Akermansia Muciniphila Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe<sup>−/−</sup> Mice

Running title: Li et al.; The anti-atherosclerotic role of A. muciniphila

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Abstract

Background—Altered composition of the gut microbiota is involved in both onset and progression of obesity and diabetes. However, the link between gut microbiota and obesity-related cardiovascular complications has not been explored. The present study was designed to investigate the role of Akkermansia muciniphila, a mucin-degrading bacterium with beneficial effects on metabolism, in the pathogenesis of atherosclerosis in apolipoprotein E-deficient (Apoe−/−) mice.

Methods and Results—Apoe−/− mice on normal chow diet or Western diet were treated with A. muciniphila by daily oral gavage for eight weeks, followed by histological evaluations of atherosclerotic lesion in aorta. Real-time PCR analysis demonstrated that the fecal abundance of A. muciniphila was significantly reduced by Western diet. Replenishment with A. muciniphila reversed Western diet-induced exacerbation of atherosclerotic lesion formation without affecting hypercholesterolemia. A. muciniphila prevented Western diet-induced inflammation in both circulation and local atherosclerotic lesion, as evidenced by reduced macrophage infiltration and expression of proinflammatory cytokines and chemokines. These changes were accompanied by a marked attenuation in metabolic endotoxemia. A. muciniphila mediated reduction in circulating endotoxin level could be attributed to induction of intestinal expression of the tight junction proteins (ZO-1 and occludin), thereby reversing Western diet-induced increases in gut permeability. Chronic infusion of endotoxin to Apoe−/− mice reversed the protective effect of A. muciniphila against atherosclerosis.

Conclusions—A. muciniphila attenuates atherosclerotic lesions by ameliorating metabolic endotoxemia-induced inflammation through restoration of the gut barrier.

Key words: atherosclerosis, gut microbiota, metabolic endotoxemia.
Introduction

The gut microbiota, a complex community of over 100 trillion microbes, plays an important physiological role in modulating host nutrition, metabolism and immunity.\(^1\),\(^2\) Altered composition and/or function of gut microbiota has been linked to a number of chronic diseases, including colon cancer, irritated bowel syndrome, colitis, obesity and diabetes.\(^3\) For example, decreased diversity within the phylum of Firmicutes in the gut microbiota is commonly found in patients with Crohn’s disease,\(^4\) and supplementation with one of the species in this phylum, Faecalibacterium prausnitzii, improves the survival rate of chemical-induced colitis in animals.\(^5\) Conversely, higher proportions of Firmicutes and lower levels of Bacteroidetes have been observed in obese individuals, and dietary intervention or bariatric surgery reverses these changes.\(^6\)

The gut microbiota modulates host physiology by producing a wide variety of metabolites or bacterial products including short chain fatty acids and endotoxins. A lower proportion of butyrate-producing bacteria has been found in autoimmune diabetes compared with the healthy controls,\(^7\) and supplementation with butyric acid improves the metabolic profiles in dietary obese murine model.\(^8\) High fat diet increases gut permeability and enhances the penetration of gut microbiota-derived endotoxins into circulation resulting in metabolic endotoxemia, and the elevated endotoxins in circulation exacerbates hepatic insulin resistance and promotes weight gain.\(^9\) Although associations between alterations in gut microbiota and many chronic diseases have been observed, it remains unclear whether such changes are the cause or the consequence of the pathologies.

Atherosclerosis, the main contributor to cardiovascular mortality, is a chronic inflammatory disease.\(^10\) Bacterial infection has been proposed as one of the triggers of
inflammation in atherosclerosis.\textsuperscript{11, 12} For example, \textit{Chlamydia pneumonia} is present in atherosclerotic lesions of patients with previous exposure, and infection with this bacterium exacerbates atherosclerosis in animals.\textsuperscript{11, 12} Bacterial DNA has been detected in atherosclerotic lesions, and the pyrosequencing result reveals that the bacteria in lesions are derived from gut and oral cavity,\textsuperscript{13} suggesting a possible involvement of gut microbiota in the development of the disease. However, the germ-free atherogenic Apoe\textsuperscript{−/−} mice which are without the colonization of gut microbiota show an worsening of atherosclerotic lesions after feeding a high cholesterol diet compared with the conventionally raised mice, and antibiotic therapy fails to elicit any beneficial effect on cardiovascular events in human trials.\textsuperscript{14-16} On the contrary, a metabolomics analysis shows that metabolism of dietary phosphatidylcholine by gut microbiota produces proatherogenic trimethylamine-N-oxide (TMAO) that can accelerate atherosclerosis in mice.\textsuperscript{17, 18} It is possible that modulation rather than elimination of the gut microbiota is a potential therapeutic strategy to prevent or slow down atherosclerotic process.

