Lactobacillus plantarum 299v probiotic supplementation in men with stable coronary artery disease suppresses systemic inflammation

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Recent trials demonstrate that systemic anti-inflammatory therapy reduces cardiovascular events in coronary artery disease (CAD) patients. We recently demonstrated Lactobacillus plantarum 299v (Lp299v) supplementation improved vascular endothelial function in men with stable CAD. Whether this favorable effect is in part due to anti-inflammatory action remains unknown. Testing this hypothesis, we exposed plasma obtained before and after Lp299v supplementation from these subjects to a healthy donor’s PBMCs and measured differences in the PBMC transcriptome, performed gene ontological analyses, and compared Lp299v-induced transcriptome changes with changes in vascular function. Daily alcohol users (DAUs) (n = 4) had a significantly different response to Lp299v and were separated from the main analyses. Non-DAUs (n = 15) showed improved brachial flow-mediated dilation (FMD) and reduced circulating IL-8, IL-12, and leptin. 997 genes were significantly changed. I.I.com decreased (1.01 ± 0.74 vs. 0.22 ± 0.51; P < 0.0001), indicating strong anti-inflammatory effects. Pathway analyses revealed downregulation of IL-1β, interferon-stimulated pathways, and toll-like receptor signaling, and an increase in regulator T-cell (Treg) activity. Reductions in GBP1, JAK2, and TRAIL expression correlated with improved FMD. In non-DAU men with stable CAD, post-Lp299v supplementation plasma induced anti-inflammatory transcriptome changes in human PBMCs that could benefit CAD patients. Future studies should delineate changes in circulating metabolites responsible for these effects.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| I.I.com      | Composite inflammatory index |
| CAD          | Coronary artery disease |
| SOCS1        | Cytokine signaling-1 |
| DAVID        | Database for annotation, visualization, and integrated discovery |
| DAU          | Daily alcohol user |
| TREX1        | DNA exonuclease 3'-repair exonuclease 1 |
| FMD          | Flow-mediated dilation |
| GBP1         | Guanyl binding protein 1 |
| IFNα         | Interferon gamma |
| JAK2         | Janus kinase 2 |
| Lp299v       | Lactobacillus plantarum 299v |
| PBMCs        | Peripheral blood mononuclear cells |
| Blimp1       | PRDM1 |
| Treg         | Regulator T-cell |

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Over the last several decades, appreciation has grown for the critical role of systemic inflammation regulated by peripheral blood mononuclear cells (including lymphocytes, monocytes, and granulocytes) in the development of atherosclerotic disease and plaque vulnerability. The mechanistic role of PBMC-driven inflammation in the development and activity of coronary artery disease has led to the completion of large randomized trials targeting pro-inflammatory cytokine and using anti-inflammatory medications that have shown some benefit in the secondary prevention of atherosclerotic events.

Emerging data also implicate the gut microbiota in the regulation of systemic inflammation. Proinflammatory gut microbiota in murine models leads to lower levels of short chain fatty acid production, increased production of pro-inflammatory cytokines including interferon gamma (IFN-γ), IL-1β, and IL-2, increased PBMC production and accelerated atherosclerosis with increased neutrophil infiltration. In humans, the relative abundance of certain genus of bacteria have been shown to be associated with coronary artery disease and carotid atherosclerosis. Metagenomic analysis suggests the gut microbiomes of patients with atherosclerosis are enriched with genes that may stimulate innate immunity, with suppressed levels of genes expressing anti-inflammatory molecules including SCFAs. The gut microbiota also influences gut barrier function which may influence the inflammatory impact of the gut microbiota and its metabolites.

Murine studies have demonstrated worsened endothelial dysfunction accompanied by increased systemic inflammatory markers and immune cell activity in two models: conventionally raised versus germ-free ApoE−/− mice, and germ-free WT mice colonized with normal gut flora versus non-colonized GF WT mice. In an LDLR-/- model, GF mice demonstrated lower carotid artery thrombus area accompanied by decreased leukocyte adhesion and decreased adhesion-dependent platelet activation ex vivo. A study in angiotensin II-infused LysM transgenic mice showed reduced eNOS glutathionylation (uncoupling) and aortic endothelial superoxide formation with LysM(+) monocyte ablation. Together, these murine data suggest the attractive hypothesis that targeting the gut microbiota with an intervention that leads to anti-inflammatory effect on PBMCs may also favorably impact the health of the vasculature in humans.

