent A was composed of 0.1% v/v formic acid, solvent B was composed of 0.1% v/v formic acid and 80% v/v acetonitrile. MS analysis was performed with a Q Exactive mass spectrometer (ThermoFisher Scientific Inc.). Full MS scan was performed from 300 to 1800 m/z, data-dependent MS/MS scan was performed for the 12 most intense ions. MS and MS/MS scan were performed with resolution of 70000 and 17500, respectively. Samples were loaded in a randomized order. In this study, 197 samples were analyzed over a period of 23 days. Result file containing the spectra data for each sample was generated as *.RAW files.

2.5 Protein identification, quantification and data analysis

Metalab software (version 1.2.0) was used in the database search, which provided an automated mass spectra analysis pipeline including peptide/protein identification and quantification, peptide taxonomy assignment, protein functional annotation[131]. The search was performed against a database based on the integrated gene catalog (IGC) which included close-to-complete sets of genes for most gut microbes[6]. Carbamidomethyl (C) was set as fixed modification, and protein N-terminal acetylation (protein N-term) and Oxidation (M) were set as variable modifications.

Analysis of changes in the gut metaproteome was based on the quantified protein groups. Label free quantification (LFQ) intensities of each protein group was first normalized by the estimated size factor calculated using the R package "DESq2"[132]. Bray-Curtis distance between samples were calculated based on the normalized intensities using the R package "vegan"[133]. For principal component analysis (PCA), protein groups were then filtered based on criteria that the protein group appears in at least one treatment in at least 3 out of the 5 tested microbiomes (60%). The intensities were then log10 transformed and PCA was performed using the R package "stats". To

remove the inter-individual metaproteome variation, the data was further processed using a Combat algorithm[134] on iMetalab.ca[135].

Taxonomy assignment of each peptide was performed based on the LCA, with abundance of each taxon calculated by summarizing the intensities of all peptides assigned to this taxon. Relative abundance of taxa on a specific taxonomic rank was calculated by normalizing to the summed abundance of all taxa on this rank. For comparison of absolute taxa abundance between samples, relative abundances were multiplied by the total microbial biomass calculated using protein concentration data. Fold changes were calculated between sweetener-treated samples and PBS control from the same microbiome.

Functional annotation of identified protein groups was carried out in Metalab software, the result included annotation to cluster of orthologous groups (COG), non-supervised orthologous groups (NOG), KEGG enzymes and gene ontology (GO). The relative abundance of each annotation was calculated based on the summed LFQ intensities of all assigned protein groups.

Clustering of sweeteners was based on the fold change of relative COG intensities, averaged across all tested sweeteners. The Euclidean distance between sweeteners was calculated and the clustering was performed with the "ward.D" method, using the R package "stats". Bootstrapping evaluation[136] of the two major clusters was performed using the R package "fpc"[137] with the number of resampling runs being 100.

Taxon-specific functional analysis was performed by combining peptide taxonomic assignment with peptide functional annotation. Peptide taxonomic assignment to LCA was performed using Metalab, as well as peptide protein assignment and protein functional annotation. In the dataset of this study, 62.8% peptides were matched to multiple proteins, while in 95.4% of those peptides,

the matched proteins have the same function with the razor protein. In this study, we assumed that the function of each peptide was the same as the function of the razor protein this peptide was matched to. In this case, each peptide had one matched LCA and one matched functional annotation.

2.6 Data visualizations

Box plots, scatterplots, and hierarchical clustering dendrograms were visualized using R packages "ggplot2"[138], "scatterplot3d"[139], and "factoextra"[140], respectively. Heatmaps were generated with R packages "pheatmap"[141], hierarchical clustering of both row and column was with the default setting.

2.7 Statistical analysis

To assess whether a microbial response was significant considering all tested microbiomes, Wilcoxon signed rank test was performed using the R package "stats", which was a non-parametric statistical hypothesis test suitable for microbiome study.

3.0 Results

3.1 Ex vivo cultured human gut microbiome altered by sweetener treatment

In this study, we included 20 common sweeteners, which covered all sweeteners approved by Health Canada (HC)[142], the U.S. Food and Drug Administration (FDA)[143], and the European Food Safety Authority (EFSA)[144] as food additives. Among the 20 sweeteners, 12 are classified as non-caloric artificial sweeteners (NAS) with diverse chemical properties and high sweetness intensities, and 8 are classified as sugar alcohols, which are carbohydrates with low digestibility[65]. To evaluate the effects of sweetener against human gut microbiome, we used the previously established RapidAIM workflow which incorporated in-vitro culturing and metaproteomics[105]. Briefly, fecal samples from 5 healthy volunteers were cultured individually in the presence of each sweetener in an anaerobic workstation for 24 hours (Figure 3.1A), positive control of the experiment included fructooligosaccharide (FOS), and kestose (KES), which are known to induce extensive responses by the human gut microbiome[145-146], and common dietary sugar glucose (GLU). Phosphate-buffered saline (PBS), which was used to dissolve all the sweeteners before inoculating into the culture media, was included as negative control. After culture, the microbial cells were collected and processed through a sample preparation workflow, before being subjected to LC-MS analysis. 1,898,894 MS/MS spectra were identified from 197 samples with an average identification rate of 33.9%, which led to 81,850 microbial peptides and 20,503 protein groups quantified.

Eight sweeteners were shown to alter the metaproteome across all five gut microbiomes, as treated samples had significantly larger Bray-Curtis distance compared with negative control (Figure 3.1B). The sweeteners inducing altered metaproteome included five sugar alcohols: maltitol (MAL), isomalt (ISO), lactitol (LAC), xylitol (XYL) and hydrogenated starch hydrolysis (HSH).

As well, three NAS including stevioside (STE) and rebaudioside A (REB) (two major glycoside components of the commercially available sweetener stevia), and monk fruit extract (MFE) (primarily composed of glycoside called mogrosides) altered the metaproteome. Principal component analysis (PCA) of all samples based on protein groups LFQ intensities showed as expected strong inter-individual variations (Figure 3.1C). We applied a Combat transformation algorism[134] to remove the individual variance in the metaproteome data. PCA of data after this transformation showed samples forming clusters by treatment, exemplified by the PBS controls, represented by squares (Figure 3.1C). Corresponding to the Bray-Curtis distance data, individual PCA of the 8 sweeteners, plus FOS as positive control, showed good separation with the PBS control (Figure 3.1D), as compared with the remaining sweeteners (Supplementary Figure S3).

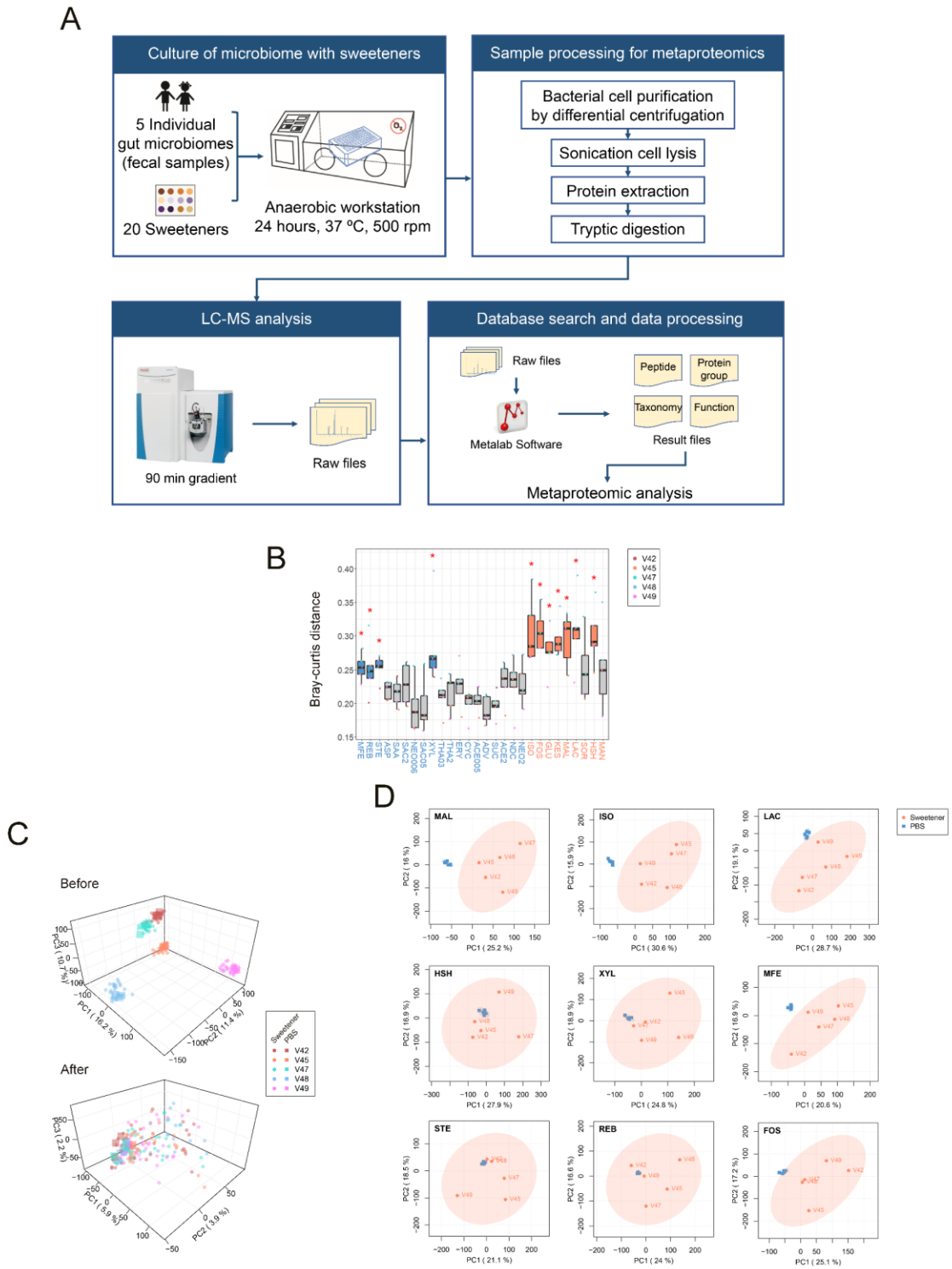


Figure 3. 1. Sweeteners induce metaproteomic changes in the gut microbiome

(A) Workflow combining in-vitro culturing and metaproteomics to study the effect of common sweeteners on the gut microbiome.