\textit{Akkermansia muciniphila}, a mucin-degrading bacterium belonging to the generum of Verrucomicrobia, has recently emerged as an important component of the gut microbial ecosystem.\textsuperscript{19} It accounts for approximately 1-3\% of the microbial community in healthy subjects and its abundance is inversely correlated with body weight in mice and humans.\textsuperscript{20, 21} Lower amount of \textit{A. muciniphila} in the gut has been observed in obese children and pregnant women.\textsuperscript{22} In high fat diet-fed mice, \textit{A. muciniphila} given by daily oral gavage reverses fat mass gain, adipose tissue inflammation and insulin resistance,\textsuperscript{21, 24} suggesting a beneficial effect in combating obesity-related metabolic disorders. However, its beneficial role against obesity-related cardiovascular diseases remains to be addressed.

In this study, we explored the relationship between the abundance of \textit{A. muciniphila} in
the gut and the severity of atherosclerosis in Apoe<sup>-/-</sup> mice. Our results showed that Western diet-induced aggravation of atherosclerotic lesion was accompanied by a marked reduction of A. muciniphila, whereas restoration of A. muciniphila by daily oral gavage substantially reduced atherosclerotic lesions in Apoe<sup>-/-</sup> mice. Therefore, we further investigated the mechanisms underlying this protective effect of gut-residing A. muciniphila.

Methods

Animal model

Mice lacking apolipoprotein E (Apoe<sup>-/-</sup>) on a C57BL background were purchased from Jackson Laboratory (Bar Harbor, MA). Eight-week-old male Apoe<sup>-/-</sup> mice were fed either a normal chow diet or Western diet (Cat no. D12079B, Research Diet, New Brunswick, NJ) ad libitum for eight weeks. Total fat mass was measured by the Minispec LF90 Body Composition Analyzer (Bruker, Billerica, MA). The fat mass at inguinal subcutaneous adipose tissue, epididymal and mesenteric white adipose tissue, and interscapular brown adipose tissue was determined by measuring the wet weight of each adipose depot after sacrificing the mice. All the animal experiments were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

Culture and administration of A. muciniphila

A. muciniphila (Cat. No. BAA-835, ATCC, Manassas, VA) were cultured anaerobically in BHI (brain-heart-infusion) broth (BD Bioscience, San Jose, CA) supplemented with 0.5% porcine mucin (Sigma-Aldrich, St Louis, MO) and 0.05% cysteine (Sigma-Aldrich). The concentration of bacteria was calculated by measuring the absorbance at the wavelength of 600 nm. 5 x 10<sup>9</sup> cfu of A. muciniphila in 200 μL of phosphate-buffered saline (PBS) was orally gavaged daily to Apoe<sup>-/-</sup> mice. In one group of experiment, A. muciniphila were heat-killed at 121°C under
pressure of 225 kPa for 15 minutes.

**Quantitative analysis of atherosclerotic lesions**

For analysis of lesion area, Oil Red O staining of the area from aorta arch to thoracic aorta were performed, whereas for analysis of atherosclerotic lesion in aortic sinus, the proximal aorta attached to heart was harvested and fixed in 4% paraformaldehyde. Serial 6-μm-thick paraffin-embedded sections from the middle portion of the ventricle to the aortic arch were collected. Sections of aorta were stained with hematoxylin and eosin for analysis of morphometric lesion. The quantification of lesion area and size were performed using ImageJ software (NIH, Baltimore, MD).