We recently reported that six weeks of supplementation with a probiotic, *Lactobacillus plantarum* 299v (Lp299v), results in improved vascular endothelial function, reduced circulating levels of interleukins 8 and 12, and increased propionic acid production in men with stable coronary artery disease. We hypothesized that a portion of the favorable impact of Lp299v is due to a systemic, genomic anti-inflammatory effect, and that a portion of these transcriptome changes would be associated with improvements in in vivo endothelial function. To test these hypotheses, we used plasma from subjects in our interventional study of Lp299v supplementation to determine whether post-probiotic supplementation plasma induces a systemic anti-inflammatory impact that could be favorable in men with CAD.

**Results**

**Study subjects.** The characteristics of the 15 subjects included in these analyses prior to and following *Lp299v* supplementation are included in Table 1. Study subjects (*n* = 15) were all men with an average age of 63 ± 7 years. Medical therapies included the following: aspirin (86%), beta blocker (73%), ezetimibe (6.7%), fibrate (6.7%), HMG-CoA Reductase Inhibitor (86.7%), P2Y12 inhibitor (40%), niacin (6.7%), and ranolazine (6.7%). Self-reported alcohol intake frequency were as follows: rarely/never (*n* = 8), several times per month (*n* = 1), once per week (*n* = 2), several times per week (*n* = 4). Supplemental Table S1 provides subject characteristics data on the four subjects who were excluded due to daily ETOH intake. Of note, post-supplementation, both the 15 non-daily drinking subjects and the 4 daily drinking subjects showed significantly increased circulating propionic acid levels. (Table 1 and Supplemental Table S1). There were no significant changes in butyric acid for either group. Acetic acid concentrations trended downward in both groups without statistically significant changes.

**Measures of brachial artery flow-mediated dilation, ILs-8,12, and leptin.** As seen in Fig. 1A–D, brachial artery flow-mediated dilation significantly increased (1A, 3.70 ± 0.51 vs. 4.58 ± 0.61%, *P* = 0.016) while IL-8 (1B, 13.8 ± 2.0 vs. 10.1 ± 1.1 pg/mL, *P* = 0.032), IL-12 (1C, 54.6 ± 8.1 vs. 33.0 ± 7.9 pg/mL, *P* = 0.042), and leptin (1D, 136 ± 25 vs. 111 ± 23, ng/mL, *P* = 0.002) all significantly decreased. These findings confirm that this subset of subjects showed similar degrees of improvements in all these parameters as previously reported for the full study cohort.

**Plasma-induced Transcriptome changes with Lp299v supplementation.** Overall, 1,443 probe sets (505 downregulated and 938 upregulated) representing 997 unique genes met our criteria for differential induction after *Lp299v* supplementation; these data are depicted as heat maps (Fig. 2) where the data are expressed as a ratio of post-supplement versus pre-supplement. As seen in Fig. 2, the pattern of changes in gene transcription of the 15 non-DAU subjects differs from DAUs (Fig. 2A). In general, the 15 non-DAU subjects exhibited reduced induction of inflammatory transcripts and increased induction of regulatory transcripts after *Lp299v* supplementation. A subset of well-annotated transcripts representing these changes is depicted in Fig. 2B. A full list of the gene transcripts that were significantly changed are included in Supplemental Table S2.