(B) Bray-Curtis distance of protein groups LFQ intensities between sweetener-treated groups and PBS control for each microbiome, boxes span the interquartile range, jitter colors indicate microbiome number. *$p < 0.05$, Wilcoxon rank sum test between each group and the average distance among control sample triplicates.

(C) PCA plot generated from protein groups LFQ intensities of all samples, before and after Combat transformation to remove inter-individual variances.

(D) Individual PCA plots of sweeteners of significant changes (indicated by Figure 1 (B)) and positive control FOS, using data after Combat transformation.

3.2 Sweetener induces taxonomic changes of ex vivo gut microbiome

The effect of sweeteners on total microbial biomass was revealed by measuring the total protein concentration in each sample after microbial cell lysis using DC assay (Supplementary Figure S4). For most sweeteners, their effect on the individual microbiomes varied, while ISO and thaumatin at 2 mg/ml (THA2) increased the total biomass in all five microbiomes.

Absolute abundance of each genus was calculated from the relative total peptide abundance of each genus measured by MS and the total microbial biomass of each sample (Figure. 3.2). Despite all sweeteners showing significant effects on the abundance of selected genus, most sugar alcohols (excluding erythritol (ERY)), STE, and all positive controls showed greater effect on microbiome composition by forming separate cluster compared with the remaining sweeteners, mostly NAS (Figure. 3.2). The features of sugar alcohol and STE induced compositional changes included promoted *Butyricicoccus* (XYL, STE, LAC), *Bifidobacterium* (ISO, sorbitol (SOR)), *Collinsella* (ISO, XYL), *Marvinbryantia* (MAL, XYL, ISO, SOR, LAC), *Blautia* (MAL, XYL, ISO), *Ruminococcus* (MAL, ISO, STE, LAC), *Phascholarctobacterium* (all), *Prevotella* (ISO, HSH), *Parabacteroides* (MAL, ISO, STE), *Dialister* (MAL, ISO), *Catonella* (XYL, ISO), and reduced

Flavonifractor (mannitol (MAN), LAC), *Roseburia* (XYL, HSH, STE, MAN), *Clostridium* (XYL, HSH, LAC), *Dorea* (HSH, STE). Several featured alterations by other NAS including promoted *Butyricicoccus* by salt of aspartame-acesulfame (SAA), REB, and THA at both 0.3 and 2 mg/ml, promoted *Bifidobacterium* by advantame (ADV), aspartame (ASP), SAA and THA03, reduced *Dorea* by saccharin (SAC) at both 0.5 and 2 mg/ml, acesulfame (ACE) at 0.05 mg/ml, neotame (NEO) at 0.06 mg/ml, reduced *Lactobacillus* by cyclamate (CYC), neohesperidin dihydrochalcone (NDC), ACE005 and NEO006.

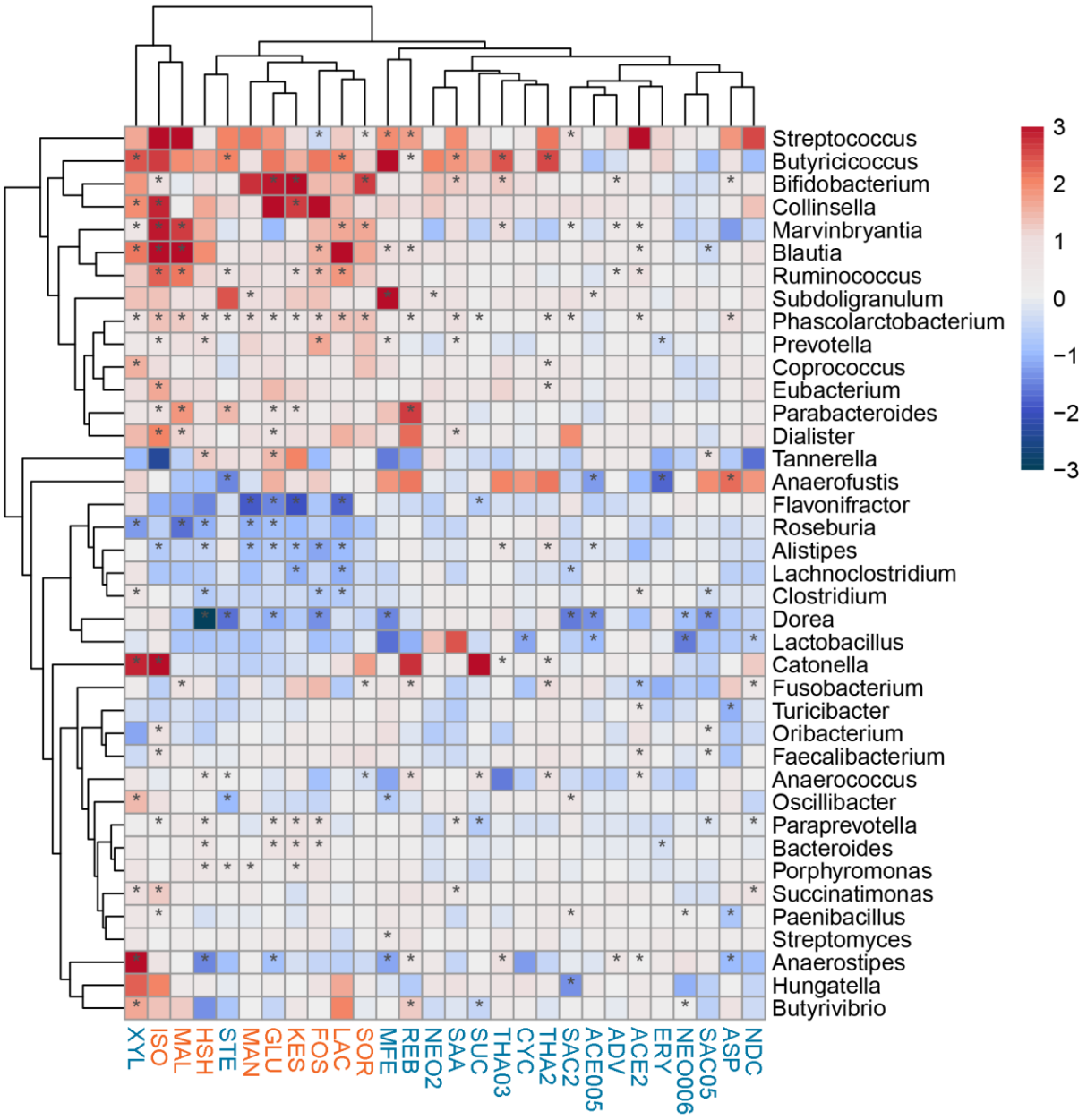


Figure 3. 2. Sweeteners induced gut microbiome compositional changes

Heatmap showing log2 fold change of absolute genus abundance of sweetener-treated samples versus PBS control. For each treatment, the averaged data of all microbiomes were used for coloring and clustering. *p < 0.05, Wilcoxon rank sum test. Genera that are detected in PBS controls in at least 4 out of the 5 microbiomes are shown.

3.3 Functional metaproteomics clusters the sweeteners into two groups

Of the identified protein groups, 93.5% had COG functional annotation. Sweeteners were categorized into two major clusters: "NAS" cluster and "CHO" cluster, based on the summed intensity of each COG (Figure. 3.3A). In the "NAS" cluster, all NAS were included, plus sugar alcohol ERY and XYL. Notably, three glycoside, MFE, STE and REB, formed a sub-cluster, and XYL formed a single element sub-cluster. The "CHO" cluster included all remaining sugar alcohols and all positive controls, all of which were carbohydrates. Bootstrapping of the two major cluster gave scores of 0.974 and 0.948, respectively, indicating high cluster robustness.

13 COG categories were found to be significantly altered by at least 2 compounds in either cluster (Figure. 3.3B). Notably, extracellular structures were significantly promoted by the "MFE, STE, REB" sub-cluster, XYL significantly promoted coenzyme transport and metabolism, while reducing cell motility, intracellular trafficking, secretion and vesicular transport, and lipid transport and metabolism.

We further investigated species level functional responses by annotating peptides to both COG categories and species (Figure 3.4). Although there were no differences in the COG functional quantitation at the microbiome level, several functions were found to be significantly altered in several species, i.e., in *Roseburia hominis,* reduced energy production and conversion by SOR and MAL, reduced signal transduction mechanisms by ASP, SAA, XYL, ERY, CYC, ACE at 2 mg/ml, MAL, LAC, HSH, promoted lipid transport and metabolism by SAA, MAL, LAC; in *Clostridium sp. FS41*, reduced carbohydrate transport and metabolism by REB, ERY, LAC, SOR, HSH, MAN. Overall, sweeteners from the "CHO" cluster were more likely to induce species level functional responses.

