Lactobacillus Plantarum 299v Supplementation Improves Vascular Endothelial Function and Reduces Inflammatory Biomarkers in Men with Stable Coronary Artery Disease

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Abstract

Rationale: A strong association has emerged between the gut microbiome and atherosclerotic disease. Our recent data suggest *L. plantarum 299v* (*Lp299v*) supplementation reduces infarct size in male rats. Limited human data are available on the impact of *Lp299v* on the vasculature.

Objective: To determine whether oral *Lp299v* supplementation improves vascular endothelial function and reduces systemic inflammation in humans with stable coronary artery disease (CAD).

Methods and Results: Twenty men with stable CAD consumed a drink containing *Lp299v* (20 billion CFU) once daily for six weeks. Following a 4-week washout, subjects were given an option of additionally participating in a 10-day study of oral liquid Vancomycin (250 mg 4x daily). Vascular endothelial function was measured by brachial artery flow-mediated dilation (FMD). Before and following *Lp299v*, plasma short-chain fatty acids, trimethylamine oxide (TMAO), and adipokine levels were measured. Additional plasma samples underwent unbiased metabolomic analyses using liquid chromatography/mass spectroscopy (UHPLC/MS). 16S rDNA sequencing was used to determine changes of the stool microbiome. Arterioles from CAD patients were obtained and endothelium-dependent vasodilation was measured by video-microscopy following intra-luminal incubation with plasma from *Lp299v* study subjects. *Lp299v* supplementation improved brachial FMD (P=0.008) without significant changes in plasma cholesterol profiles, fasting glucose, or body mass index. Vancomycin did not impact FMD. *Lp299v* supplementation

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decreased circulating levels of IL-8 (P=0.01), IL-12 (P=0.02), and leptin (P=0.0007) but did not significantly change plasma TMAO concentrations (P=0.27). Plasma propionate (P=0.004) increased while acetate levels decreased (P=0.03). Post-*Lp299v* plasma improved endothelium-dependent vasodilation in resistance arteries from patients with CAD (P=0.02).16S rRNA analysis the showed Lactobacillus genus was enriched in post-probiotic stool samples without other changes.

Conclusions: *Lp299v* improved vascular endothelial function and decreased systemic inflammation in men with CAD, independent of changes in traditional risk factors and TMAO. Circulating gut-derived metabolites likely account for these improvements and merit further study.

Clinical Trial: NCT01952834

Keywords

Clinical Studies; Endothelium/Vascular Type/Nitric Oxide; Physiology; Translational Studies; Vascular Biology; Endothelium; probiotic; gut microbiome; endothelium nitric oxide synthase; coronary artery disease

INTRODUCTION

While some progress has been made through treatment of traditional cardiac risk factors, atherosclerotic diseases remain the leading cause of morbidity and mortality in industrialized nations and a growing problem in developing countries. Therefore, there exists a significant unmet clinical need for identifying novel therapies to prevent and treat the progression of cardiovascular disease. Development of such potential therapies requires identification of additional contributory processes that contribute to cardiovascular disease so that mechanism-based interventions may be developed.

The human intestinal tract is colonized by trillions of microbes representing all three domains of life. Signals from the intestinal microbiota are important for normal development and physiology; alteration of these microbial communities (dysbiosis) in patients or animal models is associated with multiple disease states. Recent human and human-tissue based association studies suggest a strong link between the gut microbiome and the prevalence of atherosclerotic disease [1, 2]. Differences in both the human gut microbiome species balance and diversity (e.g., Firmicutes versus Bacteroides phyla prevalence) have been associated with the development of atherosclerosis and plaque stability.[3] Dietary choline intake has been mechanistically linked to atherosclerotic plaque formation through increasing circulating trimethylamine oxide (TMAO) derived from gut microbiome metabolism of choline to trimethylamine.[4–7] Mouse-based proof-of-principle studies using antibiotics, fecal transplant, high fiber diets, and acetate supplementation support the concept that the gut microbiota plays a critical role in regulating vascular function and systemic inflammation that contributes to atherogenesis.[5, 8–10]

However, to date, limited human data are available that address whether any gut microbiome-targeted intervention improves vascular endothelial function in individuals at high risk for adverse cardiovascular events. Impaired endothelial function begins before the development of atherosclerosis and predicts future adverse cardiovascular events in both

those with and without prevalent atherosclerotic disease[11–15]. Critical components of endothelial dysfunction include impaired nitric oxide bioavailability, which is readily measurable by non-invasive means and the presence of a systemic and endothelial inflammatory phenotype[15]. *Lactobacillus plantarum* supplementation decreases circulating leptin levels, systolic blood pressure, and fibrinogen levels in otherwise healthy smokers[16]. We recently demonstrated that *L. plantarum 299v* (Lp299v) supplementation or vancomycin administration reduces myocardial infarct size in hypertensive male rats[17]. Additionally, *L. plantarum* reduces LPS-mediated atherosclerotic plaque inflammation in an atherosclerotic mouse model.[18] These data support the concept that *Lp299v* may have beneficial effects on human vascular health, but need further human studies to translate the findings from mouse models and begin determining possible mechanisms of effect in humans.

We hypothesized that *Lp299v* supplementation would both improve endothelial function in humans with CAD and reduce systemic inflammation. We tested this hypothesis in a pilot study of 21 men with stable coronary artery disease (CAD) with six weeks of daily supplementation with *Lp299v*. In addition to direct measurement of *in vivo* endothelial function and markers of systemic inflammation, we used plasma obtained from these subjects preceding and following supplementation to directly test whether the favorable effects of *Lp299v* supplementation on human vascular endothelium and human mononuclear cells were a result of changes in circulating plasma composition.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Subjects.

Twenty-one men (ages 40 –75 years) with stable coronary artery disease as diagnosed by coronary angiography were recruited between 2013–2015 (ClinicalTrials.gov Identifier: NCT01952834). Participants were recruited from the Milwaukee metropolitan area by posted and distributed flyers, print media advertisements, internet-based advertisements, and physician referrals. The study protocol was approved by the Medical College of Wisconsin's Institutional Research Board, and all participants provided written informed consent prior to study participation.

Screening before enrollment included a detailed medical history including current medications, and a focused cardiac and vascular physical exam by a study physician to screen for occult non-cardiac disease and evaluate for eligibility for the study participation. Participants were eligible for the study if they were between ages 40–75 years,male, and had a known history of coronary artery disease (by either history of myocardial infarction, angiogram demonstrative of greater than or equal to 50% stenosis in at least one major epicardial coronary artery, or a previous stress test that showed evidence of ischemia that had not been revealed to be a false positive test by angiography). Given that prior animal work demonstrating the benefit of probiotic supplementation and vancomycin was performed exclusively in male rats, only men were included in the study. Individuals were excluded from participation if they met any of the following criteria: unstable angina or

myocardial infarction by history, ECG, and or enzymatic criteria within 1 month of enrollment; left ventricular dysfunction defined as left ventricular ejection fraction (LVEF %) less than 45% within 1 year of enrollment by an echocardiogram, MRI or nuclear imaging; uncontrolled resting hypertension (B.P greater than 170/100 mm Hg); chronic renal insufficiency (creatinine clearance < 60 mL/min); chronic liver disease; cancer requiring chemotherapy within five years of enrollment; cognitive impairment; implanted defibrillator or permanent pacemaker; received probiotics, prebiotics or antibiotics within 12 weeks of screening visit; and dosage changes to vasoactive medications, including HMG-CoA reductase inhibitors, in the 6 weeks prior to enrollment.

Treatment allocation.

Eligible subjects were allocated in a non-randomized manner to a six-week supplementation with 2.7 oz/day of GoodBelly StraightShot, a commercially available *Lactobacillus plantarum 299v* formulation (NextFoods, Boulder, CO) containing 20 billion colony forming units of the bacterium. One serving of GoodBelly StraightShot contains 50 calories and 13 grams of sugar. Additionally, individuals who completed the probiotic intervention had the option of additionally participating in a 10-day study of oral liquid vancomycin administration beginning approximately one month following the six-week treatment with *Lactobacillus plantarum 299v*.

Study visit procedures for probiotic intervention.

All subjects who passed a phone screen were invited to a screening visit for study eligibility. Patients fasted overnight before their study visit. Blood pressure and heart rate were measured in triplicate and averaged. Anthropometrics (height, weight, and waist circumference) were measured in metric units. Peripheral venous blood was drawn from an upper extremity vein for biomarker analyses, including conventional risk factors for cardiovascular disease (total cholesterol, LDL-cholesterol, triglycerides, serum glucose, and hemoglobin A1C); inflammatory cytokines ; circulating adipokines; and metabolomic analyses. Biomarkers were measured at baseline and the end of the probiotic intervention phase only. Stool samples were collected pre- and post-probiotic therapy for microbiome analysis. Endothelial function as measured by brachial artery reactivity was measured at baseline as well as at the end of the probiotic intervention using standard procedures in our laboratory.

