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

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Abstract

Adverse changes in the gut microbiome with aging are an emerging mediator of arterial dysfunction, which contributes to cardiovascular disease (CVD) development. We investigated the therapeutic potential of enhancing the bioavailability of gut-derived short-chain fatty acids (SCFAs; produced from dietary fiber) for improving age-related arterial dysfunction. We performed gut microbial whole-genome sequencing in young (3 months) versus old (24 months) male C57BL/6N mice to explore changes in bacterial taxonomic abundance and functional pathways with aging and relations to arterial function. We then supplemented young and old mice with the SCFA acetate in drinking water versus controls and versus a high-fiber diet

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Author Contributions

VB conceived the work. AGL, NT, NV, BZ, KL, MW, DS and VB designed the work. AGL, NT, AG, AL, NV, SM, GR, ZC, BZ, KL and VB acquired and/or analyzed data. AGL, NT, AG, GR, KL, DS and VB interpreted data. AGL, NT, and VB wrote the manuscript which was critically revised by all other authors. All authors have approved the final version of the manuscript, agree to be accountable for all aspects of the work, and qualify for authorship.

Disclosures

Rob Knight, a scientific advisory board member and consultant for BiomeSense, Inc., has equity and receives income. He is a scientific advisory board member and has equity in GenCirq. He is a consultant and scientific advisory board member for DayTwo and receives income. He has equity in and acts as a consultant for Cybele. He is a cofounder of Biota, Inc., and has equity. He is a cofounder of Micronoma, has equity, and is a scientific advisory board member. The terms of these arrangements have been reviewed and approved by the University of California, San Diego, in accordance with its conflict of interest policies.

Supplementary Materials

Supplemental information can be found here: Supplementary.

for 8–10 weeks to test the effects of these interventions on vascular function and explore potential mechanisms. Of the various differences in the gut microbiomes of old mice, lower SCFA-producing capacity (taxonomic abundance and functional pathways) stood out as a key feature related to worse arterial function after adjusting for age. Acetate supplementation and a high-fiber diet reversed ~30% of the age-related increase in aortic pulse wave velocity (stiffness) and fully restored carotid artery endothelium-dependent dilation (endothelial function) to young levels. Acetate and a high-fiber diet reduced age-related increases in systemic inflammation. We also found that improvements in endothelial function were likely mediated by suppressed early growth response-1 signaling using innovative siRNA-based knockdown in isolated arteries. There were no effects of the interventions in young mice. Acetate supplementation was comparably effective for ameliorating arterial dysfunction with aging as a high-fiber diet and thus shows promise for reducing CVD risk in older adults.

Introduction

Aging is the leading risk factor for cardiovascular diseases (CVDs)¹, largely due to the development of arterial dysfunction^{2,3}. Age-related arterial dysfunction primarily occurs due to inflammation and oxidative stress, which exacerbate each other and reduce the bioavailability of the vasodilatory and vaso-protective molecule nitric oxide (NO)². The gut microbiome, the collection of microorganisms that reside within the gastrointestinal tract, is altered with aging^{4,5}. This “dysbiosis” of the gut microbiome is now considered to be one of the hallmarks of aging⁶, as changes in gut microbiome composition and certain microbiome-derived metabolites appear to directly increase chronic low-grade inflammation⁷ and oxidative stress⁸. In particular, these changes contribute to arterial dysfunction by increasing systemic inflammation and oxidative stress^{9,10}. Therefore, interventions that improve the homeostatic balance of the gut microbiome or mimic the effects of a young, healthy gut microbiome may improve arterial dysfunction with aging and reduce the risk of CVD.

This goal could be achieved through increasing the bioavailability of short-chain fatty acids (SCFAs). SCFAs, primarily acetate, butyrate, and propionate, are produced by gut microbiome-dependent fermentation of dietary soluble fibers, with acetate being the most abundant in circulation in humans by approximately tenfold¹¹. SCFAs have gained significant attention in the biomedical research community, as they improve multiple facets of CV and overall physiological function in both murine models and humans^{12,13}. Under basal conditions, fecal levels of SCFAs, an indirect marker of SCFA production, are lower with aging in humans⁴ and mice¹⁴, and two small cohort studies in humans demonstrated that bacterial genetic pathways involved in SCFA production are lower with aging^{4,5}. Thus, enhancing the bioavailability of SCFAs may improve arterial function with aging.

Consumption of a high-fiber diet, which is known to improve CVD risk factors and to be associated with reduced CVD-related morbidity and mortality¹⁵, typically increases SCFA production in healthy individuals¹⁶. However, adherence to high-fiber diets is poor, with the average U.S. adult consuming only ~50% of the recommended dietary intake¹⁷. In addition, a potential reduction in gut bacterial SCFA-producing capacity with age suggests that high-fiber diets may be less effective for enhancing circulating levels of SCFAs in

older adults. Indeed, fecal SCFAs show only modest increases or do not change in older adults and mice in response to high-fiber diet consumption^{18,19}. Together, these observations suggest that directly supplementing SCFAs holds the greatest promise for improving arterial function with aging.

Previous literature suggests that the therapeutic effects of SCFAs are mediated at least in part via direct anti-inflammatory mechanisms, for example, by increasing production of anti-inflammatory cytokines²⁰. Another potential mechanism could be via downregulation of the transcription factor early growth response-1 (*Egr-1*). Using transcriptomics, Marques et al. identified various common genes that were differentially expressed in the hearts and kidneys of hypertensive mice following both acetate supplementation and high-fiber diet feeding²¹. Of these, downregulation of *Egr-1* stood out as being particularly relevant in the context of arterial function. *Egr-1* is expressed in endothelial and vascular smooth muscle cells and is implicated in CVD pathophysiology²². In addition, upregulation of *Egr-1* adversely affects several downstream targets that modulate arterial function, including stimulating the production of proinflammatory cytokines²³.

The primary purpose of this study was to determine the efficacy of acetate supplementation for improving age-related arterial dysfunction. Accordingly, in young and old male C57BL/6N mice, we first assessed gut microbiome composition and genes regulating bacterial functional capacity. In particular, we explored genes related to SCFA and acetate production, as studies in humans have been conducted in very few people⁵ and are confounded by diets, medications, and other environmental factors. We determined that, under controlled conditions, SCFA-related bacterial abundance and functional pathways are lower with aging. We also demonstrated that these changes in the gut microbiome correlated with impaired arterial function, whereas others (e.g., higher abundance of lipopolysaccharide [LPS]-producing bacteria) did not, thus providing rationale for supplementing acetate to improve age-related arterial dysfunction. We then performed an intervention study wherein young and old mice were supplemented with acetate in drinking water versus control water for 8 weeks and found that acetate improved arterial function in old mice. We also included a high-fiber diet group to determine the relative efficacy of acetate supplementation compared to a high-fiber diet. Finally, we explored potential mechanisms that may mediate improvements in arterial function with our interventions. We observed that our interventions reduced systemic inflammation and that reductions in *Egr-1* signaling appeared to mediate improvements in endothelial function with acetate supplementation. Overall, we identified a novel intervention, acetate supplementation, for improving arterial function with aging that could have implications for reducing CVD risk.

Methods

All the data presented in this article are available in online repositories. Raw gut microbial DNA sequences were deposited in EBI-ENA (see the Supplemental Materials). All other data were deposited in FigShare (10.6084/m9.figshare.26054122 and 10.6084/m9.figshare.26054119). Investigators were blinded to treatment groups during data collection and analyses. Further details on all procedures and analyses are provided in the Experimental Procedures section in the Supplemental Material.