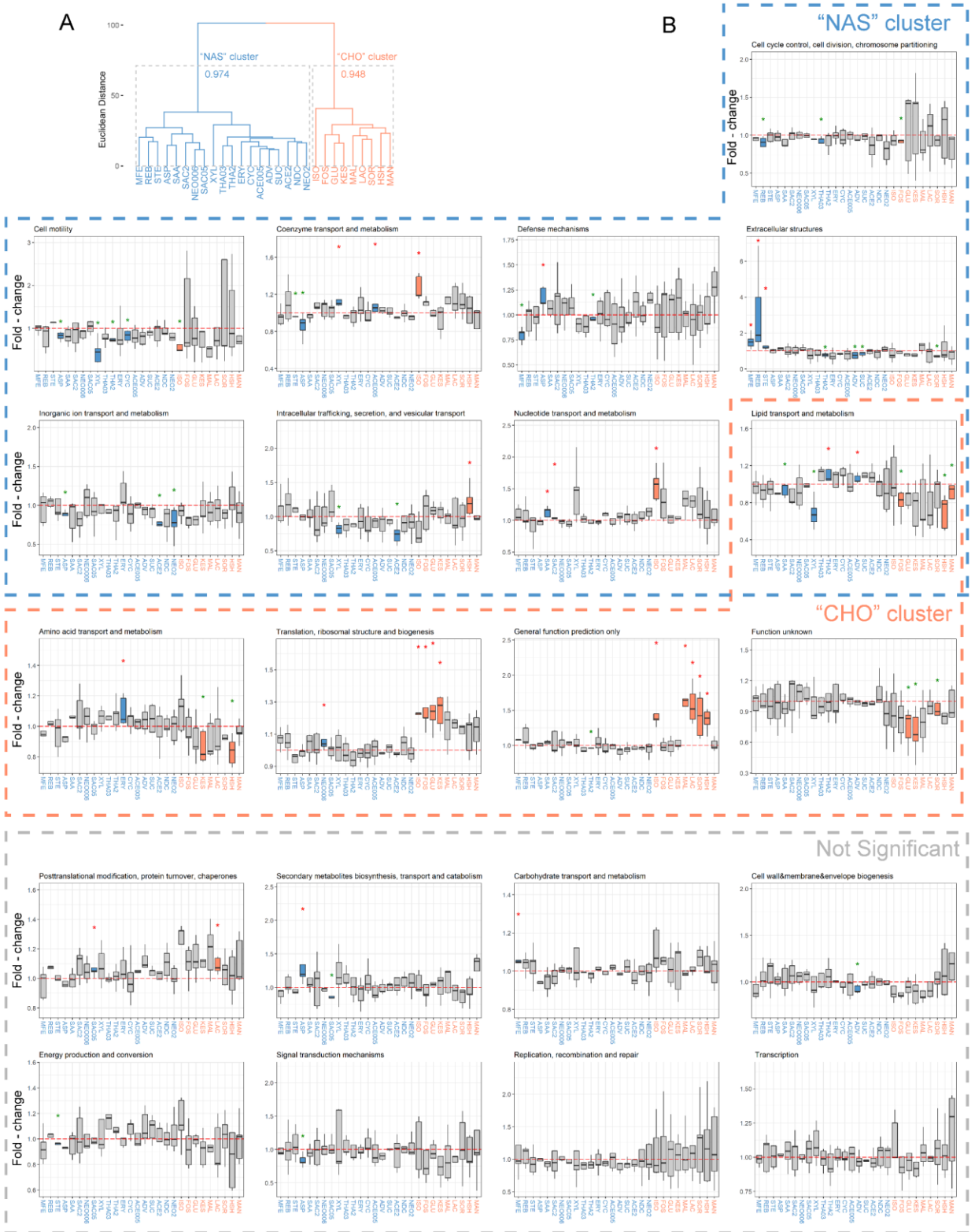

A
B
"NAS" cluster
"CHO" cluster
0.974
0.948
Euclidean Distance
"NAS" cluster
Cell cycle control, cell division, chromosome partitioning
Cell motility
Coenzyme transport and metabolism
Defense mechanisms
Extracellular structures
Inorganic ion transport and metabolism
Intracellular trafficking, secretion, and vesicular transport
Nucleotide transport and metabolism
Lipid transport and metabolism
"CHO" cluster
Amino acid transport and metabolism
Translation, ribosomal structure and biogenesis
General function prediction only
Function unknown
Not Significant
Posttranslational modification, protein turnover, chaperones
Secondary metabolites biosynthesis, transport and catabolism
Carbohydrate transport and metabolism
Cell wall&membrane&envelope biogenesis
Energy production and conversion
Signal transduction mechanisms
Replication, recombination and repair
Transcription
Fold - change

Figure 3. 3. Sweeteners induced gut microbiome functional responses

(A) Clustering of sweeteners based on induced functional responses. Euclidean distances are calculated between sweeteners based on averaged log2 fold change of COG LFQ intensities of sweetener-treated samples versus PBS control. Bootstrapping scores of the two major clusters are shown.

(B) LFQ intensity fold change between treated group and PBS control of all detected COG categories. Colored box indicates significantly changed COG categories, red and green asterisks indicate significant increase and decrease, respectively. Dashed frames include COG categories that are significantly altered by at least two sweeteners from the indicated cluster. *p < 0.05, Wilcoxon rank sum test.

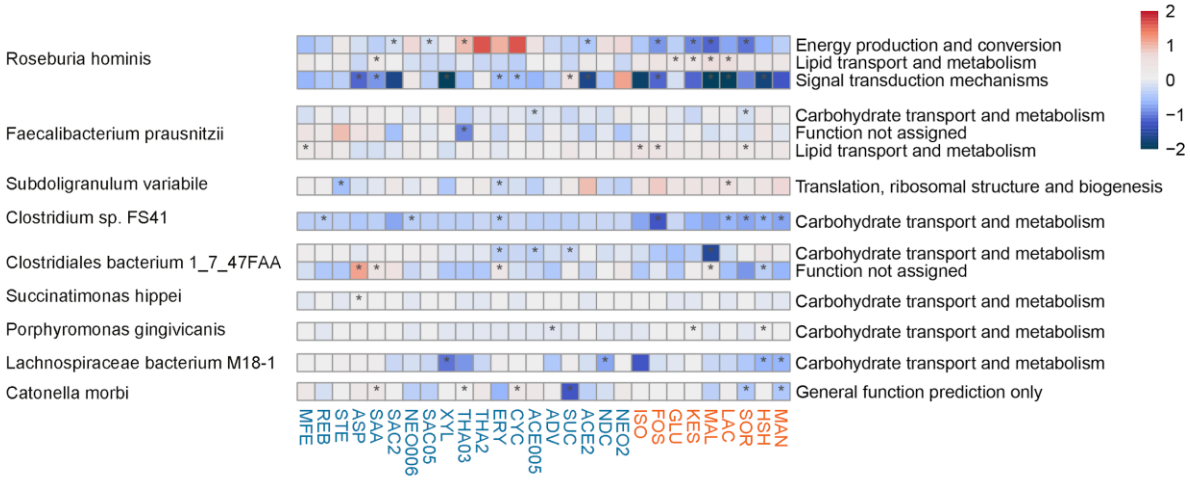


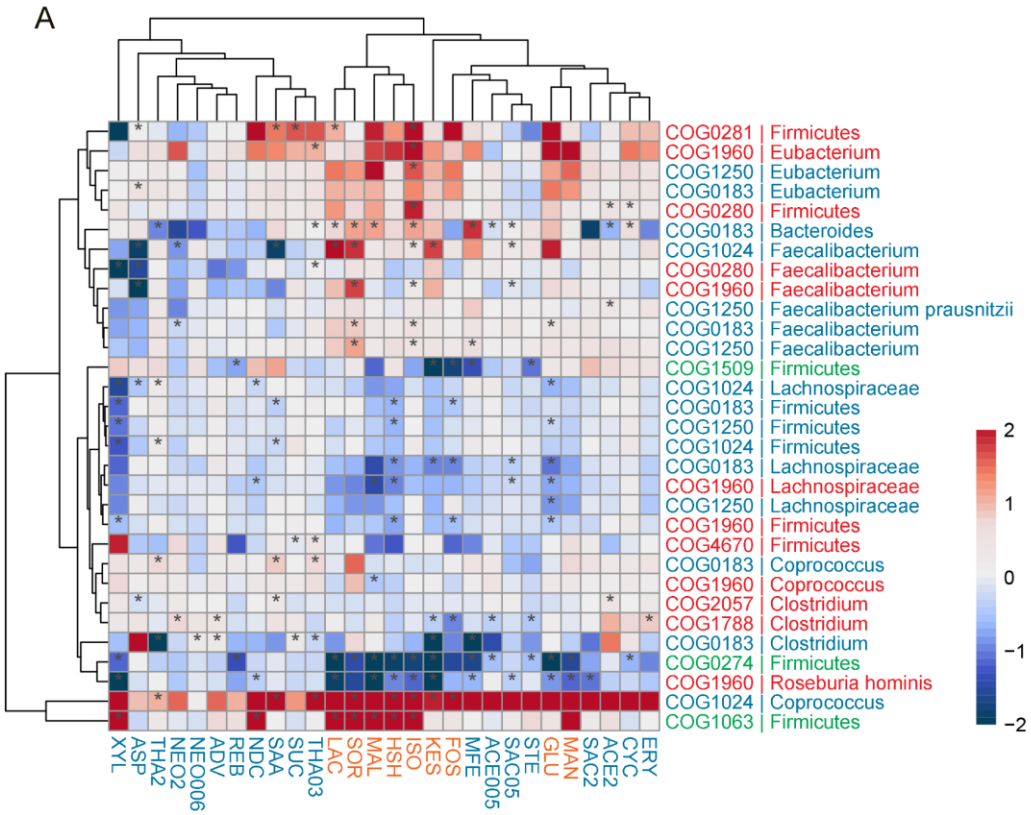
Figure 3. 4. Species level functional responses to sweetener treatment

Heatmap showing log2 fold change of relative COG category abundance in the indicated species between sweetener-treated samples versus PBS control. *p < 0.05, Wilcoxon rank sum test.

3.4 Taxon-specific butyrate production pathway alteration in response to sweeteners

Two microbial butyrate production pathways were identified in our dataset through metaproteomics, i.e., pathway starting from acetyl-CoA and L-lysine (Figure. 3.5B). We found 12 types of enzymes that were significantly altered by sweeteners, most of which were from

Firmicutes, with only one originating from *Bacteroides* (Figure. 3.5A). Notably, a group of sugar alcohols including LAC, SOR, MAL, HSH and ISO were clustered together, indicating that they induced similar butyrate production responses. Acetyl-CoA to crotonyl-CoA pathway in Firmicutes, which involved COG0183, COG1250 and COG1024, was significantly reduced by XYL, HSH and SAA. The the same pathway in *Lachonospiraceae* was significantly reduced by glucose. Acetyl-CoA to butyrate pathway through butanoyl phosphate in *Faecalibacterium*, was significantly promoted by SOR and ISO, while SOR significantly promoted the same pathway in *Eubacterium*. The sugar alcohol cluster reduced COG1960 in *Roseburia hominis*, while promoting COG1024 in *Coprococcus*. In the L-lysine to butyrate pathway of Firmicutes, the sugar alcohol cluster reduced COG0274, while promoting one subsequent enzyme COG1063; Glycoside STE, REB and MFE reduced COG1509.


