**SUPPLEMENTAL MATERIAL TO:**

**Oral Supplementation with the Short-Chain Fatty Acid Acetate Ameliorates Age-Related Arterial Dysfunction in Mice**

**Short title: Acetate Improves Age-Related Arterial Dysfunction**

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**DETAILED METHODS**

**EXPERIMENTAL PROCEDURES**

***Fecal sample collection, gDNA extraction, library preparation, and sequencing***

For fecal sample collection, mice were moved from their home cage to a clean acrylic container free of bedding and allowed to freely move around until defecation. All samples were collected in the morning (~9:00AM). Mouse fecal samples were placed in DNAase- and RNAase-free Eppendorf tubes immediately following excretion, flash frozen and stored at -80°C. Samples were shipped to collaborators in the Knight Lab at University of California San Diego. Fecal samples were transferred into 96-well extraction plates (MagMAX Microbiome Bead Plate; ThermoFisher Scientific, Waltham, MA; Cat# A42331). gDNA extraction (MagMAX Microbiome Ultra Nucleic Acid Isolation Kit; ThermoFisher Scientific; Cat # A42357) was performed using Earth Microbiome Project standard protocols1,2. Metagenomics Library Preparation Extracted gDNA was quantified (Quant-iT PicoGreen dsDNA Assay Kit; Invitrogen, Waltham, MA; Cat# P11496) and each sample was normalized based on concentration before performing a high-throughput miniaturized metagenomics library preparation (KAPA HyperPlus Kit 96rxn, 7962428001, Roche, Basal, Switzerland)3. Clean library was quantified, equal volume pooled, PCR cleaned (QIAquick PCR Purification Kit; Qiagen, Germantown, Maryland; Cat# 28106), size selected from 250-750bp (PippinHT, Sage Science, Beverly, MA), and sequenced on an iSeq 100 (Illumina Inc., San Diego, CA). Clean library was pooled a second time by utilizing the library concentration and read counts per sample generated from the iSeq to create an iSeq-normalized pool4. The iSeq-normalized pool was PCR cleaned, size selected from 300-700bp, and then sequenced on a NovaSeq 6000 with a S4 flow cell and 2x150bp chemistry (Illumina Inc.) at the Institute for Genomic Medicine at the University of California San Diego.

***Taxonomic and functional profiling of raw sequencing data***

Raw sequences were deposited in EBI-ENA (Accession: PRJEB70517), uploaded and processed in Qiita5 (Study ID: 14378). The resulting files were processed via QIIME26. Deblurred sequences were assigned a taxonomic classification using the feature-classifier / classify-sklearn plugin in QIIME27 using default parameters. Alpha diversity (species diversity within each sample) was calculated using Faith’s phylogenetic diversity (Faith’s PD)8,9, Shannon’s index9,10 and Chao19. Differences in alpha diversity were compared across ages using the Kruskal-Wallis significance test. To determine beta diversity9 (differences in overall composition between samples), differences in communities (using both unweighted and weighted UniFrac distance metrics, which considers branch length separating each pair of communities on a shared phylogenetic tree) were compared qualitatively across age groups using principal coordinate analysis (PCoA). Differences in beta diversity were compared quantitatively across age groups via PERMANOVA. Paired with this analysis, PERMDISP was also used to assess the differences in the variability and spread of data amongst the age groups. Individual OTU features were analyzed for differential abundance before treatment using an analysis of composition of microbiomes (ANCOM)11. This analysis generates a W score, which is the count of the number of sub-hypotheses that have passed for a given taxon. The W score is plotted against the centered log-ratio (CLR) which is used to address the compositional nature of relative abundance data. Lastly, differential abundance of key microbial taxa and KEGG orthologs were further probed using the QIIME2 plugins Songbird12 and Qurro13. Songbird was used to determine the log-fold change of features with respect to age and treatment. These differentials were then visualized as feature rankings (log-ratios) using Qurro. A heatmap showing the Bray-Curtis differences clustered by median in KEGG ortholog pathways between age groups was generated using QIIME2. Groupings of specific bacteria or KEGG pathways, listed in **Supplementary Table S2**, were then plotted as the natural log ratio versus the top 5% of all features present in all samples.

***Main endothelium-dependent dilation (EDD) experiments and pharmaco-dissection.***

After cannulation, carotid arteries were pressurized for 45 minutes and kept at 37°C in physiological saline solution (PSS) before the start of experiments. Vessels were pre-constricted with phenylephrine (2 μM; Sigma-Aldrich Corp., St. Louis, MO) for 5 minutes to induce basal tone before each dose-response experiment. EDD was assessed to increasing doses of acetylcholine (ACh; 10-9-10-4 mol/L; Sigma-Aldrich Corp.) for 2 minutes per dose14–16.

To determine the mechanisms of age-related impairments in vasodilatory function and improvements with the interventions, vessels were incubated with one of the following pharmacological agents prior to an additional ACh dose-response experiment: 1) the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 0.1 mM; Sigma-Aldrich Corp.) for 30 minutes, to determine the contribution of NO to the overall dilator response; or 2) the superoxide dismutase mimetic 4-Hydroxy-TEMPO,4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; 1 mM; Sigma-Aldrich Corp.) for 60 minutes, to determine reactive oxygen species-related suppression of EDD14–16.

