Supplemental material

Supplemental methods

Low-calorie sweetener (LCS) dosage.

LCS doses were chosen based on existing guidelines for safe dosage in humans and previous work in animals examining low-dose exposure to sweeteners. Weekly doses were adjusted for body weight throughout the study in dams and reflect actual human consumption of LCS. Further, our doses are ~10 times lower than the higher upper intake recommended for humans in Canada and the US and nearly 10-times lower than previous studies examining impact of LCS on animal metabolism. They reflect normal physiological intake of these sweeteners.

Glucose and Insulin Tolerance Tests.

Oral glucose tolerance tests (OGTT, 2 g/kg body weight) were performed in dams (gestation and lactation day 13-16) and in offspring at age 8 and 17 weeks. Following an overnight (12 h) fast, blood glucose was measured before (0 min) and after the oral glucose load (15, 30, 60, 90, 120 min) using One Touch® Ultra® 2 glucose meter. Two days after the OGTT, rats were fasted overnight (12 h) and an insulin tolerance test (ITT) performed with a 0.75U/kg intraperitoneal insulin injection. Blood glucose concentrations were measured before (0 min) and after (15, 30, 60, 90 120 min) insulin injection using One Touch® Ultra® 2 glucose meter. Serum insulin, leptin, ghrelin (active), and GLP-1 (active) were quantified using the Rat Gut Hormone Multiplex Kit (Millipore) from 400 µl of blood collected from the tail vein at each time point during the OGTTs. Serum IL-6 and TNF-α were quantified from the same samples using the Rat Cytokine/Chemokine Multiplex kit (Millipore).

Tissue Collection and Gene Expression.

At weaning, dams and four offspring from each litter (n=2 male and female each) were over-anesthetized with isoflurane and killed by aortic cut. Liver, small intestine, colon, and cecum were excised and weighed. Rats were decapitated and the brain excised. The nucleus accumbens (NAc) and ventral tegmental area (VTA) were isolated and stored as previously described[1]. RNA was extracted from the NAc and VTA and real-time PCR performed as previously described[2] with previously published primers[1]. Primers (tyrosine hydroxylase (TH),

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dopamine transporter (DAT), mu-opioid receptor (Mu), dopamine D1 receptor (D1) and D2 receptor (D2)) were chosen for their role in mesolimbic reward circuitry and previous work showing changes due to maternal diet[1]. Gene expression was assessed by the $\Delta\Delta_{Ct}$ method and ß-actin determined to be an appropriate housekeeping gene.

Microbiota Profiling with qPCR and Sequencing.

Maternal fecal samples were collected weekly during gestation and lactation, and offspring feces collected every 3 weeks (as a litter from 3-8 weeks and as individual rats from 9-18 weeks of age). Samples were stored at -80ºC. DNA was extracted using the FastDNA spin kit for feces (MP Biomedical). Bacteria were quantified using qPCR as previously described[3]. Primers for qPCR were chosen based on those shown to be important in obesity and from previous work examining the impact of aspartame on microbiota with chow and high-fat diets[4]. Data is expressed as relative abundance where abundance of each taxon was normalized to total bacteria.

Cecal matter was collected from dams and offspring posthumously, snap frozen, and stored at -80°C. Bacterial genomic DNA was extracted from cecal samples using FastDNA spin kit for feces (MP Biomedical) and sequenced by the Centre for Health Genomics and Informatics (University of Calgary) using the Illumina 16S rRNA sequencing platform of the V3-V4 region as described previously[5]. Sequencing data from MiSeq was demultiplexed and converted to fastq format using Illumina's bcl2fastq software. CutAdapt was used to remove primers from reads and perform initial quality trimming. Following primer removal, length sequences of shorter than 10 base pairs were removed, and quality trimming threshold was set at Q20. For sequence/OTU clustering, R package DADA2 was used. Chimeric sequences were then removed, and taxonomic assignment was performed using the RDP classifier and RDP database as reference. Phyloseq R package was then employed for further downstream analysis. Alpha diversity was measured by calculating the Shannon and Simpson index. Beta diversity was evaluated using the principle coordinate analysis on Bray-Curtis dissimilarity matrix. Differential abundance analysis between groups was carried out using LEfSe conda package.

Short Chain Fatty Acid Analysis.

Short chain fatty acids (SCFAs) contained in cecal matter (250 mg) were extracted in 500uL of 0.15 mmol/L H_2SO_4 containing internal standard (2-ethyl butyric acid) by homogenizing for two

40 second cycles using FastPrep-24™ homogenizer (MP Biomedicals, Santa Anna, CA, USA). Samples were then centrifuged at 14,000g for 15 minutes at 4°C and supernatant collected. Fatty acid derivatization was performed as previously described with modifications[6]. Briefly, 100µL of aqueous extract was transferred to a reaction tube, and 200µL of each of 3-nitrophenylhydrazine (20mmol/L), 3% Pyridine, and 1-ethyl-3-(3-dimethylamineopropyl) carbodiimide (250mmol/L) were added. The mixture was incubated at 60°C for 20 minutes. After the addition of 100uL of KOH solution, the mixture was further heated at 60°C for another 15 minutes. The sample was cooled at room temperature and then mixed with 4mL phosphate buffer mixture (0.03 mol/L phosphate buffer, pH 6.4 and 0.5mol/L HCl at 3.8:0.4 v/v). 3 mL of hexane was added, tubes were vortexed, and the supernatant was discarded; 3 mL of diethylether was added to the mixture and SCFA derivatives isolated on a shaker for 10 minutes. The supernatant was dried in a speedvac concentrator (Savant™ SPD111 SpeedVac™ Kits, Thermo Scientific™). The residue was dissolved in 1mL of 50% methanol and centrifuged at14,000g for 15 minutes at 4°C. The clear supernatant was collected and store in -20°C until assayed. 20 µL of the sample was injected in reverse-phase HPLC on a C18 column containing a column guard. The sample was eluted in a gradient of acetonitrile containing 0.05% trifluoroacetic acid (8%-100%) with a flow rate of 0.8mL/min for 30 minutes. The absorbance of the eluate was analyzed at 230nm.

References.

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