A
COG0281 | Firmicutes
COG1960 | Eubacterium
COG1250 | Eubacterium
COG0183 | Eubacterium
COG0280 | Firmicutes
COG0183 | Bacteroides
COG1024 | Faecalibacterium
COG0280 | Faecalibacterium
COG1960 | Faecalibacterium
COG1250 | Faecalibacterium prausnitzii
COG0183 | Faecalibacterium
COG1250 | Faecalibacterium
COG1509 | Firmicutes
COG1024 | Lachnospiraceae
COG0183 | Firmicutes
COG1250 | Firmicutes
COG1024 | Firmicutes
COG0183 | Lachnospiraceae
COG1960 | Lachnospiraceae
COG1250 | Lachnospiraceae
COG1960 | Firmicutes
COG4670 | Firmicutes
COG0183 | Coprococcus
COG1960 | Coprococcus
COG2057 | Clostridium
COG1788 | Clostridium
COG0183 | Clostridium
COG0274 | Firmicutes
COG1960 | Roseburia hominis
COG1024 | Coprococcus
COG1063 | Firmicutes
XYL ASP THA2 NEO2 NEO06 ADV REB NDC SAA SUC THA03 LAC SOR MAL HSH ISO KES FOS MFE ACE005 SAC05 STE GLU MAN SAC2 ACE2 CYC ERY
2
1
0
−1
−2

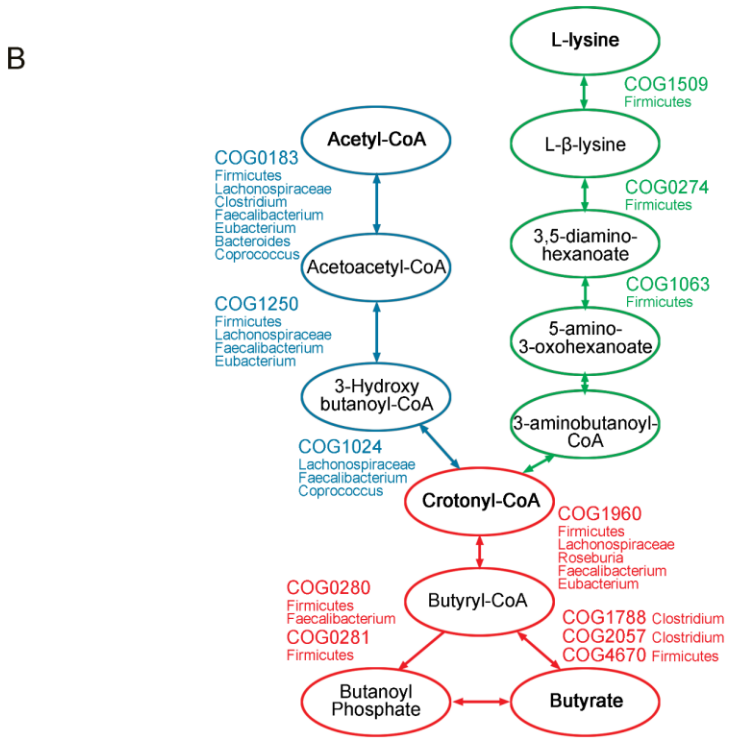

B
L-lysine
COG1509
Firmicutes
L-β-lysine
COG0274
Firmicutes
3,5-diamino-hexanoate
COG1063
Firmicutes
5-amino-3-oxohexanoate
3-aminobutanoyl-CoA
Acetyl-CoA
COG0183
Firmicutes
Lachonospiraceae
Clostridium
Faecalibacterium
Eubacterium
Bacteroides
Coprococcus
Acetoacetyl-CoA
COG1250
Firmicutes
Lachonospiraceae
Faecalibacterium
Eubacterium
3-Hydroxy butanoyl-CoA
COG1024
Lachonospiraceae
Faecalibacterium
Coprococcus
Crotonyl-CoA
COG1960
Firmicutes
Lachonospiraceae
Roseburia
Faecalibacterium
Eubacterium
Butyryl-CoA
COG0280
Firmicutes
Faecalibacterium
COG0281
Firmicutes
COG1788 Clostridium
COG2057 Clostridium
COG4670 Firmicutes
Butanoyl Phosphate
Butyrate

Figure 3. 5. Butyrate producing pathways in different taxa are altered by sweeteners

(A) Heatmap showing log2 fold change of summed COG abundance specific to taxa of sweetener-treated samples versus PBS control. For each treatment, the averaged data of all microbiomes were used for coloring and clustering. *$p < 0.05$, Wilcoxon rank sum test.

(B) Enzymes involved in butyrate production that are found to be significantly altered by sweeteners. Taxa from which those enzymes originate are listed.

4.0 Discussion

In this study, we investigated the taxonomic and functional responses of 5 individual microbiomes to 20 common sweeteners, including both NAS and sugar alcohols, using in vitro culturing and metaproteomics. Previously, studies of this scale have provided information about drug repurposing based on their unintended effect on the gut microbiome. Study by Maier et al. showed 24% of 835 human-targeted non-antibiotic drugs had antibiotic effect, with 40 affecting at least 10 out of the 40 tested bacterial strains[104]. Li et al. also showed marked compositional and functional changes in the gut microbiome in response to non-antibiotic drugs[105]. This is the first systematic study on the effects of sweeteners on a series of individual gut microbiomes.

Most of the investigated sweeteners have been shown to reach the colon intact, and may interact with the gut microbiome directly. As sweeteners are used as substitutes for dietary sugar, this study also included positive controls including common dietary sugar glucose, FOS and 1-kestose, the trisaccharide in FOS with the smallest degree of polymerization, which have been shown to induce gut microbiome compositional and functional changes[145-146]. Positive controls provide references to the extent sweeteners can alter the gut microbiome. This study showed 3 glycosides NAS, 5 sugar alcohols and all positive controls had marked effect on the metaproteome (Figure 3.1B). Based on function analysis, sweeteners are clustered into two groups, one including 6 sugar alcohols and all positive controls, which induced marked functional responses of microbiomes compared with the other cluster mostly comprising NAS (Figure 3.3A). We also identified members of the gut microbiome that contributed to altered butyrate producing pathways induced by sweeteners (Figure 3.5). To our knowledge, this study is the first sweetener study using

metaproteomic approaches, which gives a better representation of microbiome functional profile by measuring proteins that are expressed.

1. RapidAIM identified novel microbiome responses to sweeteners

Data analysis of individual compounds revealed our approach can identify novel taxonomic and functional changes of the gut microbiome in response to sweeteners. For instance, studies on the association between aspartame consumption and the development of metabolic syndrome and T2D has been conflicting[147-148]. In vivo study by Palmnas et al. in mice revealed altered gut microbiome and metabolic products in response to aspartame consumption[69], however, indirect mechanism was likely to have been involved, as aspartame, a methyl ester of aspartic acid and phenylalanine, is quickly hydrolysed into aspartic acid, phenylalanine and methanol and absorbed in the small intestine[149]. In this study, aspartame is added into the culture medium, allowing direct interaction with the gut microbiome. Observed taxonomic changes include increased *Anaerofustis,* and *Bifidobacterium*, and reduced *Turicibacter*, *Paenibacillus, Anaerostipes*, which are different from changes observed by in-vivo study (Figure 3.2). The increase of *Bifidobacterium* was also observed in another in-vitro study in the presence of dietary fibre and aspartame[70]. Increased phenylalanine metabolism and reduced phenylalanine biosynthesis were induced by aspartame and another aspartame containing sweetener SAA (Supplementary Figure S8), which indicated aspartame may serve as a source of phenylalanine to the gut microbiome. A variety of other functions, including reduced cell motility, signal transduction and mechanisms, coenzyme and inorganic ion transport and metabolism, and increased defence mechanisms (Figure 3.3B) suggested stress response from the gut microbiome upon direct exposure to aspartame.

Thaumatin is a plant-derived sweet protein composed of 207 amino acid residues, and its effects on health and the gut microbiome have not been well investigated. Thaumatin is efficiently digested in the small intestine[150]. In this study, in addition to minor direct effects on microbiome function at both 0.3 and 2 mg/mL, thaumatin at 2 mg/mL is shown to increase the total microbial biomass across all five microbiomes (Supplementary Figure S5). Thaumatin at both 0.3 and 2 mg/mL promotes *Butyricicoccus*, which is associated with beneficial effects including anti-inflammation, and reduced *Butyricicoccus* level in IBD patients[151]. Both aspartame and thaumatin can not reach the gut intact. Nevertheless, for potential compounds with beneficial colonic effect, multi-unit pellet systems comprising pH-dependent or enzyme controlled colonic coating have been used for colonic delivery[152] to avoid degradation and absorption before reaching the colon.

Saccharin consumption was associated with glucose intolerance[64]. In this study, saccharin at 0.5 mg/mL (corresponding to ADI) reduced the microbiome ABS transporters (Supplementary Figure S8), which may indicate reduced ability of the gut microbiome to utilize host-derived glucose and other saccharide, leading to glucose intolerance[153].

2. Recategorizing sweeteners into two clusters

One important finding of this study is the categorization of sweeteners based on induced gut microbiome functional responses into two major clusters.