Endothelium-independent dilation (EID; i.e., smooth muscle sensitivity to NO) was assessed to increasing doses of the NO donor sodium nitroprusside (SNP; 10-10-10-4 mol/L; Sigma-Aldrich Corp.) for 2 minutes per dose. Peak EDD or EID were determined as the highest luminal diameter obtained at any dose. Following dose-response experiments, the vessels were incubated in Ca2+-free PSS to obtain a maximal vessel diameter. All data are reported as a percent of the maximal vessel diameter14–16.

***EDD with knockdown of Egr-1***

Carotid arteries were excised at time of euthanasia, cleaned of surrounding tissue, and cannulated on glass pipette tips in a culture pressure myograph system (204CM; DMT, Inc.; 50-55mmHg) containing modified Krebs buffer (123 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 7.5 mM MgSO4, 16 mM NaHCO3, 0.026 mM EDTA, 1.18 mM KH2PO4 and 5.0 mM glucose filtered through a ≤0.45 µm sterile membrane and stored at 4°C) adjusted to pH 7.2-7.25 (resulting in a bath pH ~7.4 during incubation), bubbled with a gas mixture containing 5% CO2, 21% O2, and 74% N2 (Airgas, LLC), and kept at 37°C. Similar to previous approaches17,18, arteries were transfected intraluminally using an automated syringe driver that continuously perfused the arteries at a flow rate of 1.2 μL/min for 4 hours containing one of the following solutions.

For knockdown of Egr-1, a solution was prepared containing Opti-MEM media (ThermoFisher Scientific), either Egr-1 siRNA (ThermoFisher Scientific, Cat# AM16708) or the control scrambled oligonucleotide (SilencerTM Select Negative Control No. 2 siRNA, ThermoFisher Scientific, Cat # 4390846) were added at a final concentration of 40nM, 1.56 µL/ml of Lipofectamine 3000 (Invitrogen; used according to the manufacturer’s instructions: Document Part No. 100022234 Publication No. MAN0009872 Rev C.0), and 1% serum obtained from the same mouse. siRNA and the control scrambled oligonucleotide were reconstituted in 5x siRNA buffer according to the manufacturer’s instructions (5X siRNA Buffer, Dharmacon Reagents, Cat # B-002000-UB-100).

Following the 4-hour incubation, the solution was switched to the modified Krebs buffer containing 5% FCS at a flow rate of 0.5 μL/min for 20 hours. After 24 hours total of culturing, the baths were then replaced with PSS, and EDD and EID were assessed as described above (*Main endothelium-dependent dilation (EDD) experiments and pharmaco-dissection*), except that EDD was assessed 2x to ensure that the data were reproducible given the technical challenges of the experiment. Data were only used from vessels that had 2 repeatable dose responses (i.e., peak EDD were within ~10% of the other). Because of the duplicate EDD dose responses, pharmaco-dissection experiments were not conducted in these vessels.

***Validation of siRNA knockdown***

mRNA gene expression was measured in segments of thoracic aorta obtained from young mice (n = 6) that were treated with siRNA (siEgr-1) or the corresponding control scrambled oligonucleotide, as described above. Samples were mechanically homogenized, and RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, MD). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Transcripts of Egr-1 (forward primer: gagcgaacaaccctatgaga; Reverse primer: gggataactcgtctccacca) were analyzed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA) in 96-well plates and the PowerUp SYBR Green Master Mix (Applied Biosystems) was used as a master mix. ΔΔCt was used to quantify relative gene expression. SimpleSeq DNA sequencing (Quintara Biosciences, Cambridge, MA) was used to validate primer sequences. Knockdown data are shown in **Figure 4A.** Protein abundance of Egr-1 was not assessed as all commercially available antibodies we tested were not sufficiently specific in whole aorta segments.

***Aortic total and mitochondrial superoxide bioactivity***

Total and mitochondrial aortic superoxide bioactivity were determined by electron paramagnetic resonance (EPR) spectrometry using the spin probes 1-hydroxy-3-methoxycarbonyl-2,2,5,5- tetramethylpyrrolidine (CMH; Enzo Life Sciences, Farmingdale, NY) or 1-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine (Mito-TEMPO-H; Enzo Life Sciences), respectively.14,15 Two 1 mm (matched for volume) aortic rings were washed in warm PSS and incubated in Krebs/HEPES buffer (99 mM NaCl, 4.7 mM KCl, 1.87 mM CaCl2, 1.2 mM MgSO4, 25 mM NaHCO3, 1.03 mM KH2PO4, 20 mM NaHEPES, 11.1 mM glucose, 0.1 mM diethylenetriaminepenta-acetic acid, 0.0035 mM sodium diethyldithiocarbamate and Chelex [Sigma-Aldrich Corp.]) containing 0.5 mM CMH or MitoTEMPO-H at 37°C for 60 min. Samples were analyzed using a MS300 X- band EPR spectrometer (Magnettech, Berlin, Germany) with the following instrument parameters: BO-Field, 3350 G; sweep, 80 G; sweep time, 60 s; modulation, 3000 mG; MW atten, 7dB; gain, 5 x e^1. EPR signal was not normalized as aorta ring length was standardized across all mice.