**Ingenuity pathway analysis: regulated pathways and predicted upstream regulators.** Canonical pathways considered to be significantly changed (*|z-score|* ≥ 2 and *P* < 0.01) by exposure to post-*Lp299v* supplementation plasma are listed in Table 2. Additionally, IPA identified 101 upstream regulators whose suppression...
| Physical measurements          | Pre-Lp299v | Post-Lp299v | p Value |
|-------------------------------|-----------|------------|---------|
| Systolic blood pressure (mmHg) | 136 ± 15  | 137 ± 14   | 0.80    |
| Diastolic blood pressure (mmHg)| 75 ± 6    | 75 ± 8     | 0.84    |
| Body mass index (kg/m²)       | 31.3 ± 4.2| 31.2 ± 4.2 |         |

| Plasma biomarkers             |           |            |         |
|-------------------------------|-----------|------------|---------|
| Fasting glucose (mg/dL)       | 112 ± 45  | 112 ± 50   | 0.91    |
| Total cholesterol (mg/dL)     | 170 ± 34  | 164 ± 24   | 0.39    |
| HDL (mg/dL)                   | 49 ± 12   | 47 ± 10    | 0.40    |
| LDL (mg/dL)                   | 95 ± 31   | 89 ± 25    | 0.32    |
| Triglycerides (mg/dL)         | 130 ± 61  | 140 ± 81   | 0.44    |
| Leptin (ng/mL)                | 136 ± 98  | 111 ± 90   | 0.002   |

| Short chain fatty acids       |           |            |         |
|-------------------------------|-----------|------------|---------|
| Propionic acid (µM)           | 31.9 ± 3.5| 36.2 ± 3.6 | 0.005   |
| Butyric acid (µM)             | 0.8 ± 0.4 | 0.8 ± 0.3  | 0.99    |
| Acetic acid (µM)              | 42.1 ± 9.6| 37.2 ± 7.4 | 0.16    |

| Vascular function measurements|           |            |         |
|--------------------------------|-----------|------------|---------|
| Resting brachial diameter (mm) | 4.14 ± 0.43| 4.01 ± 0.39| 0.14    |
| Peak hyperemic shear (dynes/cm²)| 74.8 ± 14.3| 82.9 ± 15.1| 0.58    |
| Baseline peak shear (dynes/cm²) | 41.4 ± 10.4| 40.8 ± 12.2| 0.77    |
| Nitroglycerin-mediated dilation (%) | 19.9 ± 5.7 | 19.2 ± 5.1 | 0.75    |

Table 1. Subject characteristics. *All data presented as mean ± SD. N = 6 for Nitroglycerin-Mediated Dilation. N = 14 short chain fatty acid measurements. N = 15 for all other comparisons. p Values derived from Paired t-Test. †Lp299v = Lactobacillus plantarum 299v.

Figure 1. Effects of Lp299v supplementation on Brachial Artery FMD, ILs-8, 12, and Leptin in Non-Daily Alcohol Drinkers: Lp299v supplementation resulted in (A) improved brachial flow mediated dilation (FMD)% (3.70 ± 0.51 vs. 4.58 ± 0.61%, n = 15; P = 0.016) while IL-8 (B, 13.8 ± 2.0 vs. 10.1 ± 1.1 pg/mL, n = 15; P = 0.032), IL-12 (C, 54.6 ± 8.1 vs. 33.0 ± 7.9 pg/mL, n = 15; P = 0.042), and leptin (D, 136 ± 25 vs. 111 ± 23 ng/mL, n = 15; P = 0.002) all significantly decreased. All statistical tests paired t-tests. All values given as mean ± SE. *p<0.05.
Figure 2. Post-Lp299v Plasma-Induced PBMC Signatures—Stratified by Daily and Non-Daily Alcohol Drinkers: (A) 1,443 probe sets representing 997 unique genes were altered by at least 20% with a qval FDR < 0.2) in 15 individuals with stable CAD who did not report daily alcohol use (left) and four individuals with stable CAD who did report daily alcohol use (right). The mean for each group is indicated and each subsequent column is a subject. Data are expressed as a fold of change post- vs pre-supplementation with Lp299v. Red represents upregulated probe sets (n = 505); green represents downregulated probe sets (n = 938). (B) Well annotated transcripts associated with inflammatory and regulatory activity that showed significant change with Lp299v supplementation in these subjects.