Animals

The protocol was approved by the University of Colorado Boulder Institutional Animal Care and Use Committee and adhered to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals²⁴. 160 male C57BL/6N mice were obtained from Charles River (n = 35 young, received at ~8 weeks of age) or the National Institute of Aging colony maintained by Charles River (n = 125 old, received at ~20–24 months of age). Of these, 31 old mice died or were euthanized per veterinary recommendation prior to or during testing/intervention administration (24% age-related attrition), such that 35 young and 94 old mice were studied (see the Power calculations section in the Supplemental Material for details). As we have not been able to consistently show female mice exhibit age-related carotid artery endothelial dysfunction (our primary outcome), potentially due to differing hormonal patterns as compared to older women²⁵, we chose to only study male mice, which are an established model of human arterial aging^{9,10,26,27}. Mice were acclimated to our facilities for 4 weeks prior to baseline testing and provided with ad libitum access to a traditional grain-based rodent chow (Inotiv 7917; Inotiv, West Lafayette, IN; stored at room temperature) + drinking water (Boulder, CO; municipal tap water that underwent reverse osmosis and chlorination). Mice were single-housed, to prevent gut microbiome contamination due to coprophagy, in a standard facility with a 12-hour light/dark cycle.

Interventions

Mice randomly received one of three interventions beginning at 3 (young; Y) or 24 (old; O) months of age: (a) Control (YC/OC): control rodent chow (Inotiv 7917) + normal drinking water; (b) Acetate supplementation (YA/OA): control rodent chow + 100 mM calcium acetate (Spectrum Chemical Mfg. Corp., New Brunswick, NJ) in drinking water; or (c) High-fiber diet (YF/OF): custom formulated chow (Inotiv 7917 supplemented with 7.5% inulin; stored at 2°C–4°C) + normal drinking water. Mice from each age and intervention group were housed and studied simultaneously to control for confounding environmental factors on the gut microbiome. The interventions were administered for 8–10 weeks—two mice were euthanized per day for endothelium-dependent dilation (EDD) experiments (see below). Diet macro- and micronutrients are presented in Supplemental Table S1.

Calcium acetate was selected because other forms (e.g., magnesium acetate) were not well tolerated in pilot studies and it is already US Food and Drug Administration (FDA)-approved (ID: 2924927), which would facilitate later translation to humans. This concentration was selected because, in pilot studies, it was well tolerated, and a higher concentration (200 mM) did not induce greater benefits. The intervention was administered in drinking water to parallel human oral supplementation. We used normal drinking water as the control versus a calcium-containing placebo (e.g., calcium carbonate or calcium citrate) because these are not readily dissolvable in water. Furthermore, our pilot studies with calcium citrate (albeit not at a consistent dose due to solubility issues) showed no effect on arterial function outcomes in old mice relative to normal drinking water.

Overview of experimental outcomes

At baseline, aortic stiffness was assessed *in vivo* via aortic pulse wave velocity (PWV)²⁶, a corollary to carotid-femoral PWV in humans which predicts future CVD²⁸, and

fecal samples were collected for gut microbiome metagenomic sequencing²⁹. Sequence processing was conducted using the following steps: (a) adaptor removal using fastp v0.23.2³⁰; (b) host filtering using minimap2 v2.17³⁰; and (c) the obtainment of taxonomic and functional profiles using woltka v0.1.4³¹, which aligns sequences against WoLr2³¹ via bowtie v2.5.1. Further analyses of gut bacterial features were conducted using Qiita³² and QIIME2-2020.6³³.

Following the intervention, PWV was reassessed, and mice were later euthanized by cardiac exsanguination while anesthetized under inhaled isoflurane. Whole blood was collected and either heparinized, centrifuged, frozen, and stored at -80°C for analysis of plasma levels of proinflammatory cytokines, or allowed to coagulate and then centrifuged to obtain serum for use in transfection experiments.

Carotid arteries were excised and either: (a) cannulated in standard pressurized myograph chambers (111P; DMT, Aarhus, Denmark) for immediate assessment of EDD to increasing doses of acetylcholine (ACh) and endothelium-independent dilation (EID) to increasing doses of the NO donor sodium nitroprusside^{10,26}; or (b) cannulated in culture pressure myograph chambers (204CM; DMT), cultured for 24 hours with siRNA to suppress Egr-1 versus a scrambled siRNA control and 1% of the mouse's own serum (to closer mimic *in vivo* conditions), and then used to assess EDD and EID³⁴. EDD/EID were assessed in carotid (conduit) arteries to provide a corollary to brachial artery flow-mediated dilation in humans, a predictor of future CVD³⁵.

Thoracic aortas were excised, cleaned of surrounding tissue, and then either: (a) segmented into $\sim 1\text{--}1.5$ mm sections, of which two sections were used immediately to assess whole cell or mitochondrial reactive oxygen species (ROS) bioactivity (electron paramagnetic resonance spectroscopy)¹⁰, and the other sections were stored in optimal cutting temperature compound at -80°C for later assessment of adventitial collagen abundance (immunofluorescence)³⁶ and aortic diameter and intima-media thickness (IMT; light microscopy); or (b) segmented into ~ 5 mm sections, cultured for 24 or 48 hours with siRNA or a control scrambled oligonucleotide + 1% of the mouse's own serum in the lumen (ends of each section were tied off), and then either used for stress-strain testing^{9,37} or to verify knockdown of Egr-1. Stress-strain testing was assessed under passive (no active vascular smooth muscle contraction) conditions and thus isolated the structural component of arterial stiffness.

Statistical analyses

Detailed descriptions of the gut microbiome and statistical analyses are provided in the Supplemental Materials. Data are presented as mean \pm standard error of the mean (SEM) unless specified otherwise. Statistical significance was set to $\alpha = 0.05$. Statistical analyses were performed using QIIME2-2020.6, Prism version 10.0.3 (GraphPad Software, San Diego, CA), IBM SPSS Statistical Professional 28 (IBM, Armonk, NY), or RStudio version 2023.06.1 + 524 (Rstudio, Boston, MA).

Results

Gut microbiome composition and functional capacity are altered with aging, with notable reductions in SCFA-producing capacity

Gut microbiome composition and functional capacity were assessed using metagenomic sequencing of fecal samples obtained from young and old mice (samples collected prior to the intervention study presented below). Beta diversity, assessed using principal coordinates analysis of unweighted UniFrac distances, showed distinct clustering of bacterial composition between young and old mice ($P = 0.001$; Fig. 1A). Alpha diversity was not different between age groups (Faith's Phylogenetic Diversity [$P = 0.164$; Fig. 1B]; Abundance-based Coverage Estimator [$P = 0.718$]; Chao1 [$P = 0.489$]; Pielou's Evenness [$P = 0.074$]; Shannon's Index [$P = 0.062$]; and Simpson's Index [$P = 0.261$]; Supplementary Fig. S1). Next, we performed an analysis of composition of microbiomes (ANCOM) to determine differentially abundant bacterial features between groups (Fig. 1C). The abundance of several features known to contribute to SCFA production was lower with aging, including species within the genera *Prevotella*³⁸, *Lactobacillus*³⁹, and *Dorea*⁴⁰. In addition, multiple features within the core bacterial genus *Bacteroides*, some of which have been shown to contribute to SCFA production^{41,42}, were reduced with aging. Conversely, various pathogenic/opportunistic and pro-inflammatory bacteria (e.g., *Escherichia coli*⁴³ and taxa within the *Enterococcus* genus⁴⁴) were more abundant with aging.

To more thoroughly explore taxonomic changes with aging, we used the QIIME2³³ plugins Songbird⁴⁵ and Qurro⁴⁶. We selected and grouped taxa to compare across age groups based on our ANCOM results, including: key SCFA-producing, acetate-producing (subset of SCFA-producing), anti-inflammatory, and pro-inflammatory taxa. We also assessed taxa shown in previous literature to be altered with aging, including LPS-producing and mucosal-degrading taxa^{47,48}. All features selected to be included in these groupings have been established in the literature; see Supplementary Table S2 for a detailed list of all groupings explored. The abundance of key acetate-producing ($P = 0.021$), SCFA-producing ($P < 0.001$), and other anti-inflammatory bacteria (i.e., non-SCFA producers that suppress immune stimulation and increase production of anti-inflammatory cytokines) was lower in old compared to young mice ($P < 0.0001$; Fig. 1D). In contrast, the abundance of various proinflammatory ($P < 0.0001$) and LPS-producing bacteria ($P < 0.0001$) (LPS is a generally pro-inflammatory membrane component of Gram-negative bacteria) increased with aging. The relative abundance of major intestinal mucosa-degrading bacteria, which have been associated with increased intestinal permeability^{49,50}, was also elevated with aging ($P = 0.008$).