**Immunofluorescent and immunohistochemical staining**

For immunofluorescent staining, aortic sinus sections were incubated with anti-monocyte and macrophage-2 antibody (MOMA-2) (Abcam, Cambridge, UK) or anti-intercellular adhesion molecule-1 (ICAM-1) (Santa Cruz Biotechnology, Dallas, TX) antibodies, and sections of ileums were treated with anti-zona occludens protein-1 (ZO-1) or occludin (Abcam) antibodies, followed by incubation with Alex Fluor-596 or FITC conjugated secondary antibodies (Life technologies, Carlsbad, CA) and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). For immunohistochemistry staining, after the endogenous peroxidase activity had been inhibited by hydrogen peroxide (H2O2) for 20 min, sections were incubated overnight with anti-monocyte chemoattractant protein-1 (MCP-1) antibody (Santa Cruz Biotechnology), followed by staining with HRP-conjugated secondary antibody. The target proteins were visualized with 3’3-diaminobenzidine in the presence of H2O2 and sections were counterstained with Harris hematoxylin. For quantitative analysis of images, five random fields were captured from different areas of a single section, and the intensity of positive staining was analyzed by Image J software.
and calculated as the percentage of total area of lesion or villa in each field.

**RNA preparation and Real-time quantitative PCR analysis**

Total RNA was extracted from aorta or ileum using TRIzol reagent (Life Technologies) followed by reverse transcription into cDNA using an ImProm-II reverse transcription kit (Promega, Madison, WI). Quantitative real-time PCR was performed with Applied Biosystems® StepOne Real-Time PCR Systems (Life Technologies). The primers for each specific gene are listed in Supplemental Table 1.

**In vivo gut permeability assays**

Mice were orally gavaged with fluoresceinisothiocyanate (FITC) labelled dextran (DX-4000-FITC, 500mg/kg body weight, Sigma-Aldrich) after fasting for 6 hours, followed by collection of serum samples via tail vein. The concentration of DX-4000-FITC in serum was measured by a fluorescence spectrophotometer (Synergy H1, BioTek, Winooski, VT) with excitation wavelength of 485 nm and emission wavelength of 535 nm.

**Denaturing gradient gel electrophoresis**

Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (51504, Qiagen, Venlo, Netherlands) and subjected to PCR amplification targeting the V3 region of the 16S ribosome RNA (rRNA) gene with the universal primers (Supplemental Table 1). The PCR products were further separated by electrophoresis with a gradient gel from 27% to 52% using the Dcode System apparatus (Bio-Rad, Hercules, CA). Gels were stained with SYBR Green I (Life technologies) for 30 min for visualization under UV transillumination.

**Biochemical and immunological assays**

Serum levels of total cholesterol (TC), total triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL) and glucose were measured using commercially available kits.
Statistical analyses were performed using Statistical Package for Social Sciences Version 23.00

Glucose tolerance test

Apoe<sup>−/−</sup> mice were fasted overnight and glucose (2g/kg body weight) was intraperitoneally injected. Blood glucose level was determined at various time points using a glucometer (Accu-Check Performa, Roche Diagnostics, Basel, Switzerland) after the initial injection of glucose.

Chronic infusion of LPS

Osmotic pumps (Model 1004, Alzet) filled with either LPS (Cat. No. L6386, Sigma-Aldrich) or vehicle (PBS) were subcutaneously implanted into Apoe<sup>−/−</sup> mice at the 4th week of Western diet treatment, to deliver LPS at a constant rate (250 μg/kg of body weight/day) for another four weeks.

Culture of intestinal epithelial cells

Human intestinal epithelial cells, Caco-2 cells, were purchased from American Type Culture Collection (ATCC), and cultured in Minimum Essential Media (MEM, Life Technologies) with 10% FBS and antibiotics (100 U penicillin, 0.1 mg streptomycin and 0.25 μg amphotericin B per mL).