Table 2. Canonical pathways significantly impacted by Lp299v supplementation. *Lp299v = Lactobacillus plantarum 299v* genes that were differentially expressed by at least 20% between pre- and post-Lp299v plasma sample exposure with an FDR < 0.2 were considered significantly changed in this exploratory pilot study. Hierarchical clustering was performed using Genesis53. In addition, using this set of genes, we calculated the composite inflammatory index (I.I.\textsubscript{com})54. I.I.\textsubscript{com} was determined by calculating the ratio between the mean log intensity of the inflammatory genes (n = 505) versus the mean log intensity of the regulatory genes (n = 938) as described in Chen et al.54 A high score reflects greater inflammatory bias and a low score reflects greater regulatory bias. The changes in differentially expressed genes were correlated with changes in brachial FMD% using Pearson’s r with significant correlations considered for P < 0.00035 following Bonferroni correction for multiple testing. The set of genes considered to be differentially expressed were further analyzed using Ingenuity Pathway Analysis (Qiagen) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to determine up- and down-regulated pathways and functional gene clusters as well as potential upstream regulators for the transcriptome results⁴⁵.

| Canonical Pathway | p Value | z-score | Genes |
|-------------------|---------|---------|-------|
| Tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde | 0.00024 | − 2.000 | IDO1,IDO2,KMO,KYNU |
| Phospholipase C signaling | 0.00041 | 2.132 | ARHGEF7,CALM1,CHP1,FYN,GNB4,GNB5,HDAC2,HDAC9,LCP2,LYN,MARK3,MEF2A,MRAS,MYL12A,NAPEPLD,NFATC2,NFATC3,PLA2G4A,PPP1CB,PPP1R12A,PRKD3,RAP1A,RAP2B,RHOESOS2 |
| Interferon signaling | 0.00048 | − 2.828 | IFIT1,IFIT3,IFITM3,IFNB1,ISG15,JAK2,OSAS1,SOCS1 |
| PKCβ signaling in T lymphocytes | 0.0017 | 2.183 | ATM,CACNA1A,CD80,CD86,CHP1,FGFR1,FYN,IKKB,LCP2,MAP3K2,MAP3K3,MTRAS,NFATC2,NFATC3 | |
| iCOS-iCOSL signaling in T helper cells | 0.0030 | 2.309 | AKT3,ATM,CALM1,CD80,CD86,CHP1,FGFR1,IKKB,LCP2,NFATC2,NFATC3 | |
| CD28 signaling in T helper cells | 0.0060 | 2.138 | ACTR2,AKT3,ATM,CALM1,CD80,CD86,CHP1,FGFR1,FGFR1,IKKB,LCP2,NFATC2,NFATC3 | |
| RANK signaling in osteoclasts | 0.0076 | 2.111 | AKT3,ATM,CALM1,CHP1,FGFR1,IKKB,MAP3K2,MAP3K3,MAPK14,MITTENFATC2,PKR3R | |
| fMLP signaling in neutrophils | 0.0079 | 2.309 | ACTR2,ATM,CALM1,CHP1,FGFR1,IKKB,GNB4,GNB5,MRAS,NFATC2,NFATC3,PKR3R,PRKD3,RAP1A,RAP2B | |
Table 3. KEGG pathways downregulated by Lp299v. *Analysis performed using DAVID 6.8. Clustering Parameters: Threshold:2, EASE: 0.05. † Lp299v = Lactobacillus plantarum 299v.

Table 4. KEGG pathways upregulated by Lp299v. *Analysis performed using DAVID 6.8. Clustering Parameters: Threshold:2, EASE: 0.05. † Lp299v = Lactobacillus plantarum 299v.

was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10 was suggested by the patterns in gene expression changes (Supplemental Table S3), and 25 upstream regulators that appear to be activated (Supplemental Table S4). These data suggest a significant anti-inflammatory effect of Lp299v supplementation, including downregulation of interferon-signaling (including IFN-γ and IL-1β), TNF-α signaling, toll-like receptor activation (TLRs 2,3,4,7, and 9), tryptophan degradation, and upregulation of IL-10.
of Lp299v supplementation. No such impact was seen in the four DAU subjects (0.49 ± 0.78 vs 1.01 ± 1.02, P = 0.23, Fig. e 3B). Baseline I.I.com did not differ when comparing DAUs versus non-DAUs (P = 0.24).