Optional vancomycin intervention study.

After completion of the probiotic phase of the study, participants were given the option of enrolling in a 10-day oral vancomycin study that commenced following a thirty-day washout period. Vancomycin was dispensed in 250mg doses to be taken four times daily during the duration of the intervention phase. Measurements of endothelial function by brachial reactivity were made prior to and following 10 days of vancomycin administration.

Measurement of in vivo endothelial function by brachial artery reactivity.

Standard and validated technique using vascular ultrasound was used for *in vivo* measurement of endothelial function of the brachial artery in the dominant arm as previously performed in our laboratory.[19–22] Greater detail is included in the **Online Supplement**.

Stool microbiome analysis.

Stool was collected from 17 of the 20 subjects immediately before and at day 42 following consumption of *Lactobacillus plantarum*. Bacterial DNA was isolated from the stool samples, ranging in concentrations 34 ng/μl – 154 ng/μl with total masses ranging from 500 ng - 2300 ng and stored at −20ºC.The bacterial 16S rRNA genes were amplified using the degenerate forward primer: 5'-AGRGTTTGATCMTGGCTCAG-3' and the non-degenerate reverse primer:5'-GGTTACCTTGTTACGACTT-3'. Thirty-five cycles of bacterial 16S rRNA gene PCR amplification were performed. Samples were amplified to specification and moved forward for hybridization. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer.

Bacterial diversity and comparative community structure of human fecal DNA samples were characterized by Second Genome Inc. (San Francisco, CA) using the high-density G3 PhyloChip 16S rRNA microarray-based assay and bioinformatic methods. The microbiota analysis focused on calculating inter-sample distances and assessing the significance of microbiome dissimilarity [23]. Data analysis incorporated several separate stages: preprocessing and data reduction, summarization, normalization where needed, sample-to-sample distance metrics, ordination/clustering, sample classification, and significance testing. Details of the microarray analysis and data analysis are included in **Online Supplement**.

Measurement of plasma TMAO, adipokines, cytokines, and circulating adhesion molecules.

Analysis of TMAO in human plasma samples was adapted from Wang et al. 2014.[24] Details of regarding all assays for these biomarkers are included in **Online Supplement**.

Measurement of circulating short chain fatty acids.

Measurements of short-chain fatty acids in plasma samples pre- and post-probiotic supplementation were performed by the Mayo Clinic Metabolomics Research Core (U24DK100469) using a targeted mass spectrometry approach leveraging ^{13}C or ^{15}N isotope-labeled reference compounds as appropriate.

Untargeted metabolomic profiling.

Untargeted metabolite profiling was performed on paired pre- and post-probiotic supplementation plasma samples by the Mayo Clinic Metabolomics Research Core (U24DK100469) using a 6550i Funnel Accurate-Mass Quadrupole Time-of-Flight Mass Spectrometer coupled with an Agilent 1290 Infinity UHPLC system (Agilent Inc, USA). Metabolite separation was achieved with a hydrophilic interaction column and a non-polar

reversed-phase C18 column. Quality control samples composed of a subset of samples were injected during each run. Putative metabolite identification was performed using the Metlin database and MetaCore with a detection window of ±10 ppm. Only metabolites present in 80% of samples were reported and used in analyses. Pre- and post-plasma samples appropriate for this analysis were available on 18 of the 20 subjects completing the study.

Assessment of ex vivo human arteriolar endothelium-dependent vasodilation by videomicroscopy.

Human small resistance arterioles from gluteal fat pad biopsies or surgical discards were prepared and endothelium dependent vasodilation to increasing doses of acetylcholine (Ach) measured by videomicroscopy as previously described.[21, 25–27] Vessel diameters were measured after each addition of Ach by video-microscopy using digital calipers (Boeckeler Instruments, Tucson, AZ). Paired vessels from each tissue sample underwent intra-luminal exposure to plasma obtained either before or after *Lp299v* supplementation for six hours before vasomotor measurements. Exposure to intra-luminal L-NAME (100 μmol/L) was performed for 30 minutes before incubation in each type of plasma. At the end of each series of vessel study, smooth muscle reactivity was determined by adding papaverine (0.2 mmol/L). Endothelium-dependent vasodilation of additional vessels from healthy volunteers was measured prior to and following four hours of exposure to 10 μM TMAO. This TMAO concentration was selected as it represents approximately twice the concentration reported in the plasma of individuals with CAD at highest risk of repeat cardiovascular events.[28]

Statistical analyses.

Statistical analyses were performed using SPSS 22.0, SigmaPlot 12.5, and GraphPad Prism. Subject characteristics, plasma biomarkers, and measures of endothelial function were measured at baseline and following six weeks of probiotic supplementation and compared by paired t-test or Wilcoxon Signed Rank test for non-normally distributed data as appropriate. General linear models with repeated measures were used to determine if thienopyridine or beta-blocker use significantly modified the impact of *Lp299v*. Other drug classes were not tested in this manner due to either their high or low utilization in the study population making insights from such analyses limited. For the separate vancomycin sub-study, similar measurements were also compared by paired t-test before and following vancomycin therapy. The primary outcome for this study was brachial artery FMD%. Our ad hoc power analysis suggested that enrollment of 22 subjects would give us 80% power to detect a 25% increase in FMD% from baseline assuming a 20% drop-out rate at alpha=0.05. P-values of <0.05 were considered statistically significant for all comparisons except those made for the untargeted metabolomics analyses. Using the data from the untargeted metabolomics analysis, the two-factor principal component analysis was performed using Mass Profiler Professional (Agilent Inc.) to determine if there were differences in the metabolomic profile in samples obtained before and following probiotic supplementation. Details of the microarray analysis and data analysis are included in Online Supplement.

RESULTS

Subject recruitment.

A total of 23 subjects were recruited for this study. Two subjects failed screening and were excluded. One subject suffered a stroke during the study and withdrew prior to completing the probiotic portion of the study. A total of 20 subjects completed the probiotic intervention. Thirteen of the 20 subjects agreed to participate in the additional vancomycin intervention portion of the study. Baseline characteristics for these 20 subjects are delineated in **Table 1**.

Baseline Characteristics and Changes by Intervention Group.

During the probiotic intervention phase **(Table 2)**, there were non-significant downward trends in total cholesterol (172±37 to 164±32 mg/dl, P=0.18) and LDL-cholesterol (96±33 to 89±30 mg/dl, P=0.16) levels, whereas triglyceride (P=0.47), and HDL (P=0.22) levels showed no appreciable difference. Systolic blood pressure increased following probiotic supplementation (132±11 to 138±12 mmHg, P=0.04) but diastolic blood pressure (P=0.49), heart rate, weight (P=0.67), and BMI (P=0.76) remained unchanged. Vancomycin supplementation did not result in any changes in total cholesterol, LDL and HDL cholesterol, triglycerides, weight, BMI, blood pressure, and heart rate **(Table 2)**. *Vascular Measurements:* After a six-week intervention with the probiotic, brachial FMD% significantly increased overall from 3.55±1.96 % to 4.73±2.32 % (**Figure 1A-B,** P=0.004). This significant difference remained even after allosteric rescaling using the method by Atkinson et al. (P=0.008). [29] There were no significant changes in baseline and peak hyperemic shear, resting diameter, or nitroglycerin-mediated vasodilation over the probiotic intervention phase **(Table 3)**.

General linear models failed to demonstrate an impact of thienopyridine use (P=0.41) or b-blocker use (P-0.71) on our findings. The change in systolic blood pressure with *Lp299v* supplementation was not associated with a change in FMD% (r=0.09,P=0.70) or resting brachial diameter (r=−0.31, P=0.20). Vancomycin therapy (N=13) showed no significant change in FMD% (**Figure 1C,** 4.05±1.90 to 3.8±2 %, *P*=0.728). Also, resting diameter, baseline and peak hyperemic shear and nitroglycerin-mediated dilation remained unchanged **(Table 3)**.

Impact of Lp299v supplementation on overall plasma metabolite content.

A total of 11,006 metabolites were identified in the subject plasma sample. Two-thousand nine-hundred eighty-nine were identified based on known elution patterns. Results of the two-factor principal component analysis are shown in **Figure 2**. A total of 114 compounds differed between pre- and post-probiotic supplementation plasma samples at P<0.05. Twenty-nine were positively identified while 85 remain unknown. Following adjustment for multiple testing using Bonferroni correction, no individual metabolite differed significantly between time points.

Impact of post-Lp299v supplementation plasma and TMAO on endothelium-dependent vasodilation of human microvessels.