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences Version 23.00
(SPSS, Chicago, IL). Data were presented as mean± s.e.m. One-way ANOVA was applied for comparisons between multiple experimental groups, followed by post-hoc analysis using Turkey HSD for data with equal variance or Games-Howell for data with unequal variance. Data with small sample size were analyzed using the Kruskal-Wallis test, a non-parametric one-way ANOVA. An unpaired Student’s t test was applied for comparison of the two groups with normal distribution. P values less than 0.05 were accepted to indicate statistically significant differences.

Results

Oral gavage with *A. muciniphila* protected against Western diet-induced atherosclerotic lesion formation in *Apoe−/−* mice.

To explore the possible role of *A. muciniphila* in atherosclerosis, *Apoe−/−* mice fed the Western diet were treated with either live *A. muciniphila* or heat-killed *A. muciniphila*, or vehicle (PBS) by oral gavage daily for a period of eight weeks, followed by the assessment of atherosclerotic lesions. Real-time PCR analysis showed a significantly reduced amount of *A. muciniphila* in the feces of Western diet-fed *Apoe−/−* mice compared with the mice fed a normal chow diet (Figure 1A), whereas the fecal amounts of this bacteria were comparable between wild-type and *Apoe−/−* mice fed a normal chow diet (7.31±0.51 log10 bacteria/g of feces in wild type vs. 7.04±0.69 log10 in *Apoe−/−*). Daily oral gavage with 5 x 10⁹ cfu of live *A. muciniphila* was sufficient to restore the diminished level caused by the Western diet (Figure 1A).

The Western diet induced formation of atherosclerotic lesions as demonstrated by Oil Red O staining of longitudinally opened aortas and hematoxylin-and-eosin staining of aortic root regions (Figure 1B and 1C).25 Lipids accumulated in the aortic arch and at the roots of branchiocephalic, left common carotid and left subclavian arteries after eight weeks of Western diet (Figure 1B). The Western diet resulted in a 3.7-fold increase in lesion area and a 2.9-fold...
increase in lesion size in Apoe<sup>−/−</sup> mice compared with normal chow diet (Figure 1D and 1E). Treatment with A. muciniphila substantially reduced the lesion area and size by 31% and 48% in Apoe<sup>−/−</sup> mice under Western diet, respectively (Figure 1D and 1E). Administration of the same dose of heat-killed A. muciniphila did not show any improvement of lesion area or size in Apoe<sup>−/−</sup> mice (Figure 1B-E), indicating that the protective effect of this bacteria was dependent of their viability.

We next investigated whether oral gavage with A. muciniphila altered the ecosystem and changed the composition of the gut microbiota using denaturing gradient gel electrophoresis and real time polymerase chain reaction (PCR) analyses. The Western diet substantially altered the gut microbiota pattern compared with normal chow diet (Supplemental Figure 1A). This pattern change was characterized by increased amount in Proteobacteria and Firmicutes, and a decreased quantity of Bacteroidetes, Fusobacteria and Tenericutes (Supplemental Figure 1B-F), which is in line with the previously reported data on high fat diet-induced mice.<sup>1</sup> However, the daily oral gavage with A. muciniphila did not affect the overall pattern of gut microbiota and abundance of the above-mentioned bacterial species (Supplemental Figure 1A-F).

**Treatment with A. muciniphila did not alter lipid metabolism in Apoe<sup>−/−</sup> mice.**

A previous study demonstrated that treatment with A. muciniphila reduced body weight and fat mass, and improved metabolic functions during obesity.<sup>21</sup> In our atherogenic Apoe<sup>−/−</sup> model, there was no obvious change in food intake after A. muciniphila treatment (Supplemental Figure 1G) although there was a slight decrease in body weight and fat mass (Supplemental Figure 1H-J). Consistent with previous findings,<sup>25</sup> the Western diet led to hyperlipidemia in Apoe<sup>−/−</sup> mice compared with normal chow diet (Supplemental Figure 2A-D). However, the daily oral gavage with A. muciniphila did not alter serum levels of total cholesterol, total triglyceride, low-density
lipoprotein and high-density lipoprotein (Supplemental Figure 2A-D). In addition, A. muciniphila treatment did not significantly affect fasting blood glucose level or glucose tolerance in Apoe<sup>−/−</sup> mice (Supplemental Figure 2E and 2F), implying that the beneficial role of A. muciniphila against atherosclerosis was not attributed to altered lipid or glucose metabolism.