**Correlations between changes in PBMC gene expression and brachial artery endothelial function.** Of the 1373 genes differentially expressed following Lp299v supplementation, only three genes, guanylate binding protein 1 (GBP1), Janus kinase 2 (JAK2), and TNF Superfamily Member 10 (TNFSF10), were significantly correlated with changes in FMD% (Table 5). Transcription of all 3 genes was suppressed overall and the correlations all reflected an inverse association with changes in FMD%. Correlations between changes in gene expression and changes in FMD% for the additional genes in this set is included as Supplemental Table S5.

**Discussion**

In the absence of daily ETOH use, six weeks of supplementation with 20 billion colony-forming units of Lp299v in men with stable CAD resulted not only improved vascular endothelial function, but also induced changes in plasma composition to yield a strong anti-inflammatory effect. Favorable effects observed include downregulation of inflammation driven by IL-1β and TNF-a which have been successfully targeted with canakinumab (IL-1β monoclonal antibody) and colchicine to reduce secondary cardiovascular risk in large clinical trials. In addition, Lp299v supplementation broadly suppressed activation of innate immunity through TLR signaling as well as favoring suppression of inflammation through upregulation of regulatory T-cells (Tregs). Additionally, we identified suppression of transcription of three genes (GBP1, JAK2, and TNFSF10) that correlated inversely with flow-mediated dilation of the brachial artery. Whether these genes mechanistically mediate the development of inflammation-associated vascular endothelial dysfunction deserves further investigation. Lp299v’s favorable effects do not appear to extend to DAUs, suggesting that alcohol ingestion diminishes the anti-inflammatory effect of Lp299v supplementation on circulating plasma. Levels of SCFAs (acetic, propionic, and butyric acid) were similarly changed in individuals who drank daily and those who did not suggesting that these SCFAs were not mediating Lp299v’s anti-inflammatory effects. Taken together, our data support a paradigm of Lp299v contributing to improved vascular health in individuals with CAD at least in part through reductions in inflammation driven by yet-to-be identified gut-derived metabolites that should fuel future investigations.

Prior in vitro work exposing human PBMCs and L. plantarum species demonstrate the potential for L. plantarum to modulate the PBMC inflammatory response. Our initial studies with Lp299v were stimulated by the favorable effects we observed with Lp299v supplementation on infarct size and reducing pro-inflammatory leptin levels in male salt-sensitive rats. Different species of L. plantarum appear to have differing effects on the activation state and differentiation of human PBMCs in vitro. Importantly, these data also demonstrate the
in vitro effect of the direct exposure of *L. plantarum* species on PBMCs poorly predicts the in vivo response to this class of probiotics. Our data significantly extend these prior findings by focusing on highly clinically relevant human in vivo supplementation actions with Lp299v and clearly delineating this supplementation changes the composition of plasma so as to exert a systemic anti-inflammatory effect that can be observed in PBMCs. Lp299v supplementation in the present study had favorable anti-inflammatory effects on plasma composition including lowering of leptin, IL-12, and IL-8, and the reductions in the latter two may be, in part, leptin-mediated. Prior studies have mechanistically shown increases in serum leptin resulting in increases in ILs 12 and 8 in macrophages. Leptin enhanced secretion of LPS-stimulated IL-12 by peritoneal macrophages and stimulated IL-8 expression via p38 and ERK signaling pathways in M2 macrophages. Given this prior work, it is quite possible that the decrease in leptin is causal in the reductions we see in IL-12 and IL-18.