Functional capacity of the gut microbiome was compared between age groups using Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs⁵¹. KEGG orthologs are collections of genes that are clustered based on their functional similarity and are used to assess biological pathways and molecular interactions that occur within a given ecosystem (e.g., an individual's gut microbiome). We first generated a heatmap to visualize group differences in abundance of KEGG orthologs (Supplementary Fig. S2) but found no obvious differences between age groups. Similar to the differential abundance of bacterial taxa

analyses described above, differences in specific pathways may exist without obvious overall visual changes. As such, we selected KEGG orthologs based on age-related changes found in our differential abundance analysis above and previous reports^{5,52-54}. We found that KEGG orthologs associated with SCFA production were significantly less abundant in old versus young mice (Fig. 1E). Other grouped KEGG orthologs that were investigated were not different between age groups (Supplementary Fig. S2).

Overall, the reduced abundance of SCFA- and acetate-producing bacterial taxa, along with reduced SCFA-producing pathways, suggests that the biochemical capacity of the gut microbiota to ferment soluble fiber into acetate is reduced with aging. Our findings also suggest that, with aging, there is a shift from an anti-inflammatory to a more pro-inflammatory gut microbiome.

Abundance of SCFA-producing bacteria is independently related to aortic stiffness

We next explored the relation between specific gut bacterial features observed above and arterial function to identify pathways that could be viable therapeutic targets for improving arterial function with aging. We used aortic PWV as our measure of arterial function as it is noninvasive, can be measured serially *in vivo*, and was measured concomitantly with fecal sample collection (pre-intervention). Our other primary functional outcome (endothelial function) was not used in this analysis, as it was obtained only terminally/post-intervention.

We found that aortic stiffness (PWV) was ~25% higher in old compared to young mice (YC vs. OC: 334 ± 5 vs. 421 ± 7 cm/sec, $P < 0.001$), consistent with previous reports²⁶. Using *robustbase: Basic Robust Statistics* regression analyses⁵⁵, we examined the relation between aortic stiffness and groups of gut bacterial features that were altered with aging (see Fig. 1D). The abundance of SCFA-, acetate-, and LPS-producing and anti-inflammatory, pro-inflammatory, and mucus-degrading bacteria was all related to increased aortic stiffness (Table 1). After adjusting for age, there was no longer an independent relation between PWV and LPS-producing or mucus-degrading bacteria (both $P = 0.340$). In contrast, the independent relation between PWV and anti-inflammatory, pro-inflammatory, and SCFA-producing bacteria persisted (all $P = 0.072$). These findings suggest that such changes in the inflammatory potential and SCFA-producing capacity of the gut microbiome may contribute to increases in aortic stiffness with aging. As previous studies have demonstrated that reducing inflammatory signaling improves age-related arterial dysfunction⁵⁶, we sought to instead determine if increasing the bioavailability of SCFAs, specifically acetate, could improve age-related artery dysfunction.

Animal characteristics and gut microbiome composition

Next, we conducted an intervention study in which young and old mice were randomized to receive: (a) acetate; (b) a high-fiber diet; or (c) control conditions for 8–10 weeks. Water and food intake and animal characteristics are presented in Supplementary Table S3. There was no effect of age or treatment on water intake, indicating that oral acetate supplementation was well tolerated. Relative to young mice, old mice had a higher energy intake (kcal/day). Energy intake was slightly lower in the old high-fiber diet compared with control mice due to the lower caloric content of the high-fiber diet ($P = 0.025$), but there

were no group differences in consumption of grams of food per day ($P = 0.141$ vs. OC). Furthermore, there were no group differences in body mass at euthanasia, suggesting caloric needs were adequately met in these mice. There were no effects of either intervention on energy intake in young mice. Scores on a 31-point frailty index, performed to provide a composite characterization of frailty and overall declines in physical status with aging, were higher in old versus young mice, but there was no effect of either treatment. With aging, body and visceral fat masses were lower and heart and left ventricular masses were higher, whereas there were no age-related effects on kidney, liver, or spleen masses. There was an effect of the treatment group on heart and left ventricular masses, such that both were slightly higher in old acetate mice, but there were no differences between old acetate and control mice when these variables were normalized to tibia length to account for body size (both $P = 0.254$). There was an effect of treatment group on kidney mass, although pairwise comparisons were not significant (OA vs. OC and OF, both $P = 0.441$). Overall, acetate was well tolerated and did not appear to have obvious adverse side effects.

We assessed aspects of gut microbiome composition in samples collected before and at the end of the interventions. In Principal Coordinate Analysis (PCoA) analyses that compared all age/treatment groups and samples from both time points, there was a significant main effect on beta diversity ($P = 0.01$; Supplementary Fig. S3A). However, pairwise comparisons of pre versus end-intervention within each age/treatment group did not reach statistical significance (i.e., the significant main effect may have been driven by age; q value [adjusted for FDR] for pre/post intervention within all groups $= 0.068$; Supplementary Fig. S3A). Alpha diversity was altered by the high-fiber diet in young mice ($P = 0.003$) but was unaltered in all other groups (all $P = 0.587$; Supplementary Fig. S3B). SCFA- and acetate-producing taxa were also unchanged following the interventions in all groups (all $P = 0.102$; Supplementary Fig. S3C,D). Thus, we were not able to detect any appreciable changes in gut microbiome composition with acetate supplementation or a high-fiber diet.

Acetate and high-fiber diet consumption improve endothelial function in old mice

Carotid artery endothelial function (EDD) was impaired with aging (Peak EDD: OC vs. YC, $P < 0.001$; Fig. 2A), similar to previous reports^{10,27}. Both acetate and high-fiber fully improved EDD to young levels in old mice (Peak EDD: both $P < 0.001$ vs. OC; Fig. 2A), whereas neither intervention affected EDD in young mice (Peak EDD: both $P = 0.857$ vs. YC; Fig. 2A). Notably, acetate was as effective as a high-fiber diet for improving endothelial function (OA vs. OF peak EDD, $P > 0.999$). Vascular smooth muscle sensitivity to NO, that is, EID, was not different with age or treatment ($P = 0.329$; Fig. 2B), indicating that differences in EDD were largely due to differences in function of the endothelium. Carotid artery resting and maximal diameters were greater with age ($P < 0.001$), but there was no effect of either treatment ($P > 0.670$; Supplementary Table S3). There was a main effect of treatment group on artery pre-constriction to phenylephrine, but no pairwise comparisons were significant ($P = 0.039$; Supplementary Table S3). Thus, it is possible that differences in pre-constriction could have contributed to group differences in EDD, but it is unlikely for this contribution to have been substantial.

Improvements in endothelial function are due to enhanced NO bioavailability associated with reduced ROS-related suppression of EDD

The primary mechanism of impaired endothelial function (EDD) with aging is reduced NO bioavailability due to excess ROS-related oxidative stress. Therefore, we investigated whether our interventions improved endothelial function in old mice through these mechanisms. Group differences in EDD were abolished in the presence of a NO synthase inhibitor L-NAME ($P = 0.906$; Fig. 2C), suggesting that both interventions improved endothelial function by enhancing NO bioavailability.