**A. muciniphila ameliorated both aortic and systemic inflammation in Western diet-fed Apoe<sup>−/−</sup> mice.**

Increased local inflammation is one of the hallmarks during the progression of atherosclerosis. Macrophages are the major immune cells in atherosclerotic plaques, and play a key role in promoting atherosclerosis.<sup>26</sup> The number of macrophages in atherosclerotic lesions increased after Western diet feeding, and daily oral gavage with A. muciniphila significantly reduced the amount as shown by immunofluorescent staining of the macrophage marker, monocytes/macrophages antigen (MOMA) (Figure 2A and 2D). Likewise, the treatment with A. muciniphila inhibited the Western diet-induced protein expressions of intercellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), which are the major chemokines involved in promoting the adhesion of macrophages onto endothelium and subsequent transmigration into intima, respectively.<sup>27, 28</sup> (Figure 2B-D). A similar effect of A. muciniphila on suppression of the Western diet-induced mRNA expressions of F4/80 (another macrophage marker), MCP-1, ICAM-1 and tumor necrosis factor-α (TNFα) was also observed in the aortas of Apoe<sup>−/−</sup> mice (Figure 3A-D). By contrast, treatment with heat-killed A. muciniphila did not have significant effect on either macrophage accumulation or expressions of MCP-1, ICAM-1 and TNFα in the local aortic tissues (Figure 2A-D and 3A-D).

Circulating proinflammatory cytokines such as MCP-1 and interleukin-1β (IL-1β) participate in the development of atherosclerosis.<sup>29, 30</sup> The serum levels of these two pro-inflammatory
factors were significantly increased in Western diet-fed Apoe\(^{-/-}\) mice as compared with the mice on normal chow diet, whereas these Western diet-induced elevations were obviously diminished by daily oral gavage with A. muciniphila, but not the heat-killed bacteria (Figure 3E & 3F).

Similarly, the Western diet-induced increase in circulating level of soluble tumor necrosis factor receptor II (sTNFR II), a prognostic marker of fatal complication of atherosclerosis including congestive heart failure,\(^{31}\) was significantly attenuated by the administration of live A. muciniphila (Figure 3G). However, daily treatment with A. muciniphila had no obvious effect on the circulating level of adiponectin, an adipocyte-derived anti-inflammatory adipokine with anti-atherosclerotic activity (Figure 3H).

*A. muciniphila* decreased intestinal permeability and reduced the penetration of gut-derived LPS into circulation in Western diet-fed *Apoe\(^{-/-}\)* mice.

Metabolic endotoxemia is a key mediator of obesity-induced chronic inflammatory diseases, and has been suggested to be caused by the increased penetration of LPS from gut into circulation.\(^{9}\) The *in vivo* gut permeability, as determined by oral administration of fluorescent labelled dextran (DX-4000-FITC) followed by measurement of its circulating concentration, was significantly higher in Western diet-fed *Apoe\(^{-/-}\)* mice compared with the normal chow diet-fed controls, whereas the Western diet-induced increase of gut permeability was largely blocked by the treatment with live *A. muciniphila* (Figure 4A). Gut permeability is regulated by the mucus layer and tight junctions of intestine, and the former serves as the first defense preventing the adhesion of bacteria whereas the latter further blocks the intrusion of pathogens and bacterial products. Therefore, we next evaluated the effect of *A. muciniphila* on the mucin layer thickness and expression levels of the major tight junction proteins in the intestine. In line with a previous report,\(^{21}\) Western diet-fed *Apoe\(^{-/-}\)* mice showed a significant decrease in the inner mucin layer
thickness of ileum, and *A. muciniphila* treatment partially restored the thickness of this layer (Supplemental Figure 3). On the other hand, the expression of the epithelial tight junction protein, occludin was significantly reduced by Western diet feeding, but was upregulated by treatment with live *A. muciniphila* (Figure 4B, 4D and 4F). *A. muciniphila* treatment also increased the expression of another tight junction protein, zona occludens protein (ZO-1) (Figure 4C, 4E and 4G). Furthermore, the restoration of the gut barrier by *A. muciniphila* treatment led to a significant reduction in the Western diet-induced elevation of LPS levels in both mesenteric adipose tissue and circulation (Figure 4H and 4I), suggesting that *A. muciniphila* blocked LPS penetration by preserving the gut barrier.