***Plasma proinflammatory cytokines/chemokines***

Plasma levels of proinflammatory cytokines and chemokines were determined using a mouse G-series semi-quantitative cytokine array (Raybiotech, Peachtree Corners, GA; Cat#50-195-0454). Plasma was diluted 2x, and the assay was performed according to the manufacturer’s instructions. The array slides were shipped to the manufacturer for scanning and data extraction. Data were normalized to two internal proprietary positive controls and were sorted and assessed for outliers using the excel-based GS Series Software (GSM-CYT-5), which was provided by the manufacturer.

***In vivo aortic stiffness (pulse wave velocity, PWV)***

Mice were anesthetized under 1-5% inhaled isoflurane in O2 and placed supine on a heating pad (37°C) with paws secured to ECG electrodes. Heart rate was maintained between ~400-500 beats/min, and Doppler ultrasonography (Doppler Signal Processing Workstation, Indus Instruments, Webster, TX) was used to detect arterial waveforms at the transverse aortic arch and abdominal aorta19–21. Three consecutive 2-second tracings were obtained, and the distance between the two doppler probes was measured using calipers. The time delay between the ECG R-wave and each pressure wave was determined as the pre-ejection time. PWV (cm/second) was calculated as the distance between the probes (cm) divided by the difference between the pre-ejection times (Tabdominal – Tarch; seconds).

***Ex vivo aortic stiffness with knockdown of target genes***

For knockdown of Egr-1, a solution was prepared containing Opti-MEM media, 1% serum obtained from the same mouse, and either Egr-1 siRNA or the control scrambled oligonucleotide (reconstituted in 5x siRNA buffer) at a final concentration of 40 nM, 1.56 µL/ml of Lipofectamine 3000, i.e., the exact same solution as described for carotid artery culturing described above. This was injected into the lumen of the thoracic aorta (segmented as described in the main text methods), which was tied on both ends to maintain the solution intraluminally. The aorta sections were then incubated in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific) containing 10% FCS and 1% penicillin/streptomycin for 24 hours. Following the incubation period, the tied ends of the aorta were removed and the center piece was either segmented into ~1 mm sections to determine aortic elastic modulus or used to determine knockdown of Egr-1 (*EDD with knockdown of target genes - Validation of siRNA knockdown*).

Additional aorta sections were incubated under the same conditions except with a different control scrambled oligonucleotide (siGENOME Non-Targeting Control siRNA, Dharmacon Reagents, Cat # D-001206-14-20) at a final concentration of 80nM, and for 48 hours. No transfection reagent was included in this solution, as Dharmacon siRNA is engineered to enter cells without the addition of lipofectamine. These experimental conditions were used in attempt to assess the role of another target gene (free fatty acid receptor 3, FFAR3) in mediating the effects of our interventions, but we did not include the data for the experimental condition, as we were unable to confirm knockdown of FFAR3.

These duplicate segments were used to assess aortic elastic modulus via pin (force) myography (DMT620M, Inc., Arhaus, Denmark)21–23. Aorta rings were mounted onto two pins in a warm (37°C) Ca2+-free phosphate-buffered saline (PBS) bath, i.e., stiffness was assessed under passive (no active VSMC contraction) conditions and thus isolated structural component of arterial stiffness. Three rounds of pre-stretching (pins displaced to 1 mm) were performed, and the aortic diameter was increased until a force of 1mN was reached. Subsequently, the pins were incrementally displaced by 50 μm every three minutes until the vessel reached mechanical failure (i.e., a sudden drop in force). The force corresponding to each stretching interval was recorded and used to calculate stress and strain and to construct a stress-strain curve:

Strain *(λ)* = Δ*d*/*di*

Where *d* is diameter and *di* is the initial diameter.

Stress *(t)* = *(λL)/2(HD)*

Where *L* is one-dimensional load, *H* is intima-media thickness (IMT), and *D* is vessel length.

Aortic diameter and IMT were determined in ~1 mm segments of thoracic aorta that were frozen in optimal cutting temperature (OCT) compound in liquid nitrogen-cooled methyl butane and stored at -80°C. Samples were later sectioned (7 μm; Leica CM1520, Leica biosystems, Weltzar, Germany), plated on microscrope slides, and imaged using a brightfield microscope (Nikon Eclipse TS100; 4x magnification). ImageJ software (National Institutes of Health, Bethesda, MA) was used to quantify the aortic diameter and IMT. The differentiation between the aortic medial and adventitial layers was determined as the point between the regular banding patterns of the external elastic lamellae and the diffuse pattern of the adventitia.