Among old mice, total aortic (i.e., whole cell) ROS abundance was lower in old acetate versus control mice ($P = 0.058$; Fig. 2D) and was lower on average in high-fiber diet mice but not significantly ($P = 0.262$). After observing an increase in whole-cell ROS, we measured ROS derived from mitochondria, which are a key source of excessive ROS with aging. However, there were no group differences in the abundance of mitochondrial-derived ROS ($P = 0.392$; Fig. 2E). To determine if differences in ROS levels had a functional effect on endothelial function, we repeated ACh dose responses in the presence of a superoxide dismutase mimetic, TEMPOL. TEMPOL restored EDD in old control mice to young levels (Peak EDD: $P = 0.948$ vs. YC), but there were no further improvements in old acetate or high-fiber diet mice (Peak EDD: both $P > 0.999$ vs. ACh alone; Fig. 2F). Combined, these data suggest that both interventions improved endothelial function by reducing ROS from extra-mitochondrial sources (e.g., pro-oxidant enzymes) and thereby ameliorating ROS-driven suppression of endothelial function.

Improvements in endothelial function may be due to systemic inflammation and reduced Egr-1 signaling

Next, we explored potential mechanisms that may mediate the effects of acetate and a high-fiber diet on improving age-related endothelial dysfunction. Based on previous literature, we explored two plausible pathways: systemic inflammation^{20,57} and Egr-1²¹.

Systemic inflammation—We assessed circulating markers of inflammation in a subset of all old groups and young control mice using a cytokine array. Of the 40 cytokines assessed, 11 were altered across groups, and 6 others tended to be altered ($P \leq 0.100$) (Supplementary Table S4). Of note, CD30 ligand (CD30L), monocyte chemoattractant protein (MCP-1), and C-C motif chemokine ligand 17 (CCL17) were elevated in old control mice (all $P < 0.05$ vs. YC), and interleukin (IL)-15 tended to be elevated ($P = 0.093$ vs. YC), and all of these were reduced toward young levels in old acetate and high-fiber diet mice (all $P \leq 0.941$ vs. YC; Fig. 3A-D). Reductions in systemic inflammation with acetate and a high-fiber diet may reduce arterial oxidative stress, consequently increase NO bio-availability, and improve endothelial function.

Egr-1 signaling—We used siRNA to knockdown Egr-1 in paired, excised carotid arteries from a subset of old mice and assessed endothelial function (EDD) to ACh (vs. scrambled) (Fig. 4A). Egr-1 knockdown improved EDD in old control mice (general linear model main effect of siRNA condition within group: $P = 0.049$) but induced no further improvements in old acetate mice ($P = 0.887$; Fig. 4B,C), suggesting acetate-mediated improvements in

endothelial function may be due, at least in part, to reduced Egr-1 signaling. Due to technical challenges, we did not have sufficient data from arteries obtained from old high-fiber diet mice. The siRNA did not affect vascular smooth muscle function (EID) in old acetate mice (Peak EID: $P = 0.794$), but impaired vascular smooth muscle function in old control mice (Peak EID: $P = 0.044$ vs. scrambled; Supplementary Fig. S4A). Thus, endothelial function was improved by silencing Egr-1 in old control mice, despite an apparent reduction in vascular smooth muscle sensitivity to NO.

Acetate and high-fiber diet improve age-related aortic stiffening

At baseline, *in vivo* aortic stiffness (PWV) was higher with aging in all treatment groups ($P < 0.001$; Fig. 5A). There were no changes in PWV across the intervention in any young groups nor in old control mice (all $P \geq 0.311$), but acetate and high-fiber diet reduced PWV in old mice by ~40% toward young levels (both $P < 0.001$ vs. baseline and $P = 0.066$ vs. OC post-intervention).

Arterial stiffening with aging occurs through a combination of *in vivo* influences (e.g., reduced vasodilation/greater constriction and increased blood pressure) and structural changes. Structural remodeling of the arterial wall typically occurs via changes in the abundance and/or properties of aortic extracellular matrix proteins, including collagen^{3,58}. Thus, we assessed the adventitial abundance of collagen and performed stress-strain testing in aortic rings to assess markers of intrinsic aortic wall stiffness and elasticity. Because Egr-1 mediated improvements in endothelial function, we used a similar approach to determine whether this pathway could influence aortic intrinsic stiffness and elasticity. Therefore, stress-strain testing was performed after incubation with scrambled siRNA or siRNA knocking down Egr-1 in matched aortic rings from the same mice.

We first assessed intrinsic wall stiffness and elasticity in aorta rings incubated with scrambled siRNA (control condition). We pooled samples for scrambled conditions (see the Ex vivo aortic stiffness with knockdown of target genes section in the Supplemental Material) to increase the sample size. The incubation conditions did not appear to influence the structural integrity of the arteries—elastic modulus values were similar to values we obtained from freshly frozen aortas of young and old control mice of the same age/sex/strain collected at a similar time²⁶.

The elastic modulus of the high-force region of the stress-strain curve, which reflects the intrinsic stiffness of the arterial wall, tended to be different across groups ($P = 0.095$; Fig. 5C), such that intrinsic stiffness increased with aging ($P = 0.118$ YC vs. OC). This increase was reversed by acetate and a high-fiber diet, such that there were no differences between young and old acetate or high-fiber diet mice (both $P \geq 0.910$ vs. YC). The elastic modulus of the low-force region of the stress-strain curve, a marker of aortic elasticity, was different across groups ($P = 0.003$; Fig. 5D) and was reduced with aging in all groups (all $P \leq 0.012$ vs. YC; Fig. 5D). However, there was no effect of either intervention ($P \geq 0.959$ OC vs. OA and OF). There was no effect of acute Egr-1 knockdown on aortic intrinsic stiffness ($P = 0.405$; Fig. 5E) or aortic elasticity ($P = 0.772$; Fig. 5F) in any group.

Aorta diameter ($P = 0.004$) and IMT ($P < 0.001$; Supplementary Table S3) were higher with aging, but there was no effect of either intervention in young or old mice (all $P \geq 0.550$). Aortic abundance of adventitial collagen, measured via quantitative immunofluorescence, tended to be higher with aging ($P = 0.111$ YC vs. OC; Fig. 5B), but there was no effect of either intervention ($P \geq 0.535$ OC vs. OA and OF). There was no detectable signal of collagen in the media. Collectively, these results suggest that reduced *in vivo* aortic stiffness (PWV) with acetate supplementation and a high-fiber diet may be due to reduced aortic intrinsic stiffness but are not due to changes in aortic collagen abundance or acute Egr-1 signaling.

Discussion

Age-related changes in the gut microbiome contribute to arterial dysfunction^{10,59}. Thus, identifying interventions that favorably modulate gut microbiome-related pathways and improve arterial function is biomedically significant. Here, we show that gut bacterial SCFA-producing capacity is decreased with aging in mice and is related to impaired arterial function. These initial findings established enhancing SCFA bioavailability as a novel therapeutic target for improving age-associated arterial dysfunction. Accordingly, we then found that 8–10 weeks of oral supplementation with SCFA acetate or consumption of a high-fiber diet improved arterial function to a comparable extent in old mice. Our findings are the first to demonstrate the efficacy of oral supplementation with SCFAs for improving arterial function with aging. In addition, we identified reduced systemic inflammation and Egr-1 signaling as two mechanisms that may mediate these improvements. Because impairments in arterial function contribute to the development of CVDs, our findings have implications for reducing the risk of CVD with advancing age in humans.

The gut microbiome as a target for improving age-related arterial dysfunction

Changes in the gut microbiome are emerging as an important mechanism by which aging leads to arterial dysfunction⁵⁹. For example, our laboratory showed that suppression of the gut microbiome via oral antibiotics improved arterial function in old mice⁹, and initial evidence suggests that transplant of fecal microbiota between young and old mice transfers arterial phenotypes⁶⁰. The gut microbiome influences host physiology in part through the production of metabolites that can enter into the circulation and have systemic effects. A notable example is the metabolite trimethylamine N-oxide (TMAO), which is elevated in circulation with aging and can directly cause arterial dysfunction¹⁰. TMAO has been linked to CVD⁶¹, although these relations have not always been observed^{62,63}. Moreover, suppressing TMAO production prevents or reverses arterial dysfunction^{10,26,64}. Therefore, interventions targeting the gut microbiome or gut-derived metabolites may be effective for improving age-related arterial dysfunction.