As viability of *A. muciniphila* is indispensable for its anti-atherosclerotic effects, we postulate that molecules or metabolites secreted by *A. muciniphila* might affect the expression of tight junction proteins. Treatment with the inoculating medium of *A. muciniphila* culture (100 µL/mL) in Caco-2 cells, a human intestine epithelial cell line, significantly upregulated the expressions of occludin and ZO-1 compared with those treated with the plain inoculating medium. This *A. muciniphila*-induced upregulation of the tight junction proteins was independent of its characteristic as a Gram negative bacterium, as treatment with inoculating medium of *E. coli* did not show any effect. (Supplemental Figure 4A and 4B). As the short chain fatty acids butyrate and propionate have been shown to stimulate the proliferation of intestinal epithelial cells\textsuperscript{32}, we next tested their effects on expression of tight junction proteins. However, treatment with propionate or butyrate in Caco-2 cells failed to increase the expressions of either occludin or ZO-1 (Supplemental Figure 4C and 4D).

In *ApoE*\textsuperscript{-/-} mice on normal chow diet, treatment with *A. muciniphila* has no obvious effect on gut permeability (Supplemental Figure 5A), circulating levels of inflammatory markers
(MCP-1, IL-1β and sTNFRII (Supplemental Figure 5B, 5C and 5D), and atherosclerotic plaque formation (Supplemental Figure 5E and 5F), suggesting that the amount of intestinal *A. muciniphila* under normal diet is sufficient to maintain the integrity of gut barrier, thereby preventing endotoxinemia-induced systemic inflammation and atherosclerosis.

**Chronic infusion of LPS abolished the protective effect of *A. muciniphila* against Western diet-induced atherosclerosis in *Apoe<sup>−/−</sup>* mice.**

Chronic exposure to LPS can accelerate atherosclerosis via different signaling pathways, including increase in adhesion molecules, chemotactic and proinflammatory cytokines. Therefore, we hypothesized that the protective effect of *A. muciniphila* against atherosclerosis was partially mediated by decreasing serum level of LPS. To this end, *A. muciniphila* was orally gavaged daily to *Apoe<sup>−/−</sup>* mice for total eight weeks, and during the last four weeks, either LPS (250 μg/kg of body weight/day) or PBS (vehicle control) was chronically infused via subcutaneously implanted osmotic pumps (Model 1004, Alzet, Cupertino, CA) into these mice. Chronic infusion of LPS restored the serum level of LPS in *A. muciniphila*-treated mice to that similar to the Western diet-fed mice without the bacterial treatment (Figure 5A). Notably, this increased level of LPS was able to reverse the protective effect of *A. muciniphila* treatment against atherosclerosis, as demonstrated by increased lesion area (Figure 5B and 5D) and size (Figure 5C and 5E) in *Apoe<sup>−/−</sup>* mice compared with the *A. muciniphila*-treated mice without infusion of LPS. LPS infusion also augmented the number of infiltrated macrophages and the expressions of inflammatory molecules, MCP-1 and ICAM-1, in the atherosclerotic lesions of mice treated with *A. muciniphila* (Figure 6A-C). Real-time PCR analysis further demonstrated that the suppressive effects of *A. muciniphila* on mRNA expressions of F4/80, MCP-1, ICAM-1 and TNFα were reversed by LPS treatment (Figure 6D-G).
Similar to the changes in the aortas, Western diet-induced inflammation in visceral adipose tissues, as determined by real-time PCR analysis for the abundance of the macrophage marker F4/80 and the expression of the proinflammatory factors (IL-1β, MCP-1 and TNFα), was also attenuated by treatment of ApoE−/− mice with A. muciniphila, whereas such effects of A. muciniphila was abrogated by infusion with LPS (Supplemental Figure 6), suggesting that amelioration of adipose tissue inflammation by A. muciniphila was also attributed to its ability in reducing endotoxemia. Notably, the amelioration of systemic inflammation by A. muciniphila, as determined by circulating levels of MCP-1, IL-1β and sTNFRII, was also abolished by chronic infusion of LPS (Figure 6H-J).