The “CHO” cluster includes positive control glucose, FOS and 1-kestose, as well as sugar alcohols including sorbitol, mannitol, lactitol, maltitol, isomalt and hydrogenated starch hydrolysate (HSH). These compounds (excluding glucose) can not be digested by the human body. Therefore, despite incomplete absorption of sugar alcohols in the small intestine[154], they can reach the colon intact, serving as substrates for microbial fermentation to produce hydrogen gas, carbon dioxide, methane

and SCFA[155]. Compounds in this cluster are found to induce similar and marked responses in the metaproteome, composition and function of the tested microbiomes (Figure 3.1-3.5). Sugar alcohols are shown to induce gastrointestinal symptoms including bloating, laxative effect and abdominal pain[65]. In this study, despite individual differences, isomalt has been found to increase the total gut microbiome biomass (Supplementary Figure S4), to increase coenzyme and nucleotide transport and metabolism, translation, ribosomal structure and biogenesis, and reduce cell motility (Figure 3.3B). Reduction of bacterial cell motility is associated with increased susceptibility to intestinal expulsion and larger fluctuation in absolute abundance[156]. Our findings with isomalt can be used to explain the gastrointestinal symptoms associated with sugar alcohols.

Our study revealed potential taxa that metabolize compounds from "CHO" cluster. Genus including *Bacteroides*, *Bifidobacterium* and *Ruminococcus* are primary glycan degraders[20], increased presence of Bacteroidales members is also shown to oppose increased adiposity and body mass in mice consuming high-fibre diet[34]. We observed increased *Bacteroides* and *Paraprevotella* in response to FOS, 1-kestose, glucose and HSH, but not other sugar alcohols. Notably, HSH is composed of a mixture of sugar alcohols with low and high degree of polymerization, while other sugar alcohols are generated from monosaccharides or disaccharides (Supplementary Figure S1). Carbohydrate-active enzymes (CAZymes) capable of degrading oligosaccharide are coded by the increased taxa. Our study observed genus increased by at least 2 sugar alcohols include *Butyricicoccus*, *Collinsella*, *Marvinbryantia*, *Blautia*, *Bifidobacterium*, *Ruminococcus*, *Phascolarctobactobacterium*, *Prevotella*, *Paraprevotella*, *Parabacteroides*, and *Dialister*, which are potential sugar alcohol degraders. *Bifidobacterium* is a known butyrate producer, and reduced level of this genus has been associated with disorders[157]. Enriched *Prevotella* is associated with a diet rich in carbohydrates derived from plants[158]. Interestingly,

Roseburia spp. is also a known glycan degrader[158], while its abundance is reduced by mannitol, maltitol and HSH. A previous in vivo study in humans also showed reduced *Roseburia intestinalis* with the consumption of isomalt[87]. Functional study of *Roseburia hominis* in response to maltitol revealed an increase in lipid transport and metabolism, which indicated increased SCFA producing activity, while energy production and conversion, as well as signal transduction mechanisms were reduced, indicating a reduced cellular activity. This revealed the importance of taxon-specific functional analysis in profiling metabolic activities of the gut microbiome.

Two sugar alcohols, erythritol and xylitol, belonged to a different cluster with other sugar alcohols. They are featured by the shorter carbon backbones, with erythritol comprising 4 carbon atoms and xylitol comprising 5 carbon atoms (Supplementary Figure S1). Xylitol showed marked effects on the metaproteome, and the composition and function of the gut microbiome (Figure 3.1-3.3), thus the incorporation of xylitol to the "NAS" cluster indicate its distinct effects on the gut microbiome compared with other sugar alcohols and positive controls. *Coprococcus, Clostridium, Oscillibater, Anaerostipes* and *Butyrivibrio* are increased in response to xylitol and not by any other sugar alcohols (Figure 3.2). This is consistent with another single species based study which revealed xylitol was largely utilized by *Anaerostipes caccae* to produce butyrate[86]. Xylitol is also shown to promote pentose phosphate pathway (Supplementary Figure S8), the NADPH produced from this pathway is used as primary reducing agent in a variety of biosynthesis pathways, including butyrate production[159]. Erythritol is clustered with a few NAS that induce less microbial responses. This is consistent with previous in-vitro human study showing erythritol not being fermentable by human gut microbiome[160].

Three glycoside, stevioside (STE), rebaudioside A (REB) and monk fruit extract (MFE) are shown to form a sub-cluster within the "NAS" cluster. Another tested glycoside is neohesperidin

dihydrochalcone (NDC). These glycosides include sugar residue attached to another functional group via glycosidic bond. In the human gut microbiome, the sugar residue can be released by a variety of glycoside hydrolase, and potentially serve as an energy source[161], while the human body encodes few of these hydrolase, allowing consumed glycoside to reach the gut intact[161]. The hydrolysis product of STE and REB is steviol after subsequent removal of all sugar residues[161]. MFE comprises multiple mogrosides, with a series of products resulting from deglycosylation, oxidation, isomerization, and/or deoxidation found after fermentation by human gut microbiome[162]. NDC also undergoes hydrolysis to release sugar residue and yield corresponding aglycones[163]. Notably, STE, REB and MFE increased microbial function in extracellular structures, which is composed of many proteins associated with type IV pilus assembly (Figure 3.3B). Studies have been associating type IV pilus with functions including motility and molecule exchange, and they can help the establishment of gut microbiome members[164]. When enriched in beneficial members, it helps the competitive exclusion of pathogens[164]. To further prove this potential beneficial effect, taxa associated with the function change need to be identified.

3. Identifying contributor of altered butyrate producing pathway

One advantage of metaproteomics is the ability to observe taxon-specific functional microbiome changes. We further identified microbial contributors to sweeteners induced changes in butyrate producing pathway. Butyrate is a major fermentation product of the gut microbiome, which serves as an energy source, and perform important functions in maintaining gut homeostasis through inflammation and carcinogenesis protection, promote colon barrier and reduce oxidative stress[16, 157, 165]. In-vitro study by Sato et al. on the human gut microbiome also showed increased butyrate production from the fermentation of mannitol, sorbitol and xylitol[86].

We found altered butyrate producing pathways for several important butyrate producing taxa, including *Eubacterium* (*Eubacteriaceae* family), *Faecalibaterium* (*Ruminococcaceae* family), *Coprococcus* and *Roseburia* (*Lachnospiraceae* family), *Clostridium* (*Clostridiaceae* family), as well as in *Bacteroides* (Bacteroidaceae), all of which express genes encoding the pathways identified in this study[166]. Notably, in response to all sugar alcohols, COG1960, an acyl-CoA dehydrogenase is reduced in *Roseburia hominis*. The increase of butyrate production with sorbitol[86] can be explained by the increase in all enzymes involved in acetyl-CoA to butyryl-CoA pathway in *Faecalibaterium* (Figure 3.5A). Isomalt may also promote butyrate production through increased acetyl-CoA fermentation through members of Firmicutes including *Faecalibaterium* and *Eubacterium*. Xylitol reduced the total enzyme abundances involved in acetyl-CoA fermentation in Firmicutes, which was in contradiction with findings on promoted butyrate level upon xylitol fermentation[86]. The increased overall fermentation might be associated with increased enzyme activities in *Anaerostipes spp.*, enzyme of which was not identified in this study. Sugar alcohols including lactitol, sorbitol, malitol and isomalt increased the level of COG0183, acetyl-CoA acetyltransferase in *Bacteroides*, which catalyze the first step of acetyl-CoA fermentation. The increased level of acetoacetyl-CoA produced from *Bacteroides* can potentially participate in microbiome cross-feeding networks and utilized by other taxa, which is a common form of microbial metabolism[167].

4. Limitations of this study and future directions

This study provides systematic metaproteomic data of gut microbiome response to sweeteners, however, there are a few limitations associated with the experimental design and experimental

procedures. Future experiments are also required to better understand the health effect of sweeteners from a microbiome point of view.

In this study, most tested concentrations of NAS were determined based on ADI, which represented the maximum safety consumption level. However, the actual intake level of a few sweeteners is much lower. The high-level exposure of advantame and neotame in general public is 0.8% and 16% of the corresponding ADI[127], which may attribute to their high sweetness intensity (20000X and 7000-13000X sucrose equivalence[143]). High-level exposure of saccharin is also approximately 16% of ADI[168]. Also, the concentration 2 mg/mL used for sugar alcohols represents a much lower intake level compared with that in the general public[128-129]. Concentrations representative of multiple intake levels could be included in future experiments.

In this study, the absolute taxa abundance was calculated from the relative taxa abundance determined by MS, and absolute biomass determined by DC assay. While this may add to potential errors in data, and longer sample processing time, an equal-volume based approach has been used in previous study[105] where the absolute taxa abundance is directly measured by MS, and could be applied in future studies. Techniques including tandem mass tags (TMT), which allows multiplexing multiple samples in one MS analysis by labeling different sample peptides with different isotopic labeled tags[169], could also be applied in this study to greatly reduce the time for MS analysis.

The culture medium used in this study provides basic nutrition to the cultured gut microbiome, and has been shown to preserve compositional and functional profile of the cultured microbiome[101]. However, common dietary components, i.e., dietary fibre, are not present in the culture system. A recent study revealed changes in fibre fermentation by the microbiome in the presence of different

food additives[70]. Major dietary components could also be added into RapidAIM culturing assay to study the effect on microbiome fermentation capacity by sweeteners.

One key point in microbiome studies is to assess whether microbiomes are involved in the causality of metabolic syndromes (phenotypes)[62]. Only a few studies to date have revealed potential causality of food additive and metabolic syndromes. For example, fecal transplantation of saccharin consumers to germ-free mice showed transferrable glucose-intolerance and a causal relationship between saccharin consumption and induced metabolic syndrome[64]. Similar studies have been done on emulsifiers carboxymethylcellulose and polysorbate-80. In future experiments, cultured microbiome through RapidAIM could be transplanted to germ-free mice to investigate the phenotypic change sweeteners can induce, thus building a causality link between sweeteners consumption and potential health issues.