Major factors influencing the gut microbiome with aging in humans include diet, medications, and changes in the physical environment (e.g., moving to nursing homes)⁵³. Here, we examined age-related changes in the gut microbiome in a controlled setting in mice—isolating the effects of aging from environmental factors. We explored bacterial taxa and genetic pathways that could be contributing to arterial aging and observed global shifts

in the gut microbiome of old animals. Notably, we found age-related reductions in the abundance of SCFA-producing bacteria and microbial genes encoding SCFA production. There is some previous evidence for reductions in SCFAs with aging, but these data have been generated via indirect measurements (e.g., fecal SCFAs) and are confounded by experimental limitations (e.g., lack of sufficient young individuals for comparison and/or lack of controlled environmental conditions)^{4,5}. Our findings provide new, more definitive evidence that the SCFA-producing capacity of the gut microbiome is in fact decreased with aging, independent of environmental factors present in free-living humans. Furthermore, our results provide an experimental basis for exploring SCFA supplementation as a novel therapeutic for improving age-related arterial dysfunction.

We also examined other gut bacterial pathways that might be involved in arterial dysfunction with aging, including anti- and pro-inflammatory bacteria and LPS-producing and intestinal mucosal-degrading bacteria. Previous evidence suggests a shift toward a pro-inflammatory gut microbiome with aging^{65,66}, which is in line with our observations. By conducting experiments in a controlled environment, our findings contribute to the existing literature by demonstrating that this shift is attributable to biological aging rather than environmental factors. Circulating LPS is elevated in CVD, can be found in atherosclerotic plaques, and contributes to plaque instability and thrombus formation⁶⁷. Moreover, excess translocation of LPS may occur due to increases in intestinal permeability with aging and CVD, which are mediated in part by degradation of the intestinal mucosal barrier⁶⁸. We found that LPS-producing and mucosal-degrading bacteria increased with aging. However, these were not related to arterial dysfunction after controlling for age, suggesting that these functional pathways may not directly modulate age-related arterial dysfunction. LPS has been posited to be a key mechanism of pro-inflammatory changes in the gut microbiome⁶⁹, but our data suggest that reductions in SCFAs may be as, if not more, relevant of a mechanism in the context of systemic inflammation and age-related changes that occur secondary to increased inflammation (e.g., arterial dysfunction). A more comprehensive investigation of these mechanisms should be the focus of future research, including interventions that directly target LPS/LPS-producing bacteria.

Acetate supplementation and high-fiber diet consumption for improving age-related arterial dysfunction

Endothelial dysfunction and increased aortic stiffness predict and contribute to the development of CVD^{2,58}. Indeed, brachial artery flow-mediated dilation and carotid-femoral PWV, reference standard measures of endothelial function and aortic stiffness in humans, respectively, are independent predictors of future CVD risk^{28,35}. We assessed these domains of arterial function in mice using corollaries to these approaches in humans. Both acetate and high-fiber diet improved endothelial function and lowered *in vivo* aortic stiffness in old, but not young, mice. Thus, these strategies hold promise for preserving arterial health with aging.

To our knowledge, we are the first to demonstrate that supplementation with acetate improves *in vivo* arterial function in the context of aging. A prior study reported that *ex vivo* incubation with acetate protected against angiotensin II-induced endothelial dysfunction in

rat aortic rings and endothelial cells⁷⁰, and acetate supplementation improved endothelial function in a mouse model of lupus⁷¹. Other SCFAs have also been implicated in improving cardiovascular function. For example, supplementation with sodium butyrate and inulin improved diastolic blood pressure in overweight/obese individuals with type 2 diabetes⁷², and oral supplementation with the probiotic *Lactobacillus plantarum 299v* increased circulating propionate and improved endothelial function in men with coronary artery disease⁷³. It is important to acknowledge that individual SCFAs can have distinct biochemical and physiological effects⁷⁴; however, our findings suggest these different SCFAs may have similar effects on the vasculature and add to the existing literature by demonstrating improvements in arterial function with acetate supplementation.

Our data also add to the existing literature indicating that high-fiber diets improve arterial function. For example, higher habitual dietary fiber consumption has been related to lower aortic stiffness^{75,76}, and fiber supplementation improves endothelial function in ApoE^{-/-} mice⁷⁷ and a mouse model of lupus⁷¹. Our findings are the first to demonstrate that a high-fiber diet lowers aortic stiffness and improves endothelial function in the context of aging.

Importantly, acetate supplementation was as, if not more, effective as a high-fiber diet for enhancing arterial health. For example, in old mice, acetate reduced aortic ROS production to a greater extent than the high-fiber diet. This might be explained by direct oral supplementation of acetate exerting a more potent stimulus than enhancing acetate indirectly through fiber consumption, in part due to the lower SCFA-producing capacity of the gut microbiome with aging.

Overall, given the issues in adherence to dietary guidelines for fiber consumption¹⁷ and age-related reductions in the SCFA-producing capacity of the gut microbiome, our findings suggest that acetate may be a viable option for improving arterial function in older adults.

Potential mechanisms of acetate-mediated improvements in arterial function in old mice

NO bioavailability and ROS production.—Improvements in endothelial function were mediated by increased NO bioavailability. The latter was associated with reductions in arterial ROS production and reduced tonic ROS-related suppression of endothelial function, as shown by functional bioassay. These observations are consistent with previous studies showing that inulin supplementation increased NO bioavailability in ApoE^{-/-} mice⁷⁷, and *ex vivo* acetate treatment increased NO bioavailability and reduced ROS in rat aortic rings and aortic endothelial cells that were both treated with angiotensin II⁷⁰.

Systemic inflammation.—Aging is characterized by an increase in systemic inflammation⁷⁸, and changes in the gut microbiome and gut-derived metabolites appear to contribute to this pro-inflammatory phenotype⁷⁹. Our findings demonstrate that our interventions protect against age-related increases in pro-inflammatory pathways that are implicated in, and contribute to, the development of CVDs. We observed that circulating levels of various proinflammatory cytokines were elevated with aging, several of which were reduced with our interventions, including the following: (a) CD30L, a member of the tumor necrosis factor family that contributes to atherosclerosis development⁸⁰; (b) MCP-1,

which is related to reduced aortic elasticity with aging³⁶ and is heavily involved in vascular inflammation and remodeling⁸¹ and the development of atherosclerosis⁸²; (c) CCL17, a proinflammatory chemokine that is associated with increased arterial stiffness in humans; CCL17^{-/-} prevented age-related impairments in endothelial function and aortic stiffness and vascular remodeling in mice⁸³; and (d) IL-15, a pro-inflammatory cytokine that is upregulated in certain CVDs⁸⁴, including atherosclerosis, and contributes to atherosclerotic lesion development in mice⁸⁵. Consistent with these results, other SCFAs, propionate and butyrate, protect against atherosclerosis^{86,87} and aortic aneurysm⁸⁸ via systemic anti-inflammatory effects.

Egr-1 signaling.—siRNA-mediated knockdown of Egr-1 improved endothelial function in old control mice, while there were no improvements in old acetate-supplemented mice. These findings suggest that improvements in endothelial function with acetate supplementation may be mediated by lower Egr-1 signaling, consistent with a previous study showing that the cardioprotective effects of acetate and a high-fiber diet in a mouse model of hypertension were accompanied by reduced cardiac expression of Egr-1²¹. Egr-1 has been studied as a mediator of CVDs²². For example, Egr-1 is activated upon vascular injury and stimulates pro-inflammatory pathways that contribute to vascular remodeling²², and Egr-1 contributes to vascular inflammation and atherogenesis in ApoE^{-/-} mice⁸⁹. However, to our knowledge, this is the first study in which Egr-1 has been explored in the context of endothelial dysfunction with aging.