The high-force, collagen-dominant region of the elastic modulus was determined as the slope of the linear equation (r2>0.99) fitted to the final four data points on the stress-strain curve. The low-force, elastin-dominant region of the stress-strain curve was established by fitting a seventh polynomial equation to the data (r2>0.99; RStudio) and then computing the roots of the equation. The first root was considered the boundary between the very low-force region and the point at which elastin fibers are initially engaged, and the second root was considered the upper boundary of the elastin-dominant region. The elastic modulus of this region was defined as the slope of a linear equation fitted to the stress-strain data between the first and second roots where the curvature is ~0.

***Adventitial collagen-1 abundance (quantitative immunofluorescence)***

Protein abundance of collagen-1 was assessed in ~1 mm sections of thoracic aorta that were excised, frozen and stored in OCT compound, and sectioned as described above (*Ex vivo aortic stiffness with knockdown of target genes*)21,24. Samples were then plated on poly-L-lysine-coated microscope slides, fixed in 2% paraformaldehyde for 10 minutes, washed with PBS, and permeabilized for 15 minutes (0.1% Triton X-100). Slides were blocked with 5% donkey serum for 1 hour in a humidified chamber, and incubated with an anti-collagen-1 primary antibody (1:200; Southern Biotech, Birmingham, AL; Cat# 1310-01) at room temperature for 1 hour. Slides were then washed with PBS and incubated with an anti-goat fluorescent secondary antibody (1:200; AlexaFluor 647; Invitrogen) at room temperature for 30 minutes. Slides were washed, stained with DAPI (1:1000; ThermoFisher Scientific) for 5 minutes, and cured overnight at room temperature with Invitrogen ProLong Gold mounting media (Invitrogen). The slides were then imaged using an EVOS M7000 fluorescence microscope at 20x magnification under identical conditions. Images were analyzed using Invitrogen Celleste 5.0 Image Analysis Software, and the abundance of collagen-1 was determined as the average intensity (A.U.) of the collagen-positive area across n = 4-6 samples/animal. Adventitial and medial layers were assessed separately, but only adventitial collagen abundance was reported, as levels in the media were below the detection limit. Specificity of the collagen-1 antibody was determined using negative controls (secondary antibody-only conditions) in a subset of the samples.

**STATISTICAL ANALYSES**  
 Differences in animal characteristics, peak EDD with siRNA transfection, and elastic modulus with siRNA transfection were assessed using two-way analysis of variance (ANOVA) with factors of age (young or old) or condition (scrambled or siRNA) and group/treatment (control, acetate, or high-fiber diet). Differences in aortic pulse wave velocity (PWV) and carotid artery dose responses were determined using repeated measures general linear models (GLM) with factors of age or condition and group/treatment, and repeated measures of time or dose. When a significant main effect was observed for GLMs/two-way ANOVAs, pairwise comparisons were made using Šidák’s post hoc test. The relation between aortic PWV and gut microbial features were determined using *robustbase: Basic Robust Statistics* regression analyses with the ‘lmrob’ function in the ‘robustbase’ package (RStudio)25. All other outcomes were assessed using one-way ANOVA with Tukey’s post hoc test when a main effect was observed. The cytokine array results were also assessed using one-way ANOVA/Tukey’s post hoc test. Because this was a secondary outcome performed only in a subset of samples, we were underpowered to do a correction for multiple comparisons.

***Power calculations***

Power calculations were performed using G\*power 3.1 for EDD, the primary outcome variable. An effect size of 1.59 was obtained from preliminary data for the difference in peak EDD between old acetate-supplemented and control mice, which necessitated n=12 mice per group to detect differences in peak EDD with 95% statistical power using a two-side significance level of α=0.01. This sample size also ensured >90% statistical power to detect differences in aortic PWV (effect size from preliminary data = 0.97). Additional mice were studied in each group to ensure sufficient data were obtained and to account for up to 50% age-related attrition, as 27 months is the median lifespan in male C57BL/6N mice26. Additional mice were also included for the siRNA transfection experiments to ensure sufficient data were obtained because of the technical challenges of these experiments. Of the 186 mice that were obtained for this study, 26 mice were used for pilot studies to determine the optimal dose and mode of acetate delivery. Of the remaining 160 mice (35 young and 125 old), 14 old mice died or were euthanized per veterinary recommendation prior to baseline testing/intervention administration, and 17 old mice died or were euthanized per veterinary recommendation during the intervention (5 OC, 7 OA, 5 OF), i.e., 24% age-related attrition.

***Experimental randomization, inclusion and exclusion criteria, handling of outliers.***

Animals were randomly assigned to one of the experimental conditions. Data were assessed for statistical outliers (ROUT test; Q = 1%), and outliers were excluded from final analyses.

**SUPPLEMENTAL FIGURES**

**Supplementary Figure S1: Gut microbiome alpha diversity is not altered with aging in mice.** Metagenomic sequencing of fecal samples collected at baseline from young (n=17; ~3 mo.) and old (n=23; ~24 mo.) male C57BL/6N mice rarefied to a sampling depth of 1,362,029 features.Alpha diversity can be assessed using many different statistical comparisons. As such, to confirm our findings we assessed alpha diversity via **A)** Abundance-based Coverage Estimator (ACE), **B)** Chao1, **C)** Pielou's Evenness, **D)** Shannon’s Index, and **E)** Simpson’s Index.