We used plasma from 4 different subjects on five sets of subcutaneous adipose arterioles obtained from patients with CAD. As shown in **Figure 3**, treatment with post-probiotic plasma significantly improved endothelium-dependent vasodilation to acetylcholine (N=5, P=0.02 overall for pre- vs. post-*Lp299v* supplementation, P <0.004 at acetylcholine doses at $10^{-7}, 10^{-6}$, and 10^{-5} concentrations). There was no significant difference in the vasodilatory response to 2 mM papaverine (98.6±1.7 vs. 97.6±2.6 % for pre- vs. post-*Lp299v* supplementation, P=0.50). Use of L-NAME completely abrogated this improvement (**Figure 3A**). In a separate set of experiments, exposing adipose arterioles from healthy volunteers to 10 μM TMAO for 4 hours did not significantly impair endothelium-dependent vasodilation to acetylcholine (**Figure 3B,** N=4, P=0.65). Post-probiotic plasma had no effect on vasodilation of additional arterioles that were denuded of endothelium (**Figure 3C,** P=0.40, N=3)

Plasma biomarkers.

Probiotic supplementation resulted in a decrease of circulating inflammatory cytokines IL-8 by 33 % (**Figure 4A,** 14±7, to 10±4 pg/mL, *P*=0.01) and IL-12 by 21 % (**Figure 4B,** 53±29 to 42±27 pg/mL, *P*=0.02). No significant changes were seen in plasma levels of the following cytokines and circulating adhesion molecules prior to and following *Lp299v* supplementation: IL1β (median/IQR 0.83/1.37 vs. 0.66/1.45 pg/mL; P=0.37); TNF-α (median/IQL 8.80/65.10 vs. 7.30/39.30; P=0.38); IFN-γ (median/IQL 4.0/40.35 vs. 4.0/27.7 pg/mL; P=0.98); TGF-β (median/IQL 17.5/8.5 vs. 17.3/10.6 ng/mL; P=0.16); ICAM1 (23.9±5.2 vs. 24.0±7.9 ng/mL; P=0.99), and VCAM1 (47.6±15.2 vs. 47.8±18.6; P=0.95). Plasma TMAO concentration remained unchanged following probiotic supplementation [median pre-*Lp299v* 1,03 (interquartile rage 0.62–2.20) vs. vs. post-Lp299v median of 1.41 (interquartile range 0.71–4.15); P=0.27, **Figure 5A-B**). Plasma leptin levels significantly decreased post-supplementation (Figure 5C-D, 12.8±9.1 vs. 10.3±8.3 ng/mL P=0.001) while adiponectin levels remained unchanged (**Figure 5E-F,** 5.09±3.51 vs. 5.00±3.99 μg/mL, P=0.85). Plasma levels of acetic acid significantly decreased (**Online Figure IA-IB,** 44.2±11.5 vs. 37.7±7.1 μM, P=0.03) while propionic acid levels significantly increased (**Onlinge Figure IC-ID,** 31.5±.3.3 vs. 35.9 μM, P<0.001). No significant changes in butyric acid were seen **(Online Figure IE-IF)**, 0.80±0.31 vs. 0.83±0.28 μM, P=0.66).

Microbiome profiling.

Community characterization: We examined the richness, diversity and taxonomic composition of each sample. Bacterial genus richness ranged from 334 to 530, whereas archaeal genus richness ranged from 8 to 25. There was no significant change in the bacterial genus richness, and family level abundance detected in sample categories by time point of measurement (before and following *L. plantarum 299v* supplementation, **Online Figure II**). The top 9 classes represent approximately 67% of each sample's operational taxonomic units (OTUs, **Online Figure III**). None of the top 9 classes exhibited a significant change in the OTU proportion following probiotic supplementation (**Online Figure IIIB**).

Whole microbiome analysis: We analyzed beta diversity and explicit comparisons between samples, considering data from the whole microbiome. Because of the low dissimilarity within a subject's sample pairs, there was no separation of the microbiome between pre- and post-treatment samples observed in ordination and hierarchical clustering analysis using abundance metrics when paired samples were not considered. Hierarchical clustering analysis based on the abundance of 2,206 taxa revealed no separate clusters of samples from before and following probiotic supplementation **(Online Figure IIB)**. Most of the respective pre- and post-probiotic paired samples grouped with each other.

Paired comparison between pre- and post-probiotic treatment samples.

We performed a paired t-test to look for those OTUs that were significantly increased or decreased based on time-point while taking the sample parings into account. All comparisons were performed using the relative abundances of OTUs and plotted in rank abundance. There were 70 overlapping OTUs found to be significantly different between pre- and post-supplementation in their abundance. Nineteen of the 70 OTUs were classified at the genus level. Lactobacillus and Bacillus species were found enriched in post-treatment samples. Prior to adjustment for multiple comparisons, *lactobacillus reuteri* appeared more abundant in most of the post-treatment samples than pre-treatment samples from all subjects in the study (P=0.0014) (**Online Figure IIC**). OTU number 380, unclassified taxa in the Lactobacillaceae family, also appeared abundant in the post-treatment samples than pre-treatment samples from most subjects (P=0.0028) (**Online Figure IID**). Following adjusted for a false discovery rate of 5%, none of the OTUs were significantly different between sampling points. All 70 OTUs with P<0.05 in unadjusted analyses are presented in **Online Table I**.

DISCUSSION

This study suggests that the intestinal microbiota is mechanistically linked to systemic inflammation and vascular endothelial function in men with coronary artery disease. . Six-weeks of daily supplementation with GoodBelly™ StraightShot containing live, active *Lp299v* cultures significantly improved endothelium-dependent vasodilation of the brachial artery in men with stable coronary artery disease. Additionally, *Lp299v* supplementation resulted in systemic anti-inflammatory effects as evident by significantly decreased circulating levels of inflammatory cytokines IL-8 and IL-12, both known to play significant roles in leukocyte production, leukocyte, and endothelial activation, and recruitment to the vasculature.[30–33] *Lp299v* supplementation also reduced leptin levels, confirming studies performed in animal models and further supporting *Lp299v*'s anti-inflammatory effect.[34] Exposure to post-*Lp299v* supplementation plasma significantly improved endothelium-dependent vasodilation of resistance arteries from humans with CAD. Additionally, acute exposure to an elevated concentration of TMAO did not have an appreciable effect on endothelium-dependent vasodilation in healthy human vessels. Levels of short-chain fatty acids (SFCAs) changed with *Lp299v* supplementation, including a significant increase in plasma propionic acid and with a concomitant decrease in circulating acetic acid levels. Additionally, principal component analysis of plasma metabolites demonstrates that the metabolomics profile can be readily used to determine whether plasma sample was taken

before or following *Lp299v* supplementation. With respect to the stool microbiome, no changes were observed in the overall richness, diversity, or taxonomic composition of the stool microbiome. Unadjusted analyses of the OTU level suggest possible changes in species of the *Lactobacillus* genus that need to be verified with a larger sample size.

Taken together, these data demonstrate for the first time, to our knowledge, that oral supplementation with the probiotic *Lp299v* improves vascular endothelial function in men with CAD for both conduit and resistance vessels through increasing NO bioavailability while concomitantly reduces systemic inflammation. These effects were observed despite no significant favorable changes in traditional cardiovascular risk factors, suggesting that *Lp299v* supplementation may favorably impact vascular health at least in part through novel, yet-to-be-identified mediators. This concept is supported by clear differences in the plasma metabolite profiles when comparing pre- and post-*Lp299v* supplementation samples using two-factor principal component analysis. Additionally, our *in vivo* and *ex vivo* vessel data strongly suggest *Lp299v*'s favorable effects are independent of TMAO. TMAO produced as a byproduct of gut microbiome metabolism of choline has been established as both a predictor of future adverse CV events in patients with CAD and potentially as being mechanistically involved in atherogenesis, platelet activation, and endothelial inflammation and dysfunction.[7] Therefore, our data suggest gut microbiome-targeted interventions may favorably impact the vascular risk profile without an appreciable effect on TMAO production in men with CAD.

Animal studies of probiotic supplementation support the concept that this method of gut microbiome targeted therapy could have a beneficial impact on the vasculature. Rats supplemented with VSL#3 (a mixture of eight strains of probiotic from the *Streptococcus*, *Lactobacillus*, and *Bifidobacteria* genera) demonstrated reduced vascular oxidative stress and improved NO dependent vasorelaxation in a common bile duct ligation model.[35] Similar results were seen using supplementation with multiple *Lactobacillus* species in a hypertensive rat model.[36] Obese mice supplemented with *Lactobacillus coryniformis* CECT5711 on a high fat diet show improved endothelium-dependent vasodilation and reduced vascular oxidative stress.[37] To our knowledge, the only prior published work focusing on the impact of a probiotic on endothelial function in humans demonstrated a reduction in circulating VCAM-1 in 30 subjects with metabolic syndrome following 12 weeks of *Lactobacillus casei* supplementation but not in other measures of endothelial function.[38] We did not detect a change in either VCAM-1 or ICAM-1 in our study sample. Differences between the studies likely reflect differences in the study design including study duration, species of probiotic, and study duration.