Integrated multi-omics approaches, including metagenomics, metatranscriptomics, metaproteomics and metabolomics, is fully compatible with RapidAIM workflow and can provide a deeper analysis into gut microbiome composition, function and metabolic activities[113]. For instance, incorporating metagenomics and metaproteomics can not only reveal the activities of genes by measuring protein expression levels, the database generated from metagenomics result can also greatly enhance protein identification[170]; integrating metabolomics and metaproteomics allows association analysis of taxa, metabolic pathway and metabolites, providing information for a "taxa-pathway-metabolites" axis[171]. Multi-omic approaches in future studies could provide a deeper view of sweetener interaction with the gut microbiome.

5.0 Conclusion

In this study, we sought to find the taxonomic and functional responses of the human gut microbiome to 20 common sweeteners. By combining in-vitro culturing and metaproteomics, we identified sweeteners that induced marked taxonomic and functional responses from the gut microbiome. Sweeteners were regrouped into two major clusters based on induced functional responses. Finally, taxa that contributed to altered butyrate producing pathways were identified in response to different sweeteners. This work sheds light on the importance to understand the effect on the gut microbiome of supposedly inert dietary components, and allows the comparison and recategorization of sweeteners based on induced microbiome responses. This study provides important information to understand the health influence of sweeteners from a microbiome point of view.

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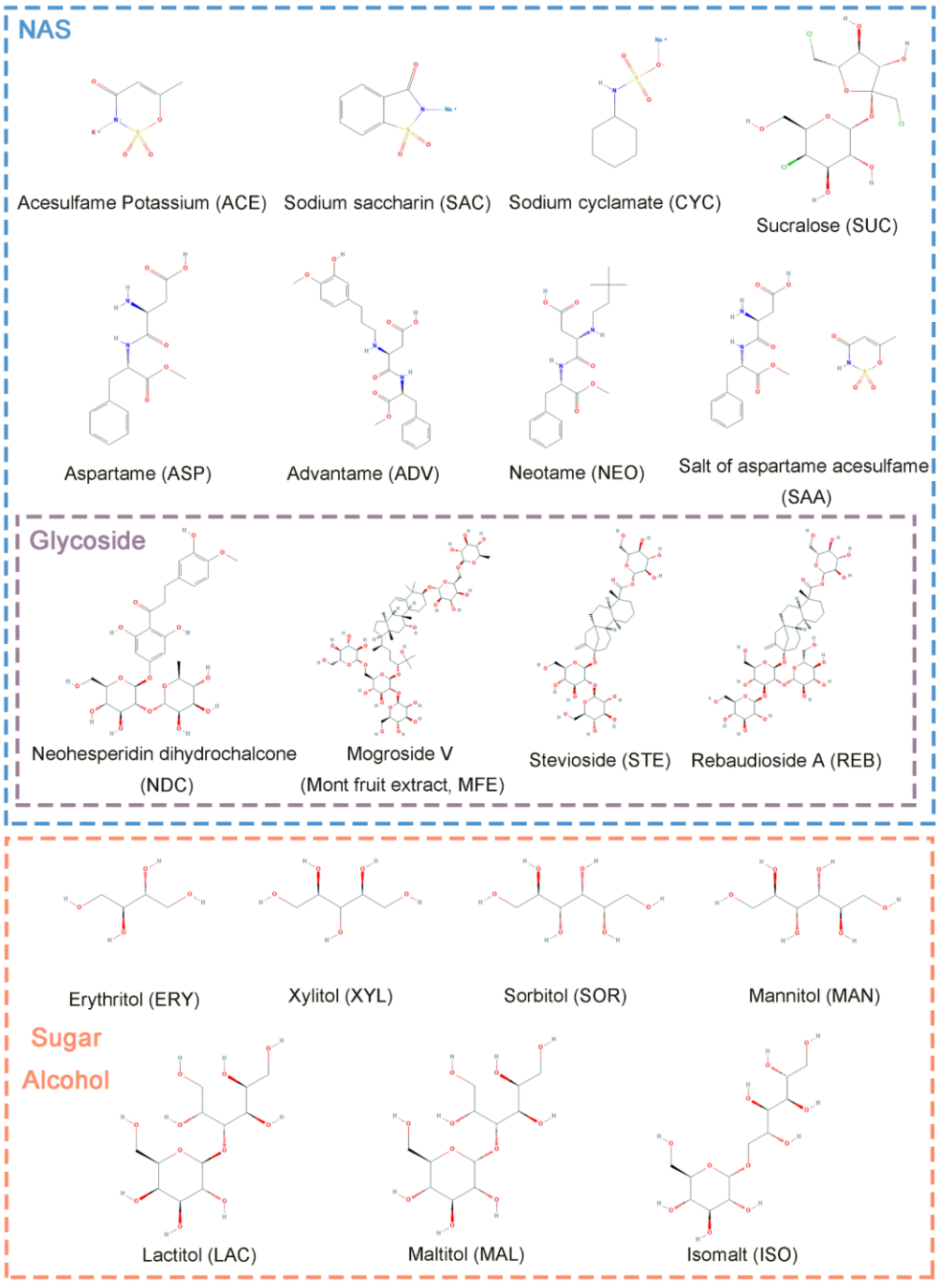
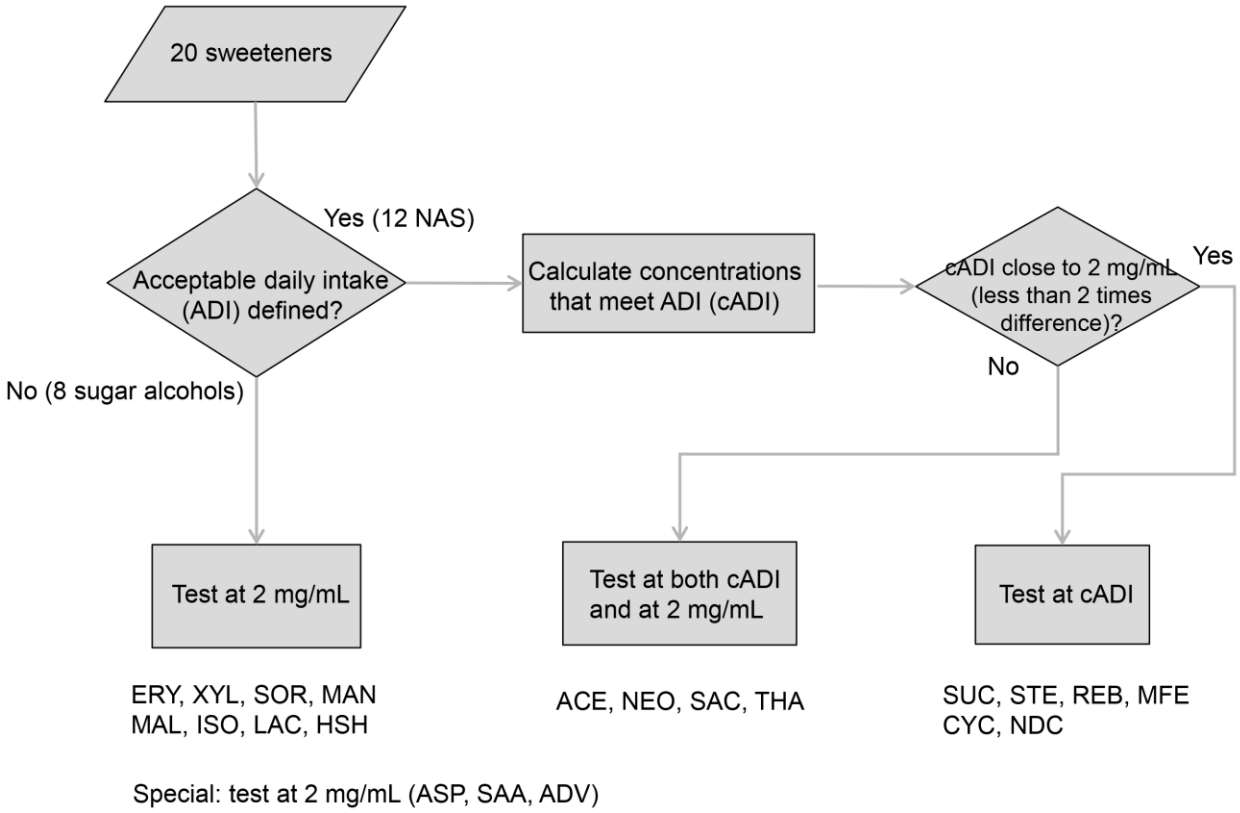
Appendices