Arterial wall modifications.—The reduction in *in vivo* aortic stiffness (PWV) with our interventions was accompanied by a reduction in aortic intrinsic mechanical wall stiffness. Aortic mechanical stiffness is largely regulated by the abundance and/or properties of extracellular matrix proteins⁵⁸. We observed no detectable change in abundance of aortic adventitial collagen, suggesting lower intrinsic stiffness with the interventions could be due to changes in *properties* of the structural proteins, for example, reduced stiffness of individual fibers or changes in their orientation/packing order^{90,91}. In contrast to our findings, a previous study in a mouse model of hypertension showed that collagen-associated cardiac fibrosis was lower in mice treated with a high-fiber diet or acetate supplementation²¹, and another report showed that genetic knockout of a key SCFA receptor increased aortic abundance of collagen⁹². The stimuli used in these studies may have caused a more potent pro-fibrotic effect compared to natural aging, potentially explaining the lack of consistent effect of our interventions on collagen abundance. In addition, we observed no effect of acutely knocking down Egr-1 on mediating improvements in intrinsic mechanical wall stiffness. Egr-1 plays a role in mediating vascular fibrosis in disease settings (e.g., plaque formation); thus, our findings are contrary to what we expected to observe based on the existing literature. However, it is plausible that the lack of effect observed in our study may have been due to the short duration of knockdown, as the signaling pathways mediating arterial wall structure are more chronic.

Experimental considerations and limitations

Overall, gut microbiome composition did not appear to be significantly affected by the interventions. Not all studies have demonstrated changes in gut microbiome composition

following SCFA supplementation^{21,87}, likely because SCFAs are byproducts, rather than substrates, of microbial metabolism. However, high-fiber diets do typically alter gut microbiome composition, and we posit that the beneficial effects of the high-fiber diet on artery function observed in the present study are due to the increased bioavailability of SCFAs, specifically acetate. Our inability to detect a difference could be due to lower sample sizes across the intervention than in our baseline analyses. Furthermore, it is possible that, even if the abundances of specific SCFA- and acetate-producing taxa were not increased, acetate bioavailability may have still been higher due to higher substrate availability and fermentation activity. However, we did not measure circulating levels of acetate because a previous study showed that acetate administered in drinking water did not increase circulating acetate beyond basal levels⁹³. This is likely due to rapid binding to receptors followed by clearance of circulating acetate, as administration via oral gavage and intraperitoneal injection resulted in only a transient increase in circulating levels that returned to baseline levels within 30 min⁹³.

In addition, only male mice were studied because female mice do not consistently exhibit age-related impairments in carotid artery endothelial function. However, whether there are sex differences in arterial function with acetate supplementation and high-fiber diets in humans should be addressed in future research.

In addition, we did not include assessments of blood pressure because invasive measures (i.e., aortic telemetry) significantly increase mortality in older animals and interfere with our assessments of carotid endothelial function (EDD) and aortic stiffness (PWV), and age-related increases in systolic blood pressure are not exhibited in this strain of mice with the tail cuff method³⁶. Indeed, we did not observe any effects of aging or either intervention on tail cuff blood pressure in an early cohort of mice, so we discontinued the measure to limit burden/stress on the animals.

Next, *in vivo* genetic knockout of Egr-1 could more definitively confirm that improvements with our interventions are *chronically* mediated via these pathways. However, Egr-1 is important for normal development⁹⁴, and genetic knockout could result in impairments that would confound aging physiology. This issue could be circumvented using inducible knockout models, which were outside the scope of feasibility for this study but should be the focus of future research. That said, an important strength of our study was that the interventions were conducted in the context of natural aging (i.e., interventions in mice were initiated at the human equivalent of ~70 y of age), which increased the translational relevance of our findings.

Perspectives

In this study, we first demonstrated that reduced gut bacterial SCFA-producing capacity is linked to age-related arterial dysfunction. Next, we identified that enhancing SCFA bioavailability via supplementation with acetate and high-fiber diet feeding shows promise for improving age-related arterial dysfunction, a key antecedent to the development of CVDs. Notably, this intervention has high potential for translation to humans because: (a) supplements are likely more adherable than high-fiber dietary interventions; (b) some forms of acetate (e.g., calcium acetate) are already FDA-approved⁹⁵; and (c) the dose of acetate

mice received was estimated (from average daily intake and body mass at sacrifice of the old acetate group and species conversion factors) to be equivalent to 10.7 g/day in a 70 kg human, which is within the range of acetate that is prescribed in patients for other indications⁹⁶. Future studies should explore the role of acetate, and potentially other SCFAs, for improving arterial function in humans and reducing the risk of developing clinical CVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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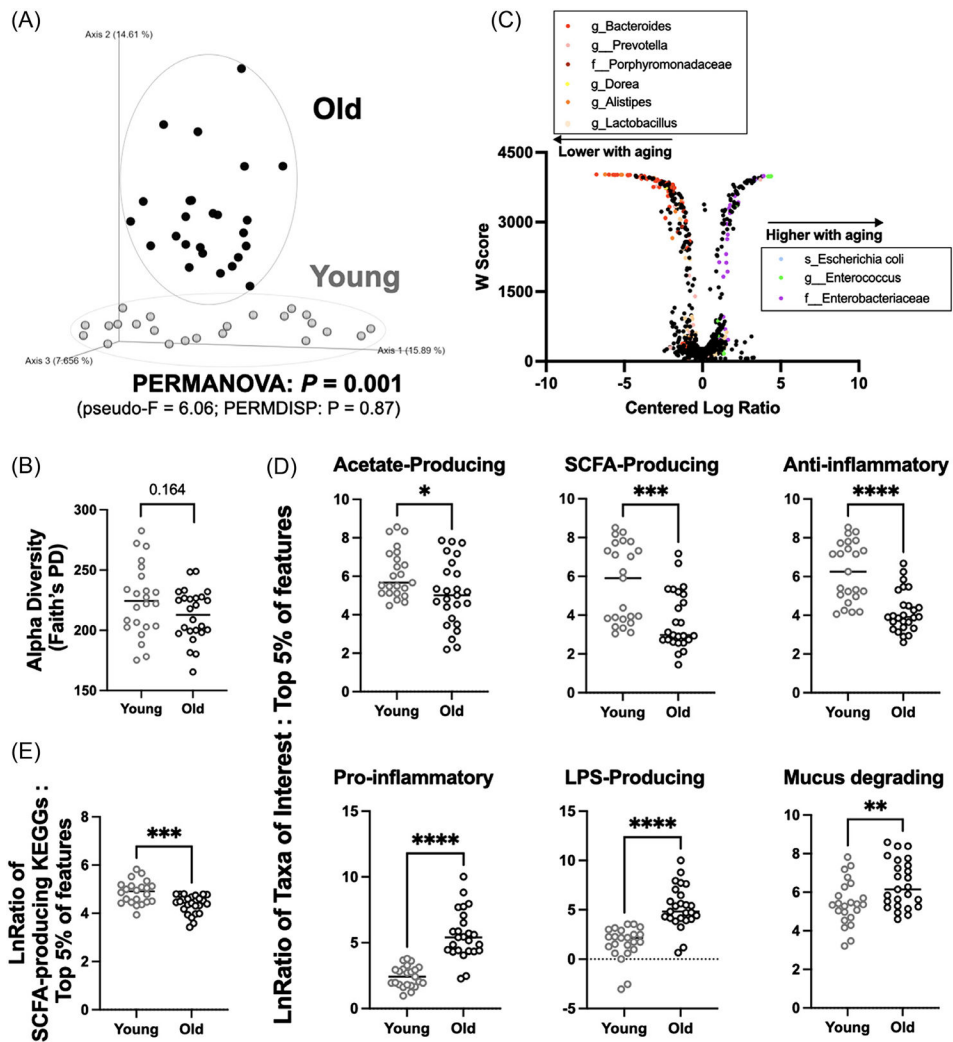


Figure 1. Gut microbiome composition and short-chain fatty acid (SCFA)-producing functional capacity are altered with aging in mice.