**Supplementary Figure S2: Gut microbial functional pathways related to lipopolysaccharide (LPS) biosynthesis, oxidative stress and maintenance of the intestinal barrier are not altered with aging.** Metagenomic sequencing of fecal samples collected at baseline from young (n=22; ~3 mo.) and old (n=25; ~24 mo.) male C57BL/6N mice. For all analyses presented in this figure, samples were rarefied to frequency of 1,981,501. **A)** QIIME2 was used to generate a heatmap of Bray-Curtis dissimilarity of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (KEGG ortholog classification names available at https://ftp.microbio.me/pub/wol2/function/kegg/) between age groups. The darker the blue the higher abundance of the KEGG ortholog.Next, a combination of the QIIME2 plugins Songbird and Qurro were used to produce differentials and create a visualization to compare log-ratios of specific KEGG ortholog pathways including **B)** LPS biosynthesis, **C)** glutathione metabolism, **D)** nicotinate and nicotinamide metabolism, and **E)** sulfur metabolism.

**Supplementary Figure S3: Gut microbiome composition at baseline and following supplementation with acetate and high-fiber diet feeding.** Metagenomic sequencing of fecal samples in young (Y) and old (O) control (C), acetate-supplemented (A), or high-fiber fed (F) male C57BL/6N mice at baseline and following the intervention period (n=10-14/group). For all analyses, samples were rarefied to a frequency of 1,362,029 which was the minimum number of reads in any sample. **A)** Beta diversity via PCoA of unweighted UniFrac distances. Baseline values are spheres and post-intervention values are rings. **B)** Alpha diversity measured as Faith’s PD. **C-D)** A combination of the QIIME2 plugins Songbird and Qurro were used to produce differentials and create a visualization to compare log-ratios of grouped short-chain fatty acid (SCFA)- and acetate-producing bacterial taxa. Panels B-D: baseline values are light gray on the left and post-intervention values are black on the right.  Filtering specifics included in the **Supplementary Table S2**.

**Supplementary Figure S4:** **Influence of early growth response-1 (Egr-1) signaling on endothelium-independent dilation (EID) in carotid arteries. A)** Peak carotid artery EID in response to the nitric oxide donor sodium nitroprusside (SNP) in arteries obtained from old (O) control (C) or acetate-supplemented (A) male C57BL/6N mice incubated for 24 hours in media containing siRNA against Egr-1 or scrambled (n=3-4/group). Data are mean ± SEM. Statistics are two-way mixed (group x condition) ANOVA with Šídák's post-hoc test. \*\*\*\**P*<0.0001.

**SUPPLEMENTAL TABLES**

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| **Supplementary Table S1: Traditional grain-based rodent chow vs. high-fiber supplemented mouse chow macronutrient comparison.** | | |
|  | **Traditional Grain-Based Rodent Chow (7917)** | **High-fiber Rodent Chow (7917 supplemented with 7.5% inulin)** |
| Energy Density (kcal / gram) | 3.0 | 2.9 |
| Protein (% kcal) | 24 | 23.1 |
| Carbohydrate (% kcal) | 62 | 63.3 |
| Fat (% kcal) | 14 | 13.6 |
| Natural Detergent Fiber (%w/w) | 13.6 | 12.7 |
| Estimated Soluble Fiber (%w/w) | ~2-3 | ~9.5-10.5 |
| The high-fiber diet was designed by Teklad Nutritionist: Derek Martin, PhD, RD, who also provided estimates for soluble fiber. Only an estimate is available as the traditional diets are not formulated for soluble fiber or tested for it, with the latter being due to lack of standard methodology for doing so. Natural detergent fiber is an estimate of insoluble fiber. A full list of the micro- and macronutrients included in Inotiv 7917 can be found on the company website (<https://www.inotivco.com/rodent-traditional-natural-ingredient-diets>). | | |