Daily alcohol ingestion appears to blunt the anti-inflammatory effects of Lp299v in our study as demonstrated by individual inflammatory indices calculated from the transcriptome data (Fig. 3). Prior work demonstrates that alcohol ingestion promotes the growth of gram negative bacteria in the intestine and increases overall gut permeability and circulating endotoxin, triggering systemic inflammation. Any of these established physiologic changes could account for the blunted anti-inflammatory effects of Lp299v in DAUs in our study. Our transcriptome data is consistent with these earlier findings, showing significant reductions in signaling pathways triggered by circulating lipopolysaccharide and peptidoglycan, including TLR4 activation, in our 15 non-DAU subjects (Supplemental Table S3). However, we had only four subjects who self-reported daily alcohol drinking with some variability in response, making our findings only hypothesis-generating. The dose and frequency relationships between alcohol intake and the anti-inflammatory impact of Lp299v supplementation, as well as the underlying mechanisms driving this mitigating effect, remain to be delineated and merit further investigation.

Over the past two decades, there has been an increasing recognition of the critical role played by activation of the innate immune system through toll-like receptors in the development of atherosclerotic disease. IPA analyses of our transcriptome data suggest Lp299v supplementation downregulates TLR signaling, including TLRs 2, 3, 4, 7, and 9. Each of these has been shown to be expressed by human coronary artery and microvascular endothelial cells with activation leading to increased expression of cell adhesion molecules and pro-inflammatory cytokines. In the peripheral human circulation, TLRs 2 and 4 are ubiquitously expressed and are enhanced in atherosclerotic plaques. TLR3 is most expressed in the carotid arteries and aorta, TLR7 in the iliac and carotid arteries, and TLR9 with overall low expression but most prominent expression in the iliac arteries. TLR 2, 3, 4, 7, and 9 stimulation have been implicated in the development of atherosclerosis through combination of promoting of lipid uptake into the plaques, monocyte activation, endothelial dysfunction, and foam cell formation. Recently TLR2 has been shown to facilitate microbiota-induced VWF expression and systemic interferon responses mice. Our data suggest Lp299v may reduce inflammation in part through reducing TLR activation in humans. Additional work will be necessary to better determine how Lp299v interacts with the innate immune system to suppress inflammation and atherogenesis.

Functional analyses of our transcriptome data support a paradigm of *Lp299v* supplementation increasing the number and activity of regulatory T-cells (*Treg*). *Treg* generally exert an anti-inflammatory influence and suppress self-antigen recognition. In mouse models of hypertension and atherosclerosis, *Treg* reduce monocyte activation and infiltration into the vasculature and atherosclerosis formation, preserve endothelium-dependent vasodilation of coronary arteries in mice through increased IL-10 expression, reduced IL-1β and TNF-α expression leading to suppression of endothelial expression of adhesion molecules like VCAM-1 and E-selectin. Our data reporting that Lp299v induces reductions in IL-1β and TNF signaling as well as upregulation of IL-10 receptor signaling in mononuclear cells are consistent with these mouse data and suggest an association between anti-inflammatory effect of *Treg* upregulation and improvements in endothelial function extend to humans with CAD. Additional work to delineate the mechanistic connections in human are warranted, particularly in light of the efficacy of IL-1β inhibition in reducing cardiovascular risk.

We identified three genes (*JAK2*, *GBP1*, and *TNFSF10*) whose transcription was significantly reduced in PBMCs by post-*Lp299v* supplementation plasma. These changes inversely correlated with increases in brachial FMD%. JAK2, a tyrosine kinase involved in cytokine signaling, increases plaque and plaque necrotic core size in hemopoietic cells and increases IL-1β from mononuclear cells challenged with lipopolysaccharide, when activated; it's suppression increases expression and activity of anti-inflammatory *Treg*. Here, the reduction in JAK2 transcription appears consistent with the increases in expression of canonical pathways involved with increased *Treg* pathways (Table 2) and as well as the decrease in expression in genes regulated by IL-1β signaling detected by our Ingenuity Pathway Analysis for upstream regulators (Supplemental Table S3). GBP1 is an IFNγ-induced GTPase found in T-cells, B-cells, and endothelial cells whose activation in the innate immune response to pathogens results in an inflammatory activation of endothelial cells and inhibited angiogenesis. TNFSF10 (TRAIL) is an apoptosis-inducing ligand expressed in T-cells and induced by IFNa and β to enhance T-cell cytotoxicity. It has been detected in CD3+ T-cells of human atherosclerotic plaques with higher concentrations in vulnerable plaques. Further studies determining the impact of *Lp299v* on endothelial cell expression of all three genes and their concomitant impact on interactions between PBMCs and endothelium, and endothelial function, are warranted.