Metagenomic sequencing of fecal samples collected at baseline from young ($n = 23$; ~3 months) and old ($n = 25$; ~24 months) male C57BL/6N mice. 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. **(B)** Alpha diversity measured as Faith's phylogenetic diversity. **(C)** Analysis of composition of microbiomes (differential abundance) depicting centered log ratio mean differences in abundance of features between young and old mice, where $W = \#$ of statistical subhypotheses that have passed for a given feature and $W > 3,000$ was considered statistically significant. **(D)** A combination of the QIIME2 plugins Songbird and Qurro was used to produce differentials and create a visualization to compare log ratios of specific grouped bacterial taxa. Groupings that were significantly different between age groups are shown here. **(E)** A similar approach was used to compare the log ratio of Kyoto Encyclopedia of Genes and Genomes ortholog pathways related to SCFA production. Filtering specifics included in the Supplementary Table S2. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

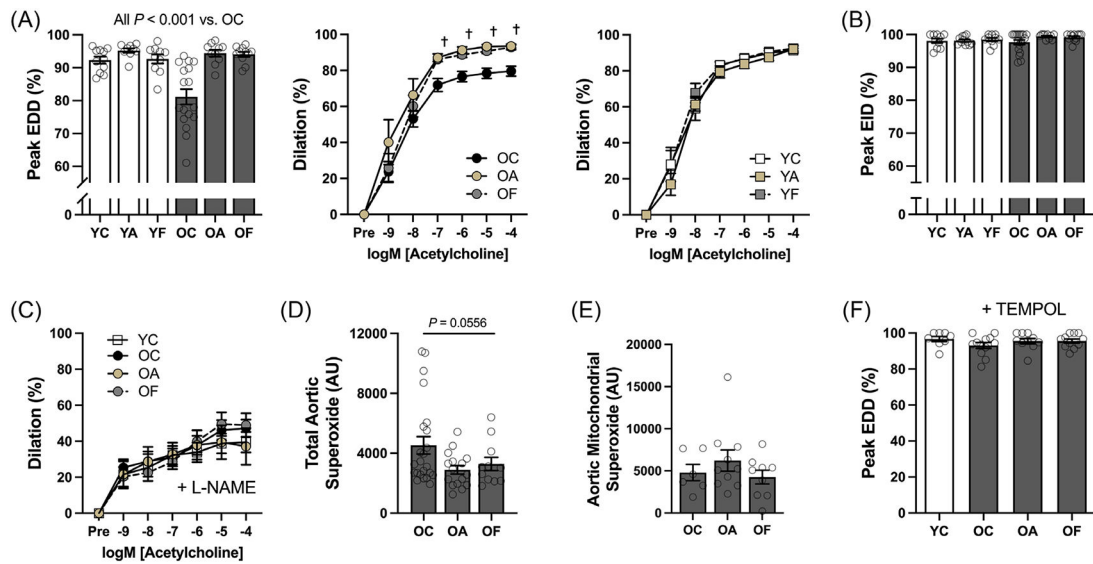


Figure 2. Acetate and high-fiber diet consumption improve age-related impairments in endothelial function by enhancing nitric oxide (NO)-mediated dilation and reducing reactive oxygen species (ROS)-related suppression of endothelium-dependent dilation (EDD). (A) Peak carotid artery EDD to acetylcholine (ACh) in young (Y) and old (O) control (C), acetate-supplemented (A), or high-fiber-fed (F) male C57BL/6N mice (n = 10–17/group), and dose responses of carotid artery EDD to ACh in old and young mice. (B) Peak carotid artery endothelium-independent dilation (EID) in response to the NO donor sodium nitroprusside. (C) Dose responses of carotid artery EDD to ACh in the presence of the NO synthase inhibitor L-NAME (NG-nitro-L-arginine methyl ester). (D,E) Whole cell (D) and mitochondrial (E) ROS production, measured in 1 mm aorta rings by electron paramagnetic resonance spectroscopy (n = 6–24/group). (F) Peak carotid artery EDD to ACh in the presence of the ROS dismutase mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL; n = 8–12/group). Data are mean ± SEM. Statistics are one-way analysis of variance (ANOVA) with Tukey's post hoc test (panels A, B, D, E and F) or two-way mixed (group x dose) ANOVA with Tukey's post hoc test (panels A and C).

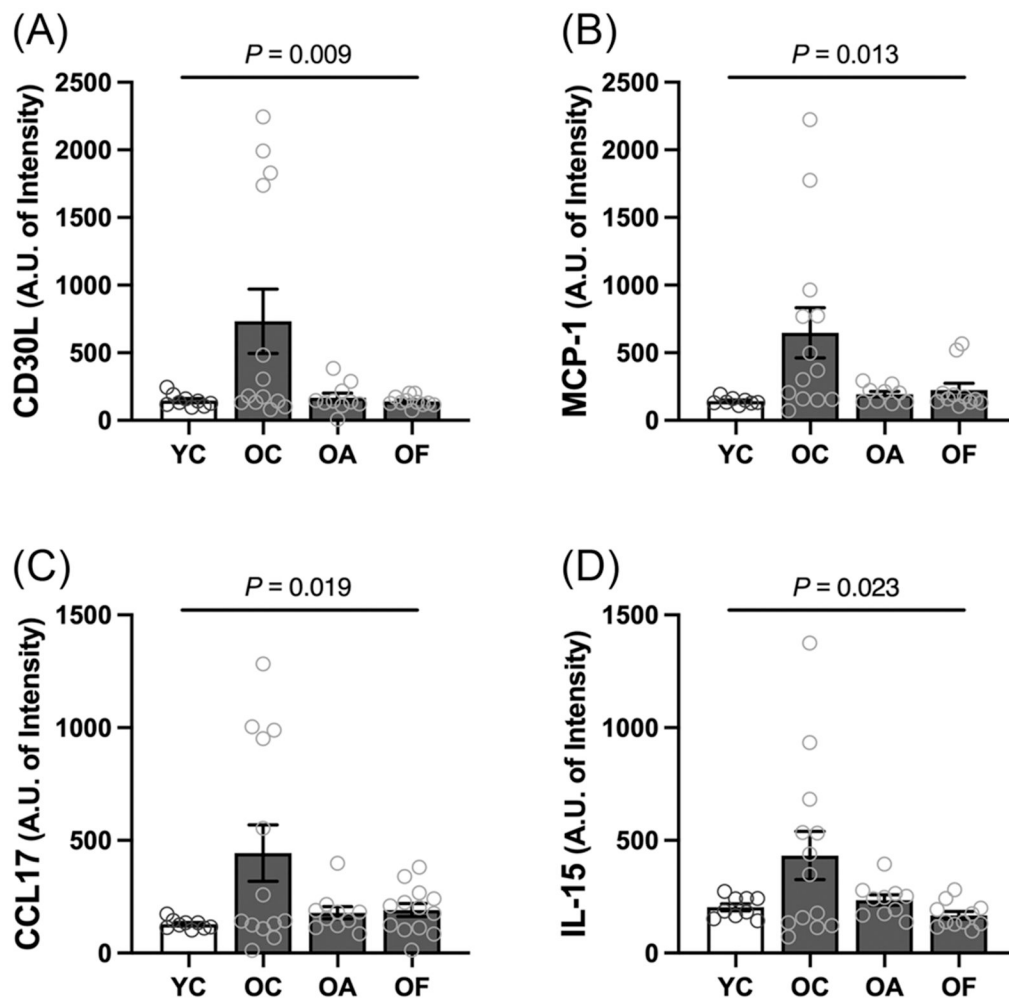


Figure 3. Systemic inflammation is increased with aging and improved with acetate supplementation and high-fiber diet feeding.

Plasma levels of the pro-inflammatory cytokines (A) CD30 ligand (CD30L), (B) monocyte chemoattractant protein-1 (MCP-1), (C) CC chemokine ligand 17 (CCL17), and (D) interleukin-15 (IL-15) in young (Y) and old (O) control (C), acetate-supplemented (A), or high-fiber-fed (F) male C57BL/6N mice. Plasma was analyzed by cytokine array and expressed as A.U. of intensity relative to an internal positive control (n = 9–13/group). Data are mean \pm SEM. Statistics are one-way ANOVA with Tukey's post hoc test.