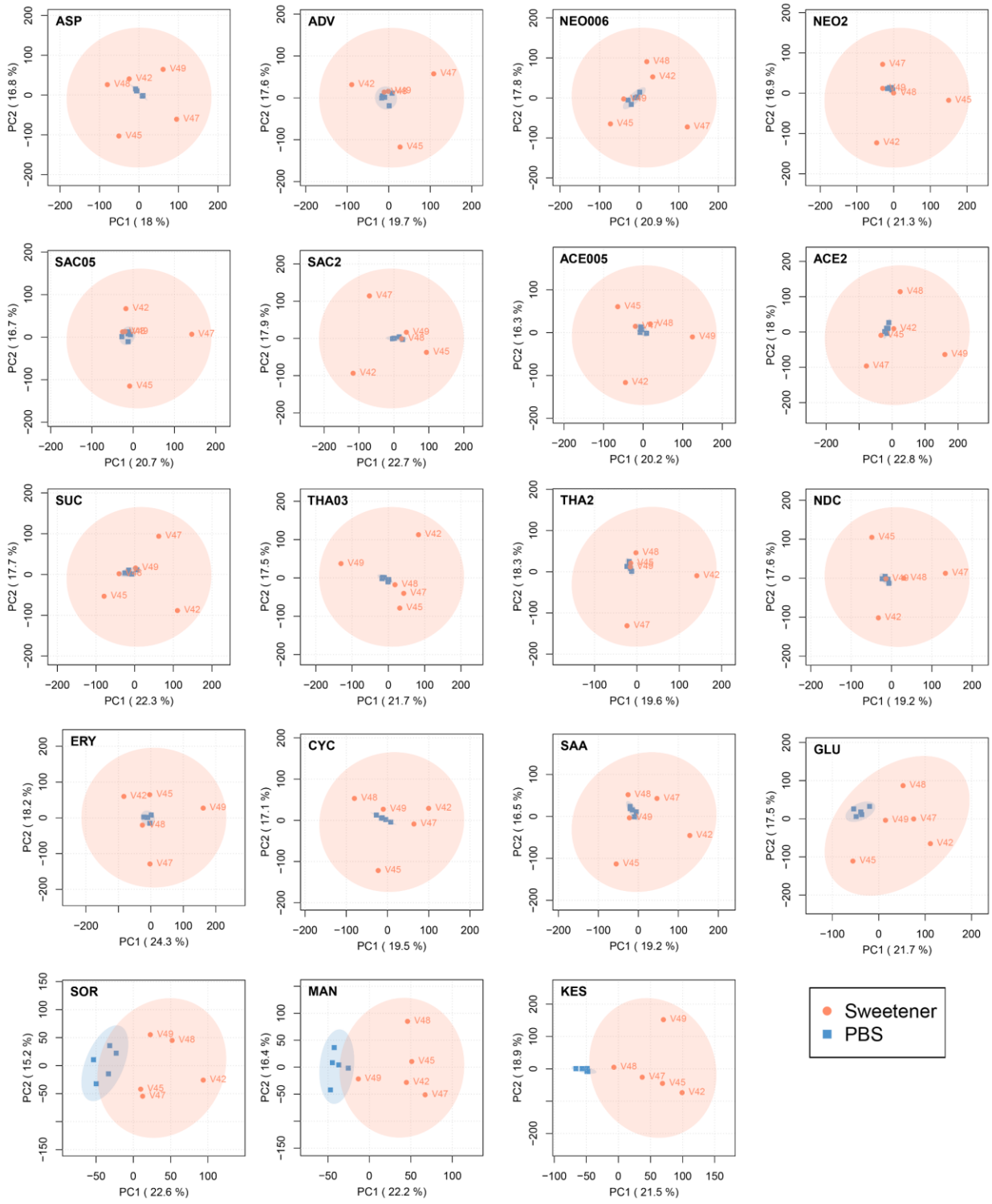


Supplementary Figure S 1. Structures of sweeteners

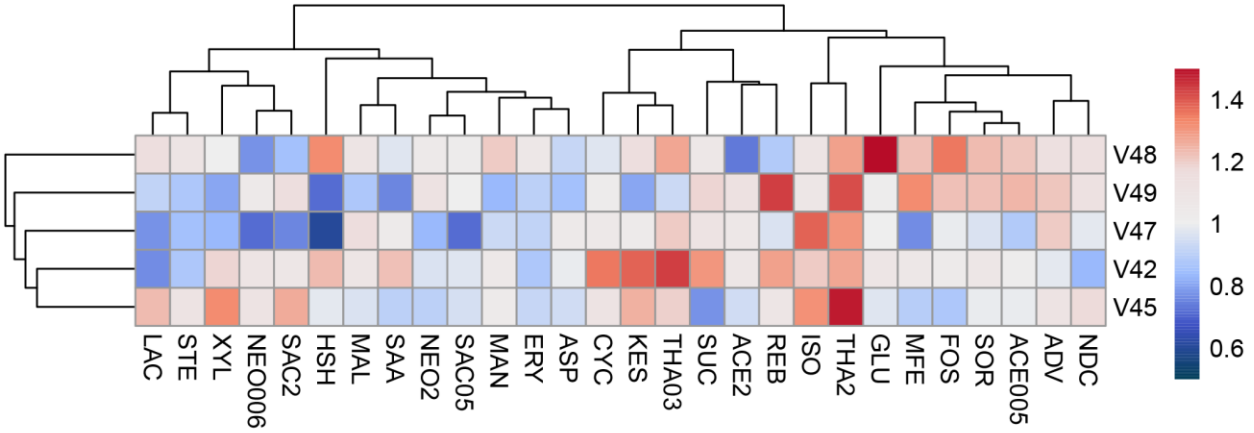
Structure information is found on PubChem (https://pubchem.ncbi.nlm.nih.gov/).



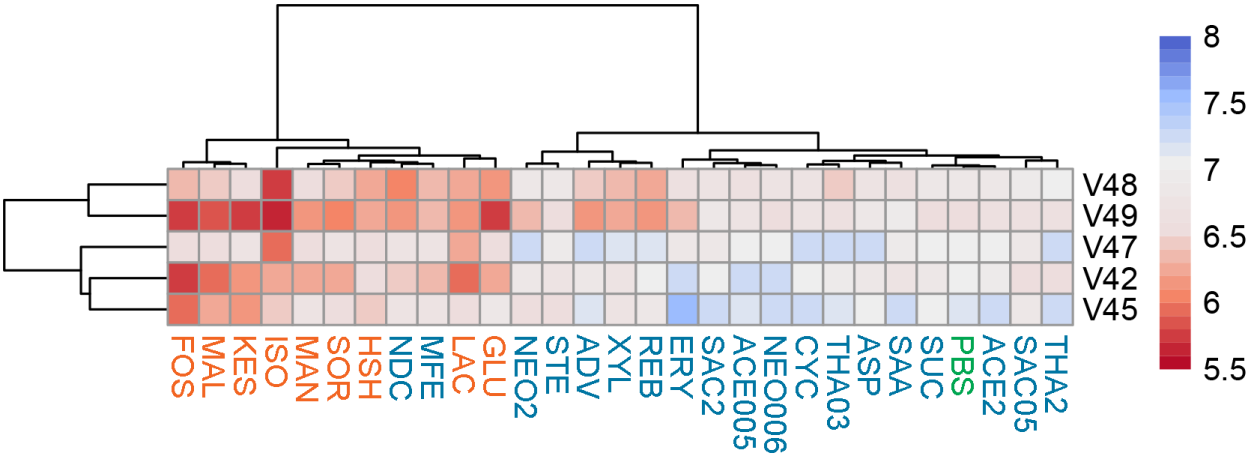
Supplementary Figure S 2. Workflow used to determine sweetener concentrations



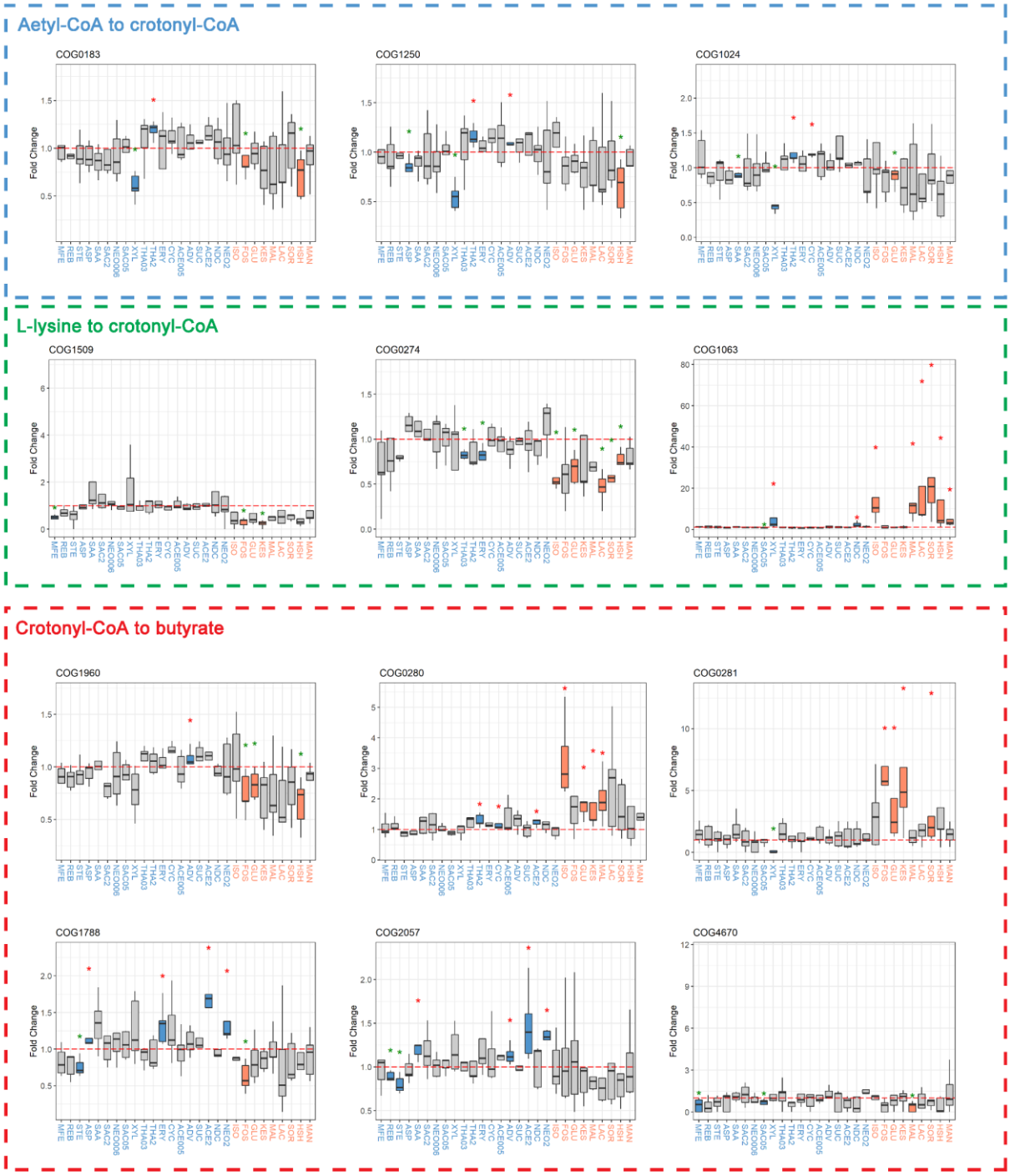
Supplementary Figure S 3. Individual PCA plots of sweeteners of non-significant changes and positive control KES, GLU