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| **Supplementary Table S2: Specific bacteria compiled to create taxa of interest and KEGG ortholog groups.** | |
| SCFA Producing Bacteria | *Faecalibacterium prausnitzi*27–29*i | Bacteroides thetaiotaomicron*30,30–35 *| Roseburia*36–38 *| Roseburia intestinalis*32,39,40 *| Eubacterium rectale*41,42 *| Eubacterium hallii*43–45 *| Bacteroides ovatus | Akkermansia muciniphila*46–51 *| Coprococcus catus | Coprococcus comes*28,52,53 *| Veillonella parvula | Veillonella atypica*53–56 *| Megasphaera elsdenii*57–59 *| Anaerostipes caccae | Anaerostipes hadrus*60–63 *| Butyricicoccus pullicaecorum*28,64,65 *| Bifidobacterium adolescentis*66,67 *| Bifidobacterium breve*68–71 *| Ruminococcus obeum | Ruminococcus flavefaciens*72–74 *| Prevotella copri*75–77 *| Prevotella ruminicola*78 |
| Acetate Producing Bacteria | *Bacteroides thetaiotaomicron*30,30–35 *| Faecalibacterium prausnitzii*30,79–82 *| Roseburia intestinalis*32,39,40 *| Eubacterium hallii*43–45 *| Bifidobacterium adolescentis*66,67 *| Prevotella copri*75–77 *| Prevotella ruminicola*78 |
| Anti-inflammatory Bacteria | *Bifidobacterium infantis | Bifidobacterium longum*83–87 *| Lactobacillus acidophilus | Lactobacillus rhamnosus*88–91 *| Faecalibacterium prausnitzii*27–29 *| Akkermansia muciniphila*46–51 *| Roseburia intestinalis*32,39,40 *| Lactococcus lactis*92–94 *| Butyricicoccus pullicaecorum*95–97 *| Bacteroides fragilis*98–100 *| Prevotella histicola*101–104 *| Roseburia hominis*105–108 *| Eubacterium rectale*109–112 |
| Pro-inflammatory Bacteria | *Escherichia coli | Fusobacterium nucleatum*113–116 *| Klebsiella pneumoniae*117–120 *| Salmonella*121–123 *| Helicobacter pylori*124–127 *| Enterococcus faecalis*128–130 *| Clostridium difficile*131–133 *| Campylobacter jejuni*134–136 *| Enterococcus faecium*137–139 *| Streptococcus pneumoniae*140–142 *| Staphylococcus aureus*143,144 |
| LPS Producing Bacteria | *Escherichia coli*145,146 *| Salmonella enterica*147,148 *| Klebsiella pneumoniae*145,146 *| Pseudomonas aeruginosa*149,150 *| Proteus mirabilis | Proteus vulgaris*151–154 *| Acinetobacter baumannii*155–158 *| Helicobacter pylori*124–127 |
| Mucosal Degrading  Bacteria | *Bacteroides fragilis | Clostridium perfringens*159,160 *| Enterococcus faecalis*128–130 *| Fusobacterium nucleatum*113–116 *| Akkermansia muciniphila*40,81,161–163 *| Bacteroides thetaiotaomicron*30,31 *| Prevotella copri*75–77 *| Ruminococcus gnavus*164–166 *| Vibrio cholerae*167–169 *| Desulfovibrio*170–173 |
| SCFA Pathways | ko00650: Butanoate metabolism174,175 | ko00640: Propanoate metabolism176 | ko00630: Glyoxylate and dicarboxylate metabolism177 | ko00620: Pyruvate metabolism176,178,179 | ko00040: pentose and glucuronate interconversions180 |
| LPS Biosynthesis | ko00540: Lipopolysaccharide biosynthesis181,182 |
| Glutathione Metabolism | ko00480: Glutathione metabolism183 |
| Nicotinate & Nicotinamide Metabolism | ko00760: Nicotinate and nicotinamide metabolism184,185 |
| Sulfur Metabolism | ko00920: Sulfur metabolism186,187 |
| We used the QIIME2 plugin Qurro to create visualizations of feature log-ratios in the context of feature rankings generated using the QIIME2 plugin Songbird. Bacterial taxa (families, genus and species) selected to represent each group needed to have been previously shown to be associated with their assigned group and had to be present in the majority of samples. KEGG orthologs were also analyzed, and we only grouped short-chain fatty acid (SCFA) pathways based on their ability to affect SCFA metabolism. Other pathways were individually analyzed. Abbreviations: Lipopolysaccharide, LPS. | |