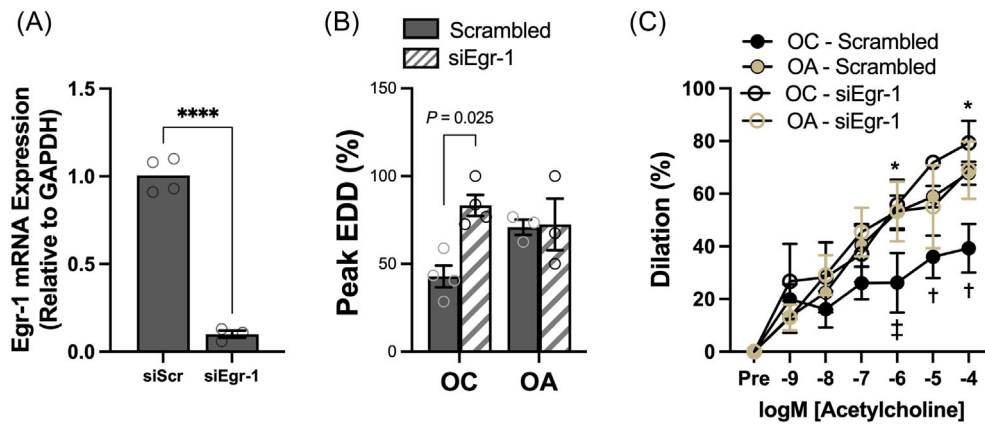


Figure 4. Improvements in age-related endothelial dysfunction with acetate supplementation may be due to reduced early growth response-1 (Egr-1) signaling.

(A) Expression quantitative polymerase chain reaction of Egr-1 assessed in thoracic aortas obtained from young ($n = 4$) male C57BL/6N mice treated with either Egr-1 siRNA or the control scrambled oligonucleotide for 24 hours. (B) Peak carotid artery EDD and (C) dose response curve to ACh 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 \pm SEM. Statistics are unpaired t-test (panel A), two-way mixed (group \times condition) ANOVA with Šídák's post hoc test (panel B), or general linear model (panel C). * $P < 0.05$ versus OC within the scramble condition. **** $P < 0.0001$. † $P < 0.05$ versus siRNA or scramble condition within group. ‡ $P < 0.1$ versus siRNA or scramble condition within group.

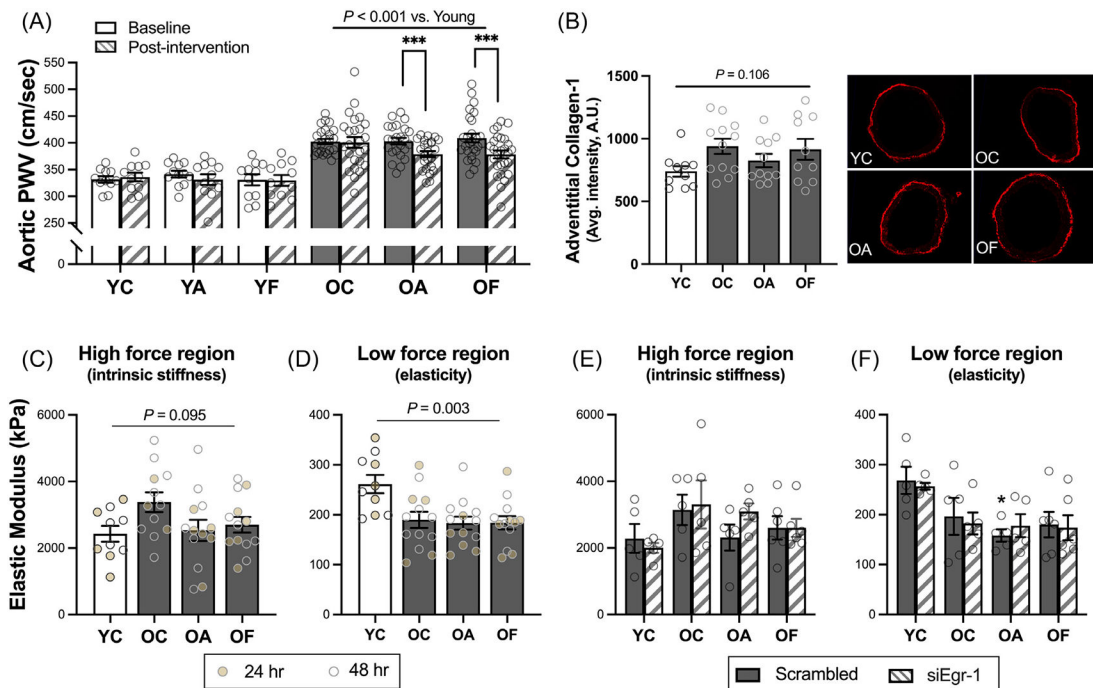


Figure 5. Acetate and high-fiber diet consumption improve age-related increases in *in vivo* aortic stiffness.

(A) *In vivo* aortic pulse wave velocity (PWV) at baseline and post-intervention in young (Y) and old (O) control (C), acetate-supplemented (A), or high-fiber-fed (F) male C57BL/6N mice ($n = 11-27$ /group). (B) Adventitial abundance of collagen-1, measured via quantitative immunofluorescence in 7 μ m segments of thoracic aorta with representative images shown to the right ($n = 10-12$ /group). (C,D) Elastic modulus of the high-force region (C; *intrinsic stiffness*) and low-force region (D; *elasticity*) of the stress-strain curve, conducted in 1–2 mm segments of thoracic aorta incubated in media containing scrambled siRNA for 24 or 48 hours ($n = 9-13$ /group). (E,F) Elastic modulus of the high-force region (E) and low-force region (F) of the stress-strain curve, conducted in 1–2 mm segments of thoracic aorta incubated in media containing siRNA specific for Egr-1 or the scrambled siRNA condition for 24 hours ($n = 5-8$ /group). Data are mean \pm SEM with individual data points. Statistics are general linear model (panel A), one-way ANOVA with Tukey's post hoc test (panels B–D), or two-way mixed (condition or age \times group) ANOVA with Šídák's post hoc test (panels E and F). * $P < 0.05$ versus YC within scramble condition. *** $P < 0.001$.

Table 1.

Robust regression analysis results.

Regression model	Estimate	Pr > ChiSq
PWV ~ Acetate-producing bacteria	-10.081	0.008 *
PWV ~ Acetate-producing bacteria + age	2.739	0.330
PWV ~ SCFA-producing bacteria	-15.45	<0.0001 *
PWV ~ SCFA-producing bacteria + age	-4.376	0.072 [†]
PWV ~ Anti-inflammatory bacteria	-17.962	<0.0001 *
PWV ~ Anti-inflammatory bacteria + age	-7.027	0.001 *
PWV ~ Pro-inflammatory bacteria	14.597	<0.0001 *
PWV ~ Pro-inflammatory bacteria + age	6.083	0.010 *
PWV ~ LPS-producing bacteria	11.404	<0.0001 *
PWV ~ LPS-producing bacteria + age	2.342	0.340
PWV ~ Mucus-degrading bacteria	8.730	0.036 *
PWV ~ Mucus-degrading bacteria + age	-2.613	0.446

LPS, lipopolysaccharide; PWV, pulse wave velocity; SCFA, short-chain fatty acid. Estimates and P values calculated from robust regressions.

Analyses were performed in R using *robustbase*⁵⁵ and the function *lmrob*. Metagenomic sequencing of fecal samples collected from young (n = 23; ~3 months) and old (n = 25; ~24 months) male C57BL/6N mice. The log ratios of relative abundance of SCFA-, acetate-, and LPS-producing as well as proinflammatory taxa, as calculated in Figure 1D, were related to *in vivo* aortic stiffness (PWV). Robust regression analyses were used to account for potential outliers and violations of assumptions (normality) that would affect standard regression analyses. *P < 0.05. †P < 0.08.

Bold values in column 3 are P < 0.05.