Our study has some limitations. This study included a small number of only male subjects; whether these findings generalize to women and hold for a large group of male subjects await further elucidation which we will be pursing though a randomized control trial (NCT03267758). We did not perform transcriptome analyses on PBMCs directly from study subjects prior to and following *Lp299v* supplementation given concerns for confounding by differences in the types and amounts of pharmacological therapies. However, our approach more clearly delineates that *Lp299v*’s anti-inflammatory effects are mediated by factors in the circulating plasma. We did not examine the direct impact of *Lp299v* on cultured endothelial cells which may reveal a direct impact of *Lp299v* supplementation on the activation state of the endothelium itself. Nevertheless, PBMCs play a critical role in the composition of plasma so as to exert a systemic anti-inflammatory effect that can be observed in PBMCs.
role in the development of coronary atherosclerosis and the stability of atherosclerotic plaques making our findings highly relevant to vascular disease. Balanced against these limitations is the novelty of the findings suggesting the anti-inflammatory effect of supplementation with probiotic Lp299v may account for part of its favorable vascular effects.

Conclusions
In this pilot study of 15 men with stable CAD, we demonstrated that six weeks of Lp299v supplementation not only improved vascular endothelial function, but also had a significant anti-inflammatory effect. This anti-inflammatory effect appears to be at least in part due to suppression of innate immune signaling and increased activity of Tregs. We additionally identified three genes (JAK2, GBP1, and TNFSF10) whose mRNA transcription suppression by Lp299v in PBMCs was inversely correlated with brachial FMD% suggesting a potential more direct role for these proteins in inflammation-induced endothelial dysfunction in humans. The changes in circulating metabolites induced by Lp299v responsible for these favorable effects remain to elucidate and hold promise for further delineating the mechanisms behind improvements in vascular health with this probiotic intervention.

Methods
Subjects and study protocol. The study protocol was reviewed and approved by the Institutional Research Board at the Medical College of Wisconsin, and all experiments were performed in accordance with their regulations and guidelines. Informed consent was given by all participants within this study. 23 men with stable CAD (ages 40–75) were recruited as previously described14. This study includes analyses on 15 of the 23 subjects that were enrolled on the initial report on the impact of Lp299v supplementation on endothelial function in men with stable CAD14. As noted in the initial report, two subjects enrolled were disqualified because they failed to pass the initial screening visit. One subject had a stroke while on protocol and did not complete the study. We excluded an additional four subjects from these study analyses who were self-reported daily alcohol users (DAUs), but did perform transcriptome analyses using their plasma to validate their exclusion. One subject did not have plasma samples from all study visits and we were therefore unable to perform transcriptome analyses for this subject. After exclusions, a total of 15 subjects were available for this set of analyses. The inclusion and exclusion criteria are identical to the previously described study protocol8. Individuals with left-ventricular ejection fraction less than 45% within one year of enrollment, uncontrolled hypertension (blood pressure > 170/110 mmHg), a creatinine clearance less than 60 mL/min, recent antibiotic or probiotic administration (within 12 weeks of enrollment), chronic liver disease, cancer requiring chemotherapy within five years of enrollment, or changes in vasoactive medications (within six weeks of enrollment) were excluded.

All subjects underwent focused medical history reviews and physical exams to screen out occult disease or instability of cardiac status that would exclude a subject. In addition, each subject was asked to self-report their daily alcohol intake in one of five categories: rarely/never, once per month, several times per month, once per week, several times per week, and daily. Subjects who passed screening and enrolled in the study underwent an initial set of measurements (heart rate and blood pressure in triplicate; height and waist circumference; IL-8, IL-12, leptin, and glycosylated hemoglobin from peripheral upper extremity venous blood, which was saved for plasma-induced regulation of mononuclear cell transcription; and endothelial function by vascular artery ultrasound). Subjects were subsequently placed on once daily supplementation with 80 ml (2.7 oz.) of GoodBelly Straight (Probi, Sweden). Each daily supplement contained 20 billion colony forming units of L.plantarum 299v. Subjects returned to the study center approximately six weeks following beginning supplementation and repeated the studies from their initial study visit.