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| **Supplementary Table S3: Food and water intake, body and tissue masses, and artery characteristics** | | | | | | | | | | |
|  | **Group** | | | | | | | **ANOVA *P* Values** | | |
|  | **YC** | **YA** | **YF** | **OC** | **OA** | **OF** | **Interaction** | | **Age** | **Treatment** |
| *n* | 11 | 12 | 12 | 34 | 26 | 28 |  | |  |  |
| Water intake (ml/day) | 3.5 ± 0.3 | 3.1 ± 0.2 | 3.3 ± 0.1 | 3.3 ± 0.2 | 3.4 ± 0.2 | 3.7 ± 0.1 | 0.260 | | 0.278 | 0.595 |
| Energy intake (kcal/day) | \*12.1 ± 0.5 | \*11.5 ± 0.2 | \*11.1 ± 0.2 | 17.0 ± 1.1 | 16.0 ± 0.8 | † 13.6 ± 0.6 | 0.356 | | <0.0001 | 0.047 |
| Food intake (g/day) | \*4.0 ± 0.2 | \*3.8 ± 0.1 | 3.8 ± 0.1 | 5.7 ± 0.4 | 5.3 ± 0.3 | 4.7 ± 0.2 | 0.387 | | <0.0001 | 0.141 |
| Frailty index | \*0.083 ± 0.011 | \*0.078 ± 0.006 | \*0.073 ± 0.010 | 0.358 ± 0.010 | 0.324 ± 0.013 | 0.330 ± 0.019 | 0.743 | | <0.0001 | 0.507 |
| Body mass at euthanasia (g) | 29.8 ± 1.1 | 30.9 ± 0.8 | 29.9 + 0.7 | 27.2 ± 0.6 | 28.5 ± 0.6 | 27.8 ± 0.6 | 0.956 | | <0.001 | 0.313 |
| Tibia length (mm) | 18.9 ± 0.3 | 19.6 ± 0.4 | 19.3 ± 0.3 | 19.2 ± 0.1 | 19.3 ± 0.2 | 19.5 ± 0.2 | 0.430 | | 0.717 | 0.166 |
| *Mass of key organs* | | | | | | | | | | |
| Heart (mg) | \*142 ± 5 | \*138 ± 2 | \*141 ± 4 | 178 ± 5 | † 195 ± 4 | ‡ 176 ± 4 | 0.092 | | <0.0001 | 0.046 |
| Heart (g) / tibia length | \*7.3 ± 0.1 | \*7.2 ± 0.1 | \*7.3 ± 0.2 | 9.3 ± 0.2 | 10.1 ± 0.2 | 9.1 ± 0.2 | 0.118 | | <0.0001 | 0.208 |
| Left ventricle (mg) | \*85 ± 4 | \*93 ± 8 | \*80 ± 4 | 106 ± 4 | 116 ± 4 | 104 ± 3 | 0.982 | | <0.0001 | 0.030 |
| Left ventricle (g) / tibia length | \*4.4 ± 0.3 | \*4.9 ± 0.3 | \*4.2 ± 0.2 | 5.6 ± 0.2 | 6.0 ± 0.2 | 5.3 ± 0.2 | 0.796 | | <0.0001 | 0.022 |
| Liver (g) | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.3 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.1 | 0.602 | | 0.183 | 0.700 |
| Kidneys (mg) | 383 ± 10 | 397 ± 10 | 360 ± 9 | 410 ± 11 | 426 ± 13 | 405 ± 8 | 0.173 | | 0.087 | 0.004 |
| Visceral fat (g) | \*0.81 ± 0.1 | \*0.89 ± 0.07 | \*0.88 ± 0.06 | 0.24 ± 0.03 | 0.26 ± 0.03 | 0.27 ± 0.03 | 0.835 | | <0.0001 | 0.535 |
| Spleen (mg) | 67 ± 3 | 66 ± 3 | 67 ± 3 | 72 ± 4 | 82 ± 5 | 70 ± 4 | 0.793 | | 0.293 | 0.821 |
| *Artery characteristics (µm)* | | | | | | | | | | |
| Carotid diameter (resting) | \*413 ± 22 | 433 ± 16 | 423 ± 15 | 481 ± 10 | 479 ± 9 | 467 ± 8 | 0.605 | | <0.0001 | 0.696 |
| Carotid diameter (max) | \*423 ± 23 | 442 ± 16 | 430 ± 16 | 488 ± 10 | 484 ± 10 | 471 ± 8 | 0.628 | | <0.0001 | 0.670 |
| Carotid constriction to phenyl-ephrine (%) | 18 ± 2 | 22 ± 3 | 16 ± 2 | 15 ± 2 | 21 ± 3 | 17 ± 2 | 0.602 | | 0.494 | 0.039 |
| Aorta diameter (resting) | 624 ± 18 | 614 ± 16 | 601 ± 14 | 644 ± 14 | 666 ± 16 | 645 ± 16 | 0.569 | | 0.004 | 0.550 |
| Aorta wall thickness | \*34 ± 1 | \*34 ± 1 | \*33 ± 1 | 46 ± 2 | 45 ± 2 | 45 ± 3 | 0.837 | | <0.0001 | 0.963 |
| Data are the mean ± SEM. Groups are young (Y) or old (O) control (C), acetate supplemented (A), or high-fiber diet fed (F) mice (ex. YA = young mouse supplemented with acetate). Statistics are two-way (age x treatment) ANOVA with Šídák’s multiple comparison's test when a significant main effect was observed. \**P*<0.05 vs. O within treatment group, †*P*<0.05 vs. C within age group, ‡*P*<0.05 A vs. F within age group. | | | | | | | | | | |