Discussion

Several recent studies have identified trimethylamine N-oxide (TMAO), a metabolite of the gut microbiota, as an independent risk factor for cardiovascular disease (CVD).17, 18, 34 Bacterial DNA from the gut microbiota has been detected in human atherosclerotic plaques.13 However, direct evidence for the causative role of altered gut microbiota (dysbiosis) in the pathogenesis of atherosclerosis is still lacking. The present study demonstrates that the Western diet-induced deterioration of atherosclerosis in ApoE−/− mice is associated with an increased level of Firmicutes, a decreased amount of Bacteroidetes, and especially a marked reduction in A. muciniphila in the gut. Replenishment of A. muciniphila by daily oral gavage reduced the size of atherosclerotic plaques in Western diet-fed ApoE−/− mice. The findings suggest a causal role of reduction in gut A. muciniphila in the Western diet-induced exacerbation of atherosclerosis.

Prebiotics such as oligofructose has been used as a food supplement to support the growth of commensal bacteria in order to maintain the general health of body.35 Several clinical trials have
reported its therapeutic effectiveness. Although the mechanism is unknown, it is generally believed that enrichment of gut microbiota by prebiotics plays an important role as lower diversity and abundance of gut microbiota is associated with certain diseases. Prebiotics-induced hypocholesterolemic effect has been reported in animals, suggesting a possible role in combating atherosclerosis. Notably, supplementation with prebiotics causes an over 100-fold enrichment in gut *A. muciniphila*. Furthermore, the metabolic benefits of dietary polyphenol, cranberry extract, and metformin are also associated with increased amount of this bacterial species in the gut. In this connection, our result showed that daily oral gavage with the monospecific genus *A. muciniphila*, without obvious change in the composition of gut microbiota, is sufficient to reduce atherosclerosis, suggesting that therapeutic interventions targeting this single genus/species in gut microbiota may represent a promising strategy for treatment and/or prevention of both metabolic and cardiovascular disorders.

Inflammation and hypercholesterolemia are the two key etiological factors for atherosclerosis. In the initial stage of atherosclerosis, injured or inflamed endothelium secretes adhesion molecules and chemokines to facilitate the recruitment and transmigration of leukocytes into the intima. The unresolved inflammation stimulates the expression of macrophage scavenger receptors and promotes the uptake of modified lipoproteins forming lipid-laden macrophages, which drives the inflammatory loop for further migration and proliferation of leukocytes and smooth muscle cells in the lesion area. Current therapeutic options for treating or preventing atherosclerosis mainly include platelet aggregation inhibitors, 3-hydroxy-3-methylglutary-CoA reductase inhibitors, antihypertensives and thrombolytic agents. However, the risks for fatal cardiovascular complications in these patients remain high due to the unsolved inflammation. Animal-based studies have reported the effectiveness of several anti-
inflammatory interventions in alleviation of atherosclerosis, including the CD40-TNF receptor-associated factor 6 (TRAF6)-specific blockade and interleukin-1 neutralization, but clinical implications of these findings remain to be confirmed.\textsuperscript{39} In this study, treatment with \textit{A. muciniphila} substantially reduced the expressions of several chemokines and adhesion molecules, MCP-1, TNF\textalpha{} and ICAM-1, along with lower aortic infiltration of macrophages and diminished atherosclerotic lesion in \textit{Apoe\textsuperscript{-/-}} mice. In contrast, \textit{A. muciniphila} had no effect on the Western diet-induced hypercholesterolemia and alterations in other metabolic profiles, suggesting that the anti-atherosclerotic effect of \textit{A. muciniphila} is mainly attributed to its anti-inflammatory activity. This notion is consistent with recent studies showing that supplementation with \textit{A. muciniphila} in obese mice reduced IL-6 and IL-1\beta{} expressions, and increased percentage of regulatory T cells in visceral adipose tissue.\textsuperscript{21, 24}