Our study significantly extends to humans the concept that probiotic supplementation can directly and favorably impact vascular endothelial function by increasing NO bioavailability, and that this effect is likely induced in large portion by changes in circulating metabolites derived from gut microbiome metabolism. The impact of any probiotic intervention, like *Lp299v*, may be genus- and species-specific and may also differ based on host factors (e.g. age, sex, obesity and prevalent disease) that influence the gut microbiome composition, and may account for differences seen between our study and Tripolt et al.[38–42] Additional work

in this area will be necessary to more precisely target specific probiotic interventions which improve vascular health in particular patient populations.

In addition to improvements in endothelium-dependent vasodilation and NO bioavailability, *Lp299v* supplementation resulted in reduced systemic levels of IL-8, IL-12, and leptin. Endothelial cells produce IL-8 which acts to recruit monocytes to the vascular wall suggesting a key role for IL-8 in vascular inflammation and atherogenesis.[43, 44] IL-12 acts to induce cytotoxic T-cell activation and monocyte activation leading to additional pro-inflammatory cytokine activation known to contribute to vascular inflammation and atherogenesis.[45, 46] The observed decrease in leptin levels is similar to a reduction observed in a prior rat study by co-authors Baker and Salzman using the same *Lp299v* formulation used in the current study.[34] The observed decrease in leptin was associated with a reduction in induced myocardial infarct size and improved myocardial recovery post-infarction. Leptin levels are known to be elevated in individuals with increased body fat mass and insulin resistance, and increased levels are associated with adipose inflammation.[47] Additionally, leptin activates multiple pro-inflammatory cells and the production of pro-inflammatory cytokines (IL-6, TNF-α, Th1 cells, mononuclear cells, NK cells) known to contribute to vascular dysfunction and atherogenesis.[48–52] While we were unable to find changes in several other inflammatory markers, overall, our findings suggest *Lp299v* supplementation may suppress systemic inflammation which may have a favorable clinical impact on men at high risk for future cardiovascular events through reductions in vascular inflammation, reduced plaque formation, and increased plaque stability.[53]

The mechanisms behind the favorable vascular effects of *Lp299v* supplementation remain unclear. The lack of change in plasma lipids and glucose levels as well as the slight increase in blood pressure seen suggest the favorable impacts of *Lp299v* on vascular function and inflammation are independent of traditional cardiac risk factors in our study of men with CAD. However, our untargeted metabolomics profiling of plasma prior to and following *Lp299v* supplementation, and our data showing improvement in endothelial function with direct, acute exposure to post-*Lp299v* plasma strongly support the concept that *Lp299v* supplementation's favorable effects are mediated by changes in circulating metabolites (or their systemically modified derivatives) originating from changes in the gut microbiome. While no single plasma metabolite significantly differed following supplementation, two-factor PCA analysis demonstrated differences of the overall metabolomic profile. Our data do not support TMAO as one of the metabolites involved in the ameliorative effects of *Lp299v* supplementation. Even when excluding the two subjects with high TMAO measurements in post-*Lp299v* measurements, no significant changes were seen (data not shown). While TMAO represents one of many potential metabolites by which gut microbiome may impact vascular function, atherogenesis, and plaque stability, the concept that additional non-TMAO metabolites are likely involved in regulation of the cardiovascular system is well-accepted.[7]

Therefore, our data suggest further studies are needed to determine the identities and mechanisms of gut-microbiome produced metabolites changed by *Lp299v* supplementation that impact vascular endothelial function and systemic inflammation. One possible contributor in our study is propionate- a short chain fatty acid (SFCA) that increased with

Lp299v supplementation. Saccharolysis and fermentation by the colonic microbiome account for the overwhelming majority of circulating SCFAs in humans.[54] Recent data implicate SFCAs as important cell signaling molecules connecting gut microbial metabolism with blood pressure and vascular endothelial function through interactions with specific G protein-coupled receptors (GPCRs) with differing affinities for specific SCFAs. Interestingly, one of these GPCRs, free fatty acid receptor 3 (FFAR3/GPR41), has recently been demonstrated to be present in mouse vascular endothelium.[55] Activation of this receptor lowers blood pressure and improves endothelium-dependent vasodilation in mice, while mice deficient in GPR41 have elevated blood pressures and impaired endothelium-dependent vasodilation.[55–57] The GPR41's EC_{50} (11.6±1.4 μM) and dose-response curve for propionate suggests the increase in propionate concentration we observed would be on the steep portion of the dose-response curve and therefore expected to have an observable biological effect.[58] FFAR2(GPR43) and FFAR3 are also expressed on human mononuclear cells, and their activation suppresses the inflammatory response in human monocytes.[59] Fit into the context of these prior works, our data suggest increases in systemic propionate bioavailability may account for a least a portion of *Lp299v*'s beneficial effects.

We did find a modest elevation in blood pressure post-*Lp299v* supplementation. These data conflict with a small prior study in healthy men and women showing *Lp299v* supplementation reduced systolic blood pressure.[60] The reason for the differences between studies is unclear but may have to do with significant differences in the populations in each study (older men with CAD with only 5% current smokers compared to a study population that is significantly younger, sex-balanced, and comprised of 100% current smokers). The size of both studies are small, suggesting further data will be necessary to fully elucidate the relationship between *Lp299v* supplementation and systolic blood pressure.

Our data have several limitations. First, this work represents a small, interventional pilot study designed to determine whether *Lp299v* supplementation might have favorable effects on vascular function and systemic inflammation in humans and warrants further study. A larger, placebo-controlled, randomized trial is necessary to validate our findings and further assess mechanisms of effect. Only men were enrolled in this pilot due to multiple reports of significant differences in the gut microbiota composition between men and women that may in part relate to sex hormones and our preliminary data derived from male rats.[34, 40–42, 61, 62] Therefore our results cannot be generalized to women, and women should be included in the larger study mentioned to provide appropriately powered sex-stratified analyses. Our results only apply to patients with stable CAD and cannot be generalized to healthier populations or those with other chronic illnesses. We did not restrict the diet of individuals in this study. While diet can influence the composition of the gut microbiome, at individual level, bacterial lineages of the gut microbiome are remarkably stable over long periods of time with a variety of diets (e.g., low fat or low carbohydrate).[62] Even in the setting of dietary interventions, the bacterial communities in each individual are more alike over time than when compared to communities in other individuals.[63–67] Due to the small sample size, we were unable to confirm significant changes in *Lactobacillus* species in our analyses after multiple testing adjustment for 2206 OTU comparisons. Thus, these findings should be considered exploratory and suggest a larger study is warranted. Balanced against these

limitations are the novelty of the findings and the novel mechanistic directions that this work suggests concerning how probiotic supplementation may impact vascular health.

In conclusion, we found that *Lp299v* supplementation results in improved endothelium-dependent vasodilation and reduced systemic inflammation in men with stable coronary artery disease. Favorable changes include increased NO bioavailability as measured by endothelium-dependent vasodilation and reduced IL-8, IL-12, and leptin levels. The mechanisms of effect appear likely related to the probiotic causing changes in gut microbiome-derived circulating metabolites, including propionate, and appear to be independent of traditional cardiovascular risk factors and not related to changes in TMAO concentrations. Overall these findings support the concept that targeted use of probiotic supplementation may be an effective method to reduce cardiovascular risk in men. Our discovery of a relationship between *Lp299v,* improved vascular endothelial function, and decreased inflammation suggests the intestinal microbiota may be a promising target for interventions to prevent and treat the progression of cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviations and Acronyms:

FMD	flow-mediated dilation
Lp299v	*Lactobacillius plantarum 299v*
NO	Nitric oxide
NMD	nitroglycerin-mediated dilation
TMAO	trimethylamine oxide

REFERENCES

1. Ettinger G, MacDonald K, Reid G and Burton JP. The influence of the human microbiome and probiotics on cardiovascular health. Gut Microbes 2014;5:719–28. [PubMed: 25529048]

2. Griffin JL, Wang X and Stanley E. Does our gut microbiome predict cardiovascular risk? A review of the evidence from metabolomics. Circ Cardiovasc Genet 2015;8:187–91. [PubMed: 25691688]

3. Emoto T, Yamashita T, Sasaki N, Hirota Y, Hayashi T, So A, Kasahara K, Yodoi K, Matsumoto T, Mizoguchi T, Ogawa W and Hirata K. Analysis of Gut Microbiota in Coronary Artery Disease Patients: a Possible Link between Gut Microbiota and Coronary Artery Disease. J Atheroscler Thromb 2016;23:908–21. [PubMed: 26947598]

4. Singh M, Bedi US, Singh PP, Arora R and Khosla S. Leptin and the clinical cardiovascular risk. Int J Cardiol 2010;140:266–71. [PubMed: 19944469]

5. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63. [PubMed: 21475195]

6. Jonsson AL and Backhed F. Role of gut microbiota in atherosclerosis. Nat Rev Cardiol 2017;14:79–87. [PubMed: 27905479]

7. Tang WH and Hazen SL. The Gut Microbiome and Its Role in Cardiovascular Diseases. Circulation 2017;135:1008–1010. [PubMed: 28289004]

8. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y and Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–84. [PubMed: 23614584]

9. Marques FZ, Nelson E, Chu PY, Horlock D, Fiedler A, Ziemann M, Tan JK, Kuruppu S, Rajapakse NW, El-Osta A, Mackay CR and Kaye DM. High-Fiber Diet and Acetate Supplementation Change the Gut Microbiota and Prevent the Development of Hypertension and Heart Failure in Hypertensive Mice. Circulation 2017;135:964–977. [PubMed: 27927713]

10. Li J, Zhao F, Wang Y, et al. Gut microbiota dysbiosis contributes to the development of hypertension. Microbiome 2017;5:14. [PubMed: 28143587]

11. Asselbergs FW, van der Harst P, Jessurun GA, Tio RA and van Gilst WH. Clinical impact of vasomotor function assessment and the role of ACE-inhibitors and statins. Vascul Pharmacol 2005;42:125–40. [PubMed: 15792930]

12. Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, Satomura K, Ohsuzu F and Kurita A. Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol 1998;82:1535–9, A7–8. [PubMed: 9874063]

13. Vita JA and Keaney JF, Jr. Endothelial function: a barometer for cardiovascular risk? Circulation 2002;106:640–2. [PubMed: 12163419]

14. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME and Green DJ. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol 2011;300:H2–12. [PubMed: 20952670]

15. Widlansky ME, Gokce N, Keaney JF, Jr. and Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol 2003;42:1149–60. [PubMed: 14522472]

16. Naruszewicz M, Johansson ML, Zapolska-Downar D and Bukowska H. Effect of Lactobacillus plantarum 299v on cardiovascular disease risk factors in smokers. Am J Clin Nutr 2002;76:1249–55. [PubMed: 12450890]

17. Lam V, Su J, Koprowski S, Hsu A, Tweddell JS, Rafiee P, Gross GJ, Salzman NH and Baker JE. Intestinal microbiota determine severity of myocardial infarction in rats. Faseb J 2012;26:1727–35. [PubMed: 22247331]

18. Kim JY, Kim H, Jung BJ, Kim NR, Park JE and Chung DK. Lipoteichoic acid isolated from Lactobacillus plantarum suppresses LPS-mediated atherosclerotic plaque inflammation. Mol Cells 2013;35:115–24. [PubMed: 23456333]

19. Kizhakekuttu TJ, Gutterman DD, Phillips SA, Jurva JW, Arthur EI, Das E and Widlansky ME. Measuring FMD in the brachial artery: how important is QRS gating? J Appl Physiol 2010;109:959–965. [PubMed: 20671033]

20. Babar GS, Zidan H, Widlansky ME, Das E, Hoffmann RG, Daoud M and Alemzadeh R. Impaired endothelial function in preadolescent children with type 1 diabetes. Diabetes Care 2011;34:681–685. [PubMed: 21289230]

21. Kizhakekuttu TJ, Wang J, Dharmashankar K, Ying R, Gutterman DD, Vita JA and Widlansky ME. Adverse alterations in mitochondrial function contribute to type 2 diabetes mellitus-related endothelial dysfunction in humans. Arterioscler Thromb Vasc Biol 2012;32:2531–2539. [PubMed: 22879582]

22. Suboc T, Strath SJ, K D, A C, N M, J W, MJ T and ME W. Relative Importance of Step Count, Intensity, and Duration on Physical Activity's Impact on Vascular Structure and Function in Previously Sedentary Older Adults. J Am Heart Assoc 2014:DOI - 10.1161/JAHA.113.000702.

23. Hazen TC, Dubinsky EA, DeSantis TZ, et al. Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 2010;330:204–8. [PubMed: 20736401]

24. Wang Z, Levison BS, Hazen JE, Donahue L, Li XM and Hazen SL. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. Anal Biochem 2014;455:35–40. [PubMed: 24704102]

25. Wang J, Alexanian A, Ying R, Kizhakekuttu TJ, Dharmashankar K, Vasquez-Vivar J, Gutterman DD and Widlansky ME. Acute Exposure to Low Glucose Rapidly Induces Endothelial Dysfunction and Mitochondrial Oxidative Stress: Role for AMP Kinase. Arterioscler Thromb Vasc Biol 2012;32:712–720. [PubMed: 22207730]

26. Dharmashankar K, Welsh A, Wang J, Kizhakekuttu TJ, Ying R, Gutterman DD and Widlansky ME. Nitric oxide synthase-dependent vasodilation of human subcutaneous arterioles correlates with noninvasive measurements of endothelial function. Am J Hypertens 2012;25:528–34. [PubMed: 22337207]

27. Tanner MJ, Wang J, Ying R, Suboc TB, Malik M, Couillard A, Branum A, Puppala V and Widlansky ME. Dynamin-related protein 1 mediates low glucose-induced endothelial dysfunction in human arterioles. Am J Physiol Heart Circ Physiol 2017;312:H515–H527. [PubMed: 27923790]

28. Li XS, Obeid S, Klingenberg R, et al. Gut microbiota-dependent trimethylamine N-oxide in acute coronary syndromes: a prognostic marker for incident cardiovascular events beyond traditional risk factors. Eur Heart J 2017;38:814–824. [PubMed: 28077467]

29. Atkinson G and Batterham AM. The percentage flow-mediated dilation index: a large-sample investigation of its appropriateness, potential for bias and causal nexus in vascular medicine. Vasc Med 2013;18:354–65. [PubMed: 24172228]

30. Boekholdt SM, Peters RJ, Hack CE, Day NE, Luben R, Bingham SA, Wareham NJ, Reitsma PH and Khaw KT. IL-8 plasma concentrations and the risk of future coronary artery disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. Arterioscler Thromb Vasc Biol 2004;24:1503–8. [PubMed: 15178568]

31. Zittermann SI and Issekutz AC. Basic fibroblast growth factor (bFGF, FGF-2) potentiates leukocyte recruitment to inflammation by enhancing endothelial adhesion molecule expression. Am J Pathol 2006;168:835–46. [PubMed: 16507899]

32. Strasly M, Cavallo F, Geuna M, Mitola S, Colombo MP, Forni G and Bussolino F. IL-12 inhibition of endothelial cell functions and angiogenesis depends on lymphocyte-endothelial cell cross-talk. J Immunol 2001;166:3890–9. [PubMed: 11238633]

33. Bussolino F, Ziche M, Wang JM, Alessi D, Morbidelli L, Cremona O, Bosia A, Marchisio PC and Mantovani A. In vitro and in vivo activation of endothelial cells by colony-stimulating factors. J Clin Invest 1991;87:986–95. [PubMed: 1705569]

34. Lam V, Su J, Koprowski S, Hsu A, Tweddell JS, Rafiee P, Gross GJ, Salzman NH and Baker JE. Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J 2012;26:1727–35. [PubMed: 22247331]

35. Rashid SK, Idris-Khodja N, Auger C, Alhosin M, Boehm N, Oswald-Mammosser M and Schini-Kerth VB. Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: role of the angiotensin system. PLoS One 2014;9:e97458. [PubMed: 24832090]

36. Gomez-Guzman M, Toral M, Romero M, Jimenez R, Galindo P, Sanchez M, Zarzuelo MJ, Olivares M, Galvez J and Duarte J. Antihypertensive effects of probiotics Lactobacillus strains in spontaneously hypertensive rats. Mol Nutr Food Res 2015;59:2326–36. [PubMed: 26255877]

37. Toral M, Gomez-Guzman M, Jimenez R, Romero M, Sanchez M, Utrilla MP, Garrido-Mesa N, Rodriguez-Cabezas ME, Olivares M, Galvez J and Duarte J. The probiotic Lactobacillus coryniformis CECT5711 reduces the vascular pro-oxidant and pro-inflammatory status in obese mice. Clin Sci (Lond) 2014;127:33–45. [PubMed: 24410749]

38. Tripolt NJ, Leber B, Blattl D, Eder M, Wonisch W, Scharnagl H, Stojakovic T, Obermayer-Pietsch B, Wascher TC, Pieber TR, Stadlbauer V and Sourij H. Short communication: Effect of supplementation with Lactobacillus casei Shirota on insulin sensitivity, beta-cell function, and markers of endothelial function and inflammation in subjects with metabolic syndrome--a pilot study. J Dairy Sci 2013;96:89–95. [PubMed: 23164226]

39. Dominianni C, Sinha R, Goedert JJ, Pei Z, Yang L, Hayes RB and Ahn J. Sex, body mass index, and dietary fiber intake influence the human gut microbiome. PLoS One 2015;10:e0124599. [PubMed: 25874569]

40. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD and Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102:11070–5. [PubMed: 16033867]

41. Haro C, Rangel-Zuniga OA, Alcala-Diaz JF, Gomez-Delgado F, Perez-Martinez P, Delgado-Lista J, Quintana-Navarro GM, Landa BB, Navas-Cortes JA, Tena-Sempere M, Clemente JC, Lopez-Miranda J, Perez-Jimenez F and Camargo A. Intestinal Microbiota Is Influenced by Gender and Body Mass Index. PLoS One 2016;11:e0154090. [PubMed: 27228093]

42. Le Chatelier E, Nielsen T, Qin J, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500:541–6. [PubMed: 23985870]

43. Srinivasan S, Yeh M, Danziger EC, Hatley ME, Riggan AE, Leitinger N, Berliner JA and Hedrick CC. Glucose regulates monocyte adhesion through endothelial production of interleukin-8. Circ Res 2003;92:371–377. [PubMed: 12600878]

44. Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., Luster AD, Luscinskas FW and Rosenzweig A. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999;398:718–23. [PubMed: 10227295]

45. Lee TS, Yen HC, Pan CC and Chau LY. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 1999;19:734–42. [PubMed: 10073981]

46. Sprague AH and Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol 2009;78:539–52. [PubMed: 19413999]

47. Harle P and Straub RH. Leptin is a link between adipose tissue and inflammation. Ann N Y Acad Sci 2006;1069:454–62. [PubMed: 16855173]

48. Iikuni N, Lam QL, Lu L, Matarese G and La Cava A. Leptin and Inflammation. Curr Immunol Rev 2008;4:70–79. [PubMed: 20198122]

49. Yang WH, Liu SC, Tsai CH, Fong YC, Wang SJ, Chang YS and Tang CH. Leptin induces IL-6 expression through OBRl receptor signaling pathway in human synovial fibroblasts. PLoS One 2013;8:e75551. [PubMed: 24086566]

50. Tang CH, Lu DY, Yang RS, Tsai HY, Kao MC, Fu WM and Chen YF. Leptin-induced IL-6 production is mediated by leptin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, Akt, NF-kappaB, and p300 pathway in microglia. J Immunol 2007;179:1292–302. [PubMed: 17617622]

51. Wassmann S, Stumpf M, Strehlow K, Schmid A, Schieffer B, Bohm M and Nickenig G. Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin II type 1 receptor. Circ Res 2004;94:534–41. [PubMed: 14699015]

52. Santos-Alvarez J, Goberna R and Sanchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. Cell Immunol 1999;194:6–11. [PubMed: 10357875]

53. Ridker PM, Everett BM, Thuren T, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med 2017;377:1119–1131. [PubMed: 28845751]

54. Occurrence Bugaut M., absorption and metabolism of short chain fatty acids in the digestive tract of mammals. Comp Biochem Physiol B 1987;86:439–72. [PubMed: 3297476]

55. Natarajan N, Hori D, Flavahan S, Steppan J, Flavahan NA, Berkowitz DE and Pluznick JL. Microbial short chain fatty acid metabolites lower blood pressure via endothelial G protein-coupled receptor 41. Physiol Genomics 2016;48:826–834. [PubMed: 27664183]

56. Miyamoto J, Hasegawa S, Kasubuchi M, Ichimura A, Nakajima A and Kimura I. Nutritional Signaling via Free Fatty Acid Receptors. Int J Mol Sci 2016;17:450. [PubMed: 27023530]

57. Pluznick JL. Renal and cardiovascular sensory receptors and blood pressure regulation. Am J Physiol Renal Physiol 2013;305:F439–44. [PubMed: 23761671]

58. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M and Detheux M. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 2003;278:25481–9. [PubMed: 12711604]

59. Ang Z, Er JZ, Tan NS, Lu J, Liou YC, Grosse J and Ding JL. Human and mouse monocytes display distinct signalling and cytokine profiles upon stimulation with FFAR2/FFAR3 short-chain fatty acid receptor agonists. Sci Rep 2016;6:34145. [PubMed: 27667443]

60. Naruszewicz M, Johansson ML, Zapolska-Downar D and Bukowska H. Effect of Lactobacillus plantarum 299v on cardiovascular disease risk factors in smokers. Am J Clin Nutr 2002;76:1249–55. [PubMed: 12450890]

61. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ and Danska JS. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science 2013;339:1084–8. [PubMed: 23328391]

62. Ley RE, Turnbaugh PJ, Klein S and Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022–3. [PubMed: 17183309]

63. Nelson KE, Weinstock GM, Highlander SK, et al. A catalog of reference genomes from the human microbiome. Science 2010;328:994–9. [PubMed: 20489017]

64. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, Knights D, Gajer P, Ravel J, Fierer N, Gordon JI and Knight R. Moving pictures of the human microbiome. Genome biology 2011;12:R50. [PubMed: 21624126]

65. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI and Knight R. Bacterial community variation in human body habitats across space and time. Science 2009;326:1694–7. [PubMed: 19892944]

66. Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK and Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PloS one 2010;5:e9836. [PubMed: 20352091]

67. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK and Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012;489:220–30. [PubMed: 22972295]

NOVELTY AND SIGNIFICANCE

What Is Known?

- The connection between gut microbiome composition and activity and atherosclerotic vascular disease has been increasing recognized in recent years.

- Animal studies suggest supplementation with oral probiotics may have favorable effects on the vasculature.

What New Information Does This Article Contribute?

- Oral supplementation with *L. plantarum 299v* (*Lp299v*), a probiotic, improved vascular endothelial function and inflammation in men with stable coronary artery disease.

- The favorable effects of *Lp299v* occur without favorable changes in traditional cardiovascular risk factors or trimethylamine oxide (TMAO) levels.

- The favorable effects of *Lp299v* appear to be mediated through changes in circulating metabolites that lead to an increase in nitric oxide (NO) bioavailability.

The interaction between the gut microbiome, vascular regulation, and atherosclerosis has only recently been recognized. Limited human data are available to determine whether any gut microbiome-targeted intervention has the potential modifies cardiovascular risk in patients with coronary artery disease. We demonstrated, for the first time, that supplementation with *L. plantarum 299v* (*Lp299v*) improved endothelium-dependent vasodilation and reduced systemic inflammation in men with stable coronary artery disease. These favorable effects of *Lp299v* occurred without favorable changes in traditional cardiovascular risk factors. Additionally, we found supplementation did not change plasma TMAO, the gut metabolite most commonly associated with cardiovascular risk. The findings suggest that *Lp299v*-induced changes in gut-derived metabolites may be responsible for the favorable effects. Our discovery of a relationship between *Lp299v*, improved vascular endothelial function, and decreased inflammation suggests the target the intestinal microbiota with *Lp299v* could be a promising new method to reduce the activity and progression atherosclerotic disease.