Measurement of endothelial function by vascular ultrasound. Measurements of vascular endothelial function were performed using high-resolution vascular ultrasound as previously described14,45–48. All image acquisition and analyses were performed by trained technicians. Our measurements are highly reproducible between different operators (average intra-class correlation between four technicians for ten studies of 0.97 [0.92–0.99, P < 0.001] and a single rater intraclass correlation coefficient of 0.88 [0.74–0.97, P < 0.001].

Measurement of circulating IL-8, IL-12, and leptin plasma levels. Serum cytokines and leptin levels were measured as described previously4. Sample collections were taken from all subjects between the hours of 08:00 AM and 12:00 PM on the day of study visit. IL-8 and IL-12 measurements were performed by Eve Technologies (Calgary, Canada). Human plasma leptin was measured using a quantitative ELISA kit (R&D Systems, Minneapolis, MN, #DLP00) on EDTA-preserved plasma both pre- and post-probiotic treatment; assays were read with a SpectraMax M5e Microplate Reader (Molecular Devices, Sunnyvale, CA).

Plasma-induced regulation of mononuclear cell transcription. Using a validated assay49, we incubated 200 µL of pre- and post-Lp299v supplementation plasma from each subject in separate wells of Costar 24-well plates seeded with 5 × 10^5 UPN119 PBMCs (Cellular Technology LTD, Shaker Heights, OH). Wells also contained RPMI 1,640 medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin to a total volume of 500 µL. RNA from PBMCs was subsequently isolated, labeled, and hybridized to the Affymetrix U133 + 2.0 array as previously described50,51. Affymetrix microarray data were uploaded to NCMI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/).

Statistical analyses. All data analyses were performed using SPSS 24.0, SigmaPlot 12.5, and GraphPad Prism. Subject characteristics prior to and following Lp299v supplementation were compared by paired t-test,
Wilcoxon signed-rank test, or chi-square tests as appropriate. Changes in brachial artery parameters and inflammatory cytokines were compared by paired t-test or the signed-rank test as appropriate. For these data, P < 0.05 was considered significant. For the plasma-induced transcriptome data, genes that were differentially expressed by at least 20% between pre- and post-Lp299y plasma sample exposure with an FDR of 0.2 were considered significantly changed in this exploratory pilot study. Hierarchical clustering was performed using Genesis. In addition, using this set of genes, we calculated the composite inflammatory index (I.I.com). I.I.com was determined by calculating the ratio between the mean log intensity of the inflammatory genes (n = 505) versus the mean log intensity of the regulatory genes (n = 938) as described in Chen et al. A high score reflects greater inflammatory bias and a low score reflects greater regulatory bias. The changes in differentially expressed genes were correlated with changes in brachial FMD% using Pearson’s r with significant correlations considered for P < 0.00035 following Bonferroni correction for multiple testing. The set of genes considered to be differentially expressed were further analyzed using Ingenuity Pathway Analysis (Qiagen) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to determine up- and down-regulated pathways and functional gene clusters as well as potential upstream regulators for the transcriptome results.

Data availability
Transcriptome microarray data is publicly available in the Gene Expression Omnibus (GEO) repository (accession number GSE156357). All other data analyzed for this study can be found within this published manuscript and the supplementary materials.

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**Author contributions**

B.C.H. drafted the work and was involved in data analysis and interpretation. V.K.P. and S.T. participated in data acquisition, data analyses, and revised the draft. A.A. contributed to data acquisition and draft revision. S.J. contributed to data analyses, interpretation, and draft revision. N.H.S. and M.J.H. contributed to study design, data interpretation, and draft revision. M.E.W. contributed to study conception, design, data analyses and interpretation, and substantially revised the draft.

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**Competing interests**

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**Additional information**

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