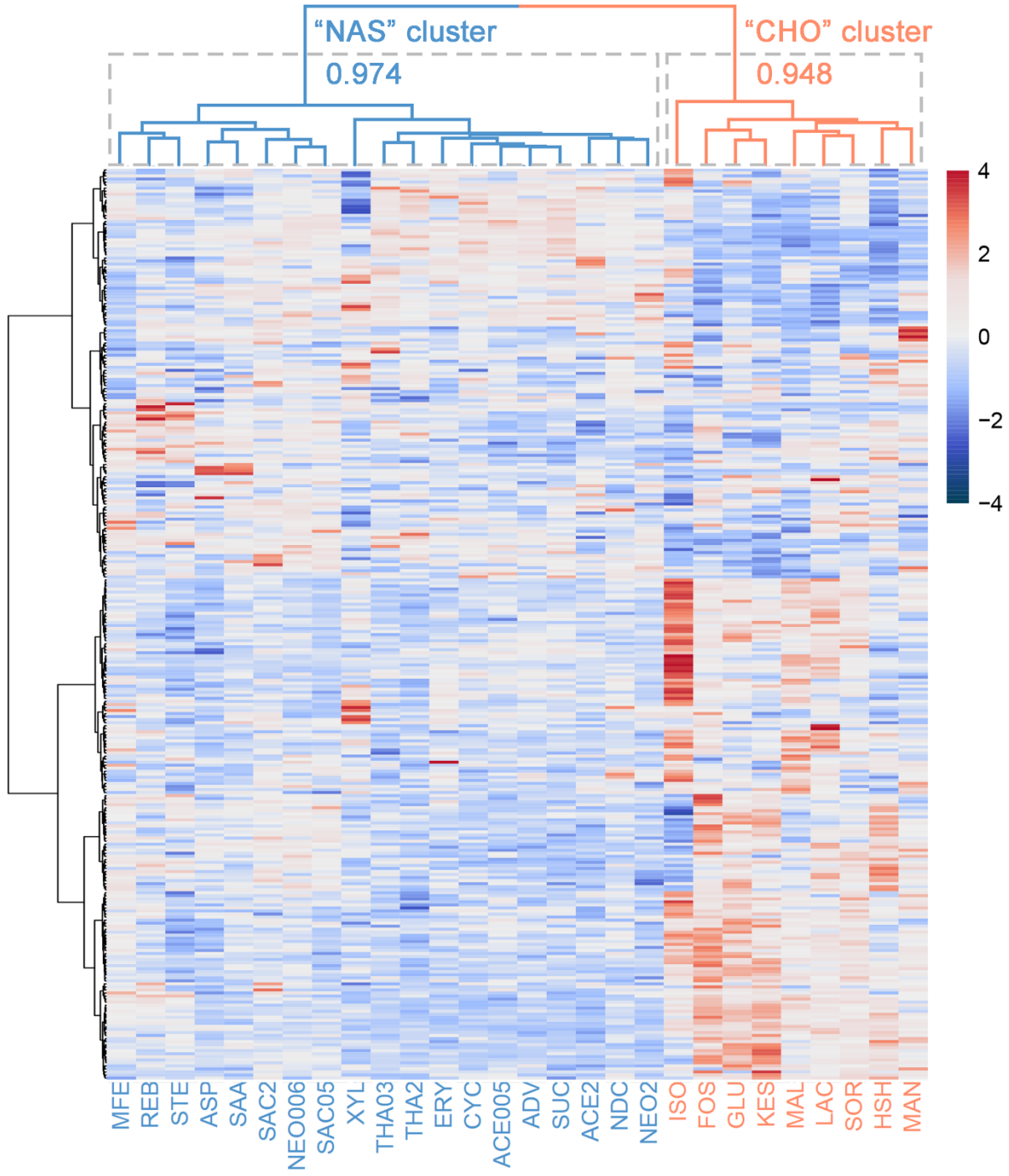
Supplementary Figure S 4. Fold change of total protein abundance of each sample versus PBS control



Supplementary Figure S 5. pH of supernatant after culturing



Supplementary Figure S 6. Fold change of total abundances of butyrate production related enzymes



Supplementary Figure S 7. Hierarchical clustering of sweeteners based on induced microbial functional responses

Heatmap color is based on averaged COG intensity of all microbiomes.

Supplementary Figure S 8. Fold change of total abundances of KEGG pathways

Supplementary Figure S8 (*Continued*)

Supplementary Figure S8 (*Continued*)

Supplementary Figure S8 (*Continued*)

Supplementary Table S 1. Summary of sweeteners

Sweetener*	Classification	Abbreviation in this paper	PubChem CID	Molecular Weight (g/mol)	Approved by
Acesulfame K	NAS	ACE	11074431	201.24	HC, EFSA, FDA
Aspartame	NAS	ASP	134601	294.3	HC, EFSA, FDA
Advantame	NAS	ADV	10389431	458.5	HC, EFSA, FDA
Neotame	NAS	NEO	9810996	378.5	HC, EFSA, FDA
Saccharin (Sodium saccharin dihydrate)	NAS	SAC	517320	241.2	HC, EFSA, FDA
Sucralose	NAS	SUC	71485	397.6	HC, EFSA, FDA
Stevia extract (Stevioside)	NAS	STE	442089	804.9	HC, EFSA, FDA
Stevia extract (Rebaudioside A)	NAS	REB	6918840	967	HC, EFSA, FDA
Monk fruit extract	NAS	MFE	N/A	N/A	HC, FDA
Thaumatin	NAS	THA	N/A	N/A	HC, EFSA, FDA
Cyclamate (Sodium cyclamate)	NAS	CYC	23665706	201.22	EFSA
Neohesperidin Dihydrochalcone	NAS	NDC	30231	612.6	EFSA
Salt of Aspartame-Acesulfame	NAS	SAA	25130065	457.5	EFSA
Sorbitol (D-Sorbitol)	sugar alcohols	SOR	5780	182.17	HC, EFSA, FDA
Mannitol (D-Mannitol)	sugar alcohols	MAN	6251	182.17	HC, EFSA, FDA
Isomalt	sugar alcohols	ISO	88735	344.31	HC, EFSA, FDA
Maltitol	sugar alcohols	MAL	493591	344.31	HC, EFSA, FDA
Lactitol (Lactitol monohydrate)	sugar alcohols	LAC	3067270	362.33	HC, EFSA, FDA
Xylitol	sugar alcohols	XYL	6912	152.15	HC, EFSA, FDA
Erythritol (meso-Erythritol)	sugar alcohols	ERY	222285	122.12	HC, EFSA, FDA
Hydrogenated starch hydrolysates	sugar alcohols	HSH	N/A	N/A	HC, EFSA, FDA
Glucose (D-Glucose)	dietary sugar (positive control)	GLU	5793	180.16	N/A
1-Kestose	oligosaccharide (positive control)	KES	440080	504.4	N/A
Fructooligosaccharide	oligosaccharide (positive control)	FOS	N/A	N/A	N/A

Supplementary Table S1 (*Continued*)

Sweetener*	Supplier and catalog number	Acceptable Daily Intake (ADI) (mg/kg bw/d)	cADI without considering proportion reaching the colon (mg/mL)	Proportion that reaches the colon (%)	Concentration in medium (mg/mL)
Acesulfame K	TCI A1490	15 (FDA)	0.053	1[172]	0.05 (AE005) and 2 (ACE2)
Aspartame	Alfa Aesar J61523	50 (FDA)	17.575	0[125]	2
Advantame	Sigma-aldrich 80054	32.8 (FDA)	10.31	89.5[127]	2
Neotame	Sigma-aldrich 49777	0.3 (FDA)	0.067	63.7[173]	0.067 (NEO006) and 2 (NEO2)
Saccharin (Sodium saccharin dihydrate)	J&K 926097	15 (FDA)	0.527	10[174]	0.5 (SAC05) and 2 (SAC2)
Sucralose	Alfa Aesar J66736	5 (FDA)	1.494	85[175]	1.5
Stevia extract (Stevioside)	TCI S0594	4 (FDA)	1.406	100[174]	1.4
Stevia extract (Rebaudioside A)	TCI R0095	4 (FDA)	1.406	100[174]	1.4
Monk fruit extract	Sigma USP 1445492	6.8 (FDA)**	2.39	100[176]	2.4
Thaumatin	TCI T1144	1.1 (FDA)***	0.3867	0[126]	0.39 (THA03) and 2 (THA2)
Cyclamate (Sodium cyclamate)	Alfa Aesar A18666	7 (EFSA)	1.55	63[177]	1.6
Neohesperidin Dihydrochalcone	TCI N0675	5 (EFSA)	1.758	100[163]	1.8
Salt of Aspartame-Acesulfame	Sigma USP 1043750	20.46 (EFSA)****	7.192	0[177]	2
Sorbitol (D-Sorbitol)	TCI S0065	N/D	N/A	75[154]	2
Mannitol (D-Mannitol)	TCI M0044	N/D	N/A	75[154]	2
Isomalt	Sigma-aldrich PHR1769	N/D	N/A	90[154]	2
Maltitol	TCI M0797	N/D	N/A	60[154]	2
Lactitol (Lactitol monohydrate)	J&K 126721	N/D	N/A	98[154]	2
Xylitol	TCI X0018	N/D	N/A	50[154]	2
Erythritol (meso-Erythritol)	TCI E0021	N/D	N/A	10[154]	2
Hydrogenated starch hydrolysates	CarboMer Inc. 68425-17-2	N/D	N/A	60[154]	2
Glucose (D-Glucose)		N/A	N/A	N/A	2
1-Kestose		N/A	N/A	N/A	2
Fructooligosaccharide		N/A	N/A	N/A	2

*names of the compounds that were used to represent the sweetener are shown in the brackets.

**The estimated 90th percentile intake of MFE in the general population by the FDA is used here, as its ADI is not specified[178].

*** The highest estimated exposure level of THA in the general population by the EFSA is used here, as its ADI is not specified[150].

****The ADI of SAA is calculated based on ADI of both ASP and acesulfame, as specified by the EFSA[179].