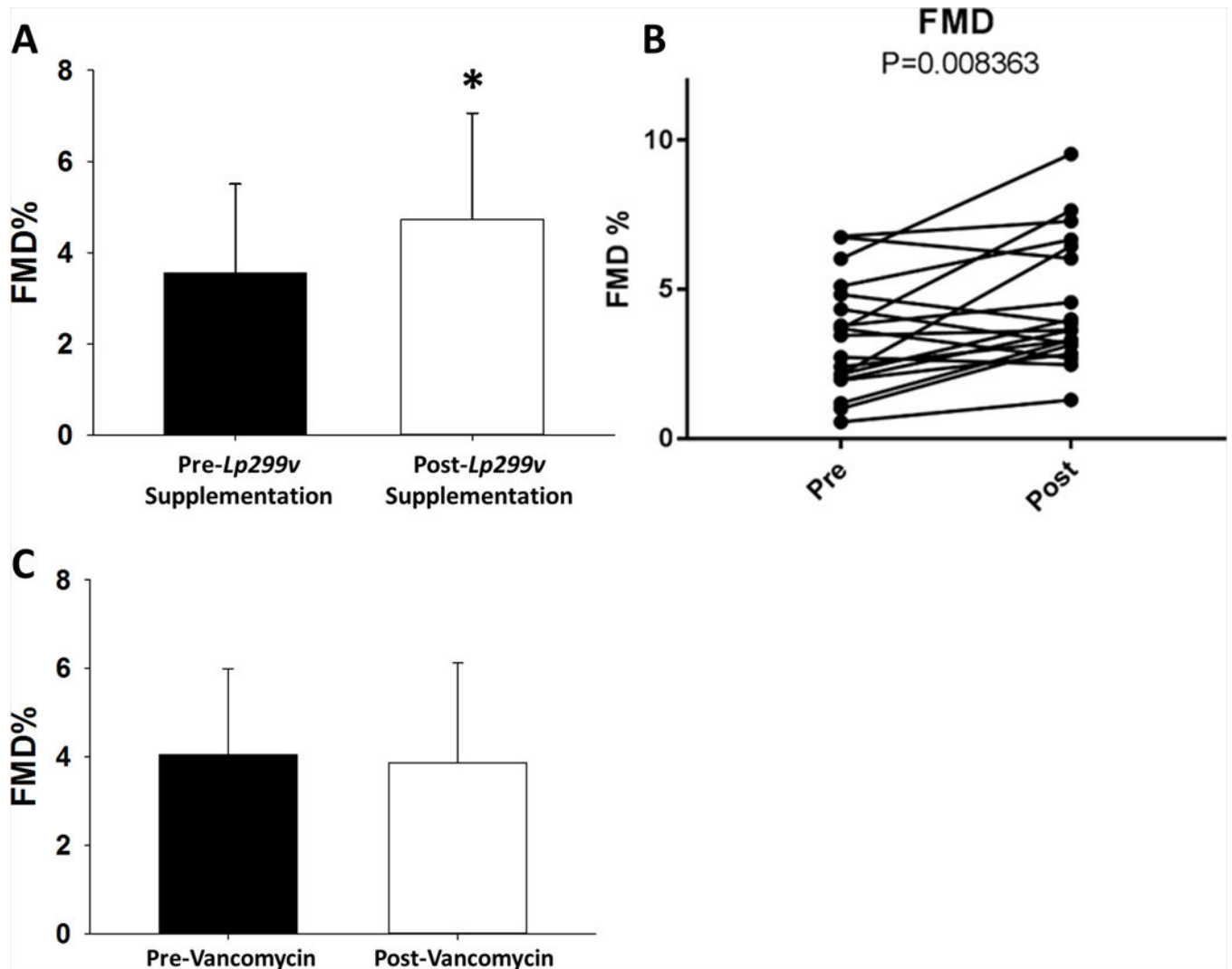**Figure 1:**

Brachial FMD% significantly improved following six weeks of *Lp299v* supplementation (3.55+1.96 to 4.73+2.32%, P=0.008) **(A)**. Individual changes in brachial FMD% are depicted **(B)**. There was no significant change in FMD% following ten days of oral vancomycin (4.05+1.90 to 3.8+2 %, P=0.73) **(C)**. FMD- Flow-Mediated Dilation
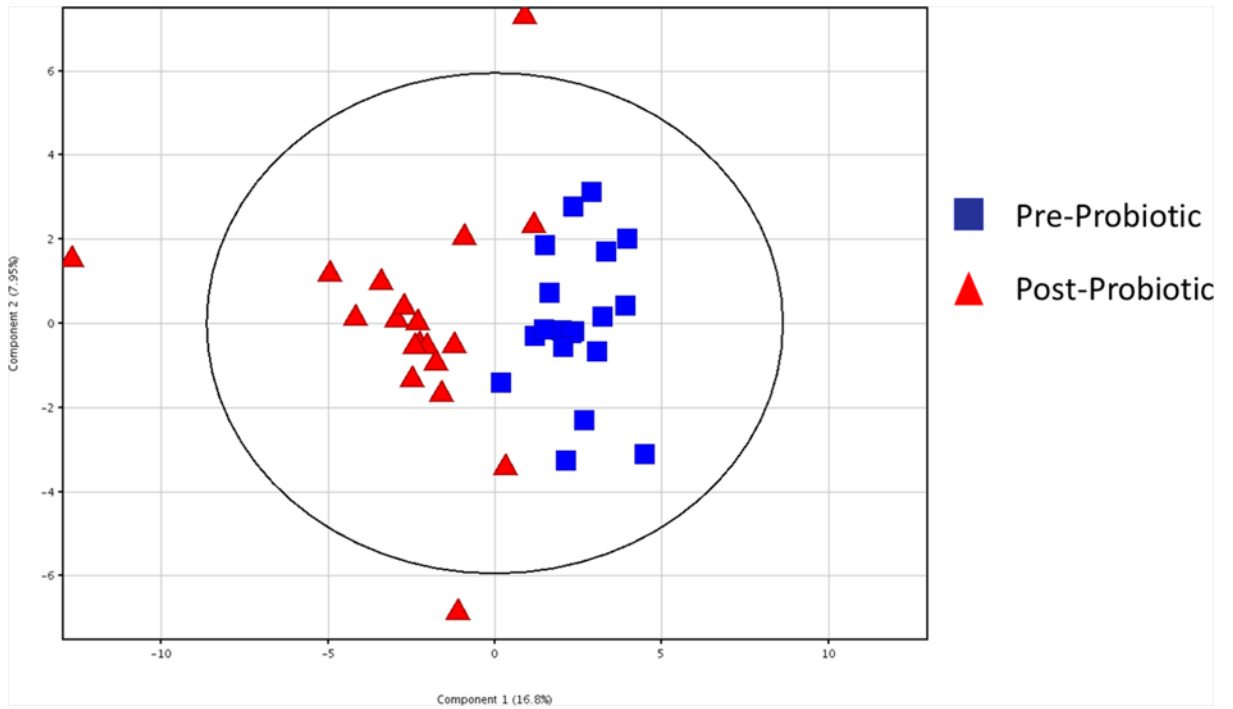
Figure 2:
Two-way Principal Component Analysis of untargeted metabolite profiling performed on paired pre- and post-probiotic supplementation plasma samples. Squares represent pre-*Lp299v* samples and triangles represent post *Lp299v* samples.
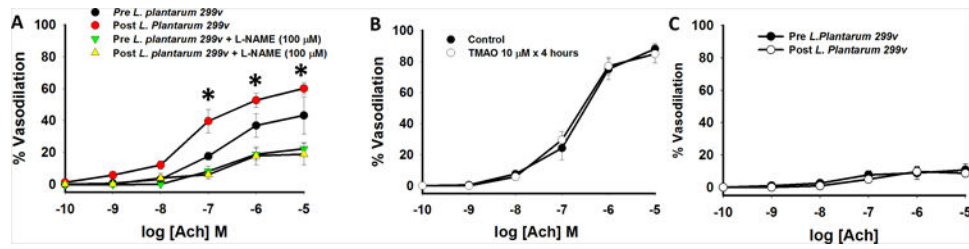
Figure 3:

Six hours of intra-luminal exposure to post *Lp299v* plasma significantly improved endothelium-dependent vasodilation of resistance arterioles from subjects with CAD (N=5 for all experiments, P=0.02 overall, *–P<0.004 at the indicated concentration of Ach). This improvement was completely blocked by eNOS-inhibitor L-NAME **(A).** Four hours of intra-luminal exposure with 10μM TMAO has no significant impact on endothelium-dependent vasodilation to Ach (N=4, P=0.65) in arterioles from healthy subjects **(B).** Endothelial denudation abrogated the acetylcholine vasodilatory response of human arterioles exposed to pre- and post-*Lp299v* plasma equivalently (P=0.40). Ach- acetylcholine.
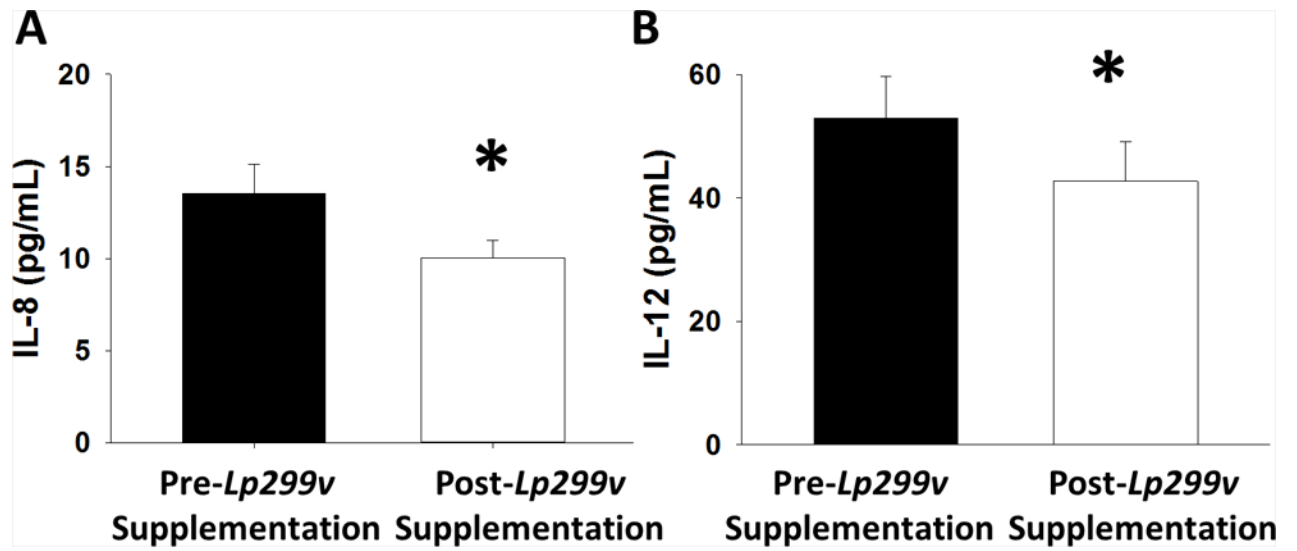
Figure 4:

Impact of *Lp299v* supplementation on systemic inflammatory cytokines. Circulating plasma levels of IL-8 were significantly reduced by *Lp299v* supplementation (14±7, to 10±4 pg/mL, *P*=0.01) **(A).** *Lp299v* similarly reduced systemic IL-12 levels (53±29 to 42±27 pg/mL, *P*=0.02) **(B).**
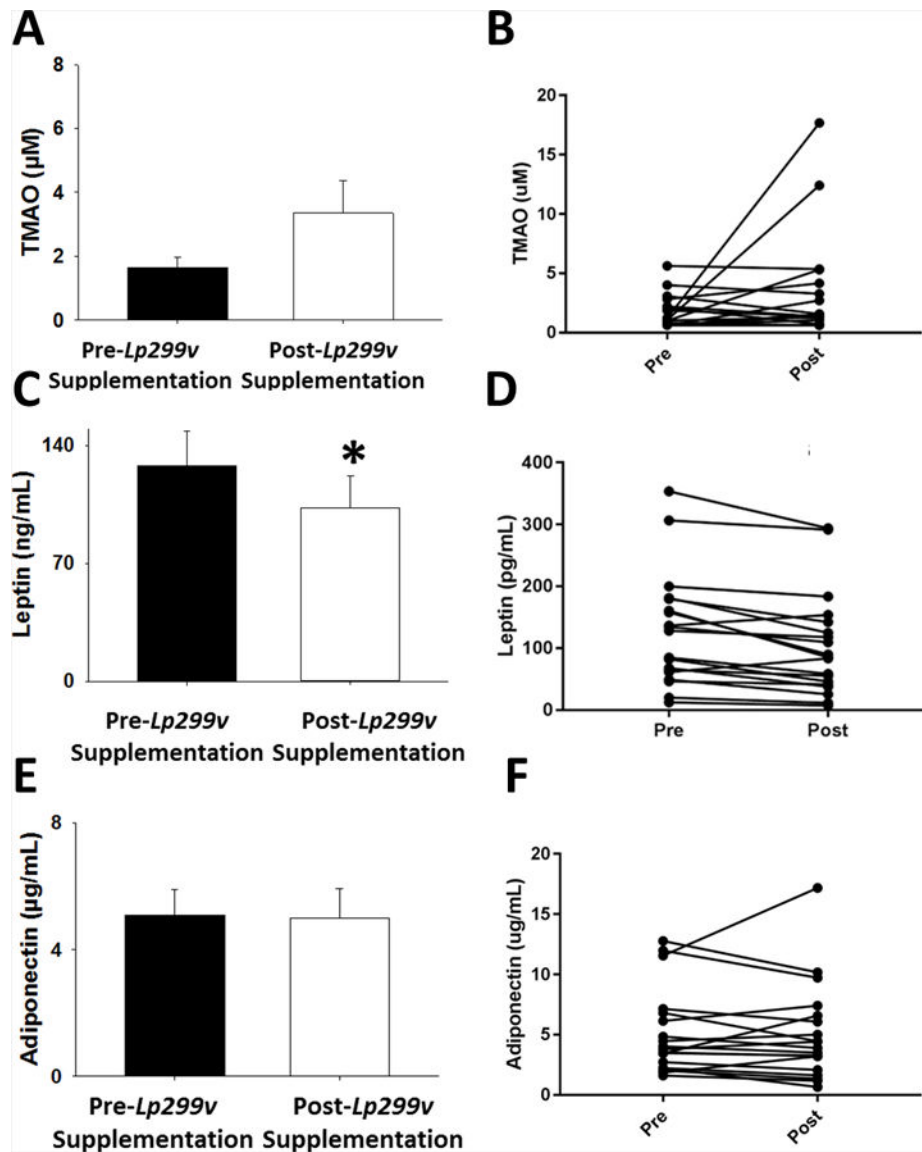
Figure 5:
Impact of *Lp299v* supplementation on circulating TMAO and adipokine concentrations. Plasma TMAO concentration remained unchanged following probiotic supplementation (1.64±1.39 vs. 3.35±4.47 μM, P=0.27) **(A** and **B).** Plasma leptin levels significantly decreased post-supplementation (2.8±9.1 vs. 5.00±3.99 μg/mL, P=0.85).

Table 1:

Demographics and Characteristics of Study Participants

Table 1 Baseline Demographics	
Age (yr)	63±7
Sex (% Male)	100
History of Coronary Artery Disease (%)	100
History of Myocardial Infarction (%)	30
History of Percutaneous Coronary Intervention (%)	80
Diabetes Mellitus (%)	15
Hypertension (%)	50
Hyperlipidemia (%)	70
HMG-CoA Reductase Inhibitors (%)	85
Smoking Status (%current/%past)	5/45
Weight (kg)	98.4±13.0
Waist Circumference (cm)	109.3±9.5
Body Mass Index (kg/m^2)	31.1±4.1
Medication Use (% taking medication class indicated):	
Aspirin	75
Thienopyridines	45
Beta-Blockers	65
ACE Inhibitors	25
Angiotensin II Receptor Blockers	15
HMG CoA Reductase Inhibitors	85
Ezetimibe	15
Fish Oil	25
Long-Acting Nitrates	0
Ranolazine	5

Table 2:

Subject Characteristics Before and Following Study Interventions

Baseline Characteristics	Pre-Probiotic (N=20)	Post-Probiotic (N=20)	
Weight, kg	98 ± 3	99 ± 13	P = 0.67
Waist Circumference, cm	109 ± 9	110 ± 9	P = 0.39
BMI, kg/m2	31 + 4	31 + 4	P = 0.76
Heart Rate, per minute	66 ± 8	68 ± 8	P = 0.38
Systolic Blood Pressure, mmHg	132 ± 11	138 ± 12	P = 0.039*
Diastolic Blood Pressure, mmHg	76 ± 8	77 ± 8	P = 0.49
Total Cholesterol, mg/dl	172 ± 37	164 ± 32	P = 0.18
LDL-Cholesterol, mg/dl	96 ± 33	89 ± 30	P = 0.16
HDL-Cholesterol, mg/dl	50 ± 14	48 ± 13	P = 0.22
Triglycerides, mg/dl	128 ± 59	135 ± 74	P = 0.47
	Pre-Vancomycin (N=13)	Post-Vancomycin (N=13)	
Weight, kg	102 + 14	102 + 14	P = 0.48
Waist Circumference, cm	112 + 10	112 + 10	P = 0.47
BMI, kg/m2	32 + 5	32 + 5	P = 0.49
Heart Rate, per minute	66 + 7	67 + 7	P = 0.15
Systolic Blood Pressure, mmHg	132 + 16	134 + 18	P = 0.74
Diastolic Blood Pressure, mmHg	73 + 7	75 + 8	P = 0.38
Total Cholesterol, mg/dl	160 + 40	155 + 32	P = 0.33
LDL-Cholesterol, mg/dl	88 + 34	83 + 29	P = 0.29
HDL-Cholesterol, mg/dl	50 + 14	51 + 12	P = 0.65
Triglycerides, mg/dl	105 + 45	96 + 25	P = 0.46

BMI indicates body mass index; LDL low-density lipoprotein; HDL, high-density lipoprotein

Table 3:

Measurements of Vascular Function Before and Following Study Interventions

	Pre-Probiotic (N=20)	Post-Probiotic (N=20)	
Resting Diameter, mm	3.94 + 0.38	3.87 + 0.42	P = 0.35
Flow Mediated Dilation, mm	0.14 + 0.08	0.18 + 0.08	P = 0.008*
Peak Hyperemic Shear dynes/cm2	76 + 14	80 + 16	P = 0.29
Baseline Peak Shear dynes/cm2	41 + 10	40 + 11	P = 0.79
Nitroglycein Mediated Dilation, %	22.8 + 6.3	22.0 + 7.6	P = 0.87
	Pre-Vancomycin (N=13)	**Post-Vancomycin (N=13)**	
Resting Diameter, mm	4.05 + 0.06	4.01 + 0.43	P = 0.73
Flow Mediated Dilation, mm	0.16 + 0.07	0.16 + 0.09	P = 0.77
Peak Hyperemic Shear dynes/cm2	76 + 23	82 + 23	P = 0.32
Baseline Peak Shear dynes/cm2	39 + 8	42 + 10	P = 0.04
Nitroglycein Mediated Dilation, %	19.5 + 5.9	17.5 + 4.3	P = 0.17