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| --- | --- | --- | --- | --- | --- |
| **Supplementary Table S4: Plasma levels of inflammatory cytokines.** | | | | | |
| **Cytokine** | **YC** | **OC** | **OA** | **OF** | **ANOVA *P* Value** |
| **bFGF** | 288 ± 41 | 305 ± 34 | 310 ± 67 | 312 ± 43 | 0.9846 |
| **BLC** | 1241 ± 228 | \* 7112 ± 1312 | \* 6161 ± 1323 | 3293 ± 933 | **0.0026** |
| **Eotaxin** | 17347 ± 1028 | 21228 ± 1425 | 18823 ± 1168 | 20813 ± 1155 | 0.1357 |
| **Eotaxin-2** | 1021 ± 109 | 1015 ± 187 | 926 ± 145 | 948 ± 134 | 0.9647 |
| **Fas L** | 286 ± 47 | 469.3 ± 107 | 317 ± 57 | 353 ± 75 | 0.3857 |
| **G-CSF** | 724.5 ± 112 | 2935 ± 711 | 2474 ± 503 | 2142 ± 524 | 0.0527 |
| **GM-CSF** | 284 ± 34 | 421 ± 107 | 285 ± 37 | 273 ± 33 | 0.3199 |
| **ICAM-1** | 1181 ± 141 | 1273 ± 134 | 1369 ± 150 | 1190 ± 106 | 0.7492 |
| **IFN-γ** | 463 ± 44 | 877 ± 174 | 513 ± 49 | 778 ± 152 | 0.0873 |
| **IL-1α** | 1389 ± 170 | 1408 ± 107 | 1221 ± 191 | 1572 ± 158 | 0.4625 |
| **IL-1β** | 126 ± 7 | 182 ± 24 | 130 ± 9 | 177 ± 26 | 0.101 |
| **IL-2** | 160 ± 16 | 304 ± 64 | 192 ± 19 | 226 ± 29 | 0.0764 |
| **IL-3** | 643 ± 85 | 635 ± 100 | 654 ± 65 | 548 ± 82 | 0.7979 |
| **IL-4** | 129 ± 7 | 141 ± 14 | 139 ± 18 | 113 ± 13 | 0.3993 |
| **IL-5** | 197 ± 35 | 614 ± 150 | 229 ± 30 | 984 ± 498 | 0.2451 |
| **IL-6** | 442 ± 83 | 1226 ± 339 | 780 ± 75 | 655 ± 86 | 0.0906 |
| **IL-7** | 59 ± 16 | 97 ± 19 | \* 162 ± 34 | 131 ± 22 | **0.0279** |
| **IL-10** | 114 ± 12 | 178 ± 19 | 116 ± 18 | 176 ± 24 | **0.0435** |
| **IL-12p40** | 124 ± 19 | 130 ± 23 | 92 ± 21 | 130 ± 34 | 0.7069 |
| **IL-13** | 168 ± 14 | 262 ± 58 | 168 ± 16 | 185 ± 22 | 0.181 |
| **IL-15** | 203 ± 16 | 433 ± 107 | 234 ± 23 | † 167 ± 17 | **0.0236** |
| **IL-17** | 359 ± 70 | 660 ± 190 | 426 ± 78 | 335 ± 35 | 0.1995 |
| **IL-21** | 1150 ± 76 | 1140 ± 112 | 1226 ± 70 | 1135 ± 75 | 0.8696 |
| **KC** | 179 ± 38 | \* 485 ± 117 | 301 ± 40 | † 205 ± 40 | **0.0211** |
| **Leptin** | 560 ± 122 | \* 197 ± 58 | \* 121 ± 21 | \* 127 ± 28 | **<0.0001** |
| **LIX** | 215 ± 29 | 224 ± 32 | 150 ± 9 | 261 ± 26 | 0.0858 |
| **MCP-5** | 362 ± 59 | 891 ± 195 | 757 ± 185 | 629 ± 136 | 0.1624 |
| **M-CSF** | 96 ± 16 | 94 ± 24 | 79 ± 26 | 96 ± 16 | 0.9368 |
| **MIG** | 98 ± 35 | 150 ± 42 | 181 ± 37 | 194 ± 39 | 0.3129 |
| **MIP-1γ** | 30547 ± 1499 | 30284 ± 2797 | 29508 ± 2568 | 30917 ± 1746 | 0.9781 |
| **PF-4** | 24177 ± 1798 | 23788 ± 1541 | 25342 ± 1870 | 24331 ± 1435 | 0.9247 |
| **RANTES** | 356 ± 42 | 1204 ± 438 | 689 ± 246 | 805 ± 209 | 0.2457 |
| **TCA-3** | 513 ± 101 | 388 ± 43 | 343 ± 13 | 468 ± 65 | 0.2489 |
| **TNF RI** | 30339 ± 1735 | 29477 ± 2884 | 38131 ± 2981 | 37378 ± 3000 | 0.0656 |
| **TNF RII** | 11776 ± 989 | 13187 ± 795 | \* 17383 ± 2126 | 15801 ± 923 | **0.0183** |
| **TNFα** | 236 ± 22 | 310 ± 56 | 258 ± 35 | 318 ± 53 | 0.5912 |
| Data are mean ± SEM and are expressed as A.U. of intensity relative to an internal positive control. Plasma was analyzed by cytokine array. n = 9-13/group. Statistics are one-way ANOVA with Šídák’s multiple comparison's test when a significant main effect was observed. \*P<0.05 vs. YC, †P<0.05 vs. control within old mice. Abbreviations: Y, young; O, old; C, control; A, acetate-supplemented; F, high-fiber diet fed; bFGF, basic fibroblast growth factor; BLC, B lymphocyte chemoattractant; Fas L, Fas ligand; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IL-, interleukin-; KC, keratinocyte chemoattractant; LIX, lipopolysaccharide-induced CXC chemokine; MCP-5, monocyte chemoattractant protein-5; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by γ; MIP-1γ macrophage inflammatory protein-1γ; PF-4, platelet factor 4; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; TCA-3, T cell activation gene 3; TNF R, tumor necrosis factor receptor. | | | | | |

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