Metabolic endotoxemia, defined as a two- to three-fold elevation of circulating endotoxin/LPS level, has been proposed as an initiating factor of obesity-related cardio-metabolic dysfunction.\textsuperscript{9} LPS, a ligand of toll-like receptor 4 (TLR4), is a potent stimulus of inflammation.\textsuperscript{33} A clinical study revealed that subclinical endotoxemia is a strong risk factor for carotid atherosclerosis, and weekly injection of LPS worsens the formation of atherosclerosis in animal models.\textsuperscript{41, 42} A positive correlation between serum LBP level and carotid intima thickness, and an association of toll-like receptor 4 polymorphisms with decreased atherosclerosis have been found in human.\textsuperscript{43, 44} TLR4 is expressed in various vascular cells including endothelial cells, focal leukocytes and macrophages, its activation by LPS not only stimulates the release of pro-inflammatory molecules from these cells but also inhibits cholesterol efflux from macrophages thus facilitates foam cell formation. In obesity, inflammatory factors in adipose tissue are the major contributors to systemic inflammation.\textsuperscript{45} LPS can also act on TLR4 in
adipocytes to stimulate the production of proinflammatory adipokines and further reinforce the systemic inflammation. Furthermore, LPS-induced inflammatory cytokines in perivascular adipose tissue, which surrounds almost all the blood vessels, can act in a paracrine manner to exacerbate vascular inflammation and atherosclerosis. Inactivation of the LPS pathway by deletion of TLR4 or the downstream cytosolic adaptor, myeloid differentiation factor-88, or prevention of its transport by inhibiting LPS binding protein (LBP) reduces aortic lesions in Apoe<sup>-/-</sup> and low-density lipoprotein receptor deficient (Ldlr<sup>-/-</sup>) mice. Notably, all these models showed a reduction of lesion area and lesional lipid content without any significant alteration of plasma cholesterol levels, which is similar to our findings of A. muciniphila treatment. Here, we show that chronic infusion of LPS reversed the effect of A. muciniphila on alleviation of Western diet-induced local and systemic inflammation, indicating the anti-atherogenic effect of A. muciniphila is mediated by limiting the LPS level in the bloodstream and ameliorating metabolic endotoxemia.

Penetration of LPS into the bloodstream is controlled by the integrity of gut barrier. Administration of A. muciniphila has been shown to prevent the thinning of mucus layer in dietary-induced obese mice. The mucus layer is enriched with various mucins which forms a hydrated gel layer covering the mucosal surface to prevent adhesion of harmful bacteria. However, the primary control of the gut barrier relies on an intact epithelium where tight junctions sealing the space between individual epithelial cells maintain the epithelial integrity. Tight junction is a multiple protein complex including occludin, claudins and ZOs. Loss of occludin leads to an increase in gut permeability, whereas deficiency of ZO-1 can interrupt the assembly of tight junction by inhibiting the recruitment of other components. The expressions of the two tight junction proteins, occludin and ZO-1 were increased in the ileum of Apoe<sup>-/-</sup> mice.
after administration with \textit{A. muciniphila}, and treatment with inoculating medium of \textit{A. muciniphila} directly stimulated the expression of these tight junction proteins in intestinal epithelial cells. These findings suggest an additional mechanism of preserving gut barrier by \textit{A. muciniphila}. However, how gut-residing \textit{A. muciniphila} increases the expression levels of these tight junction proteins remains to be determined.

In summary, our study uncovered a key link between gut microbiota, gut permeability and vascular system (Supplemental Figure 7). The Western diet-induced atherosclerosis is partly caused by a reduction of \textit{A. muciniphila} in gut, resulting in compromised gut barrier and increased endotoxemia, which in turn exacerbates vascular inflammation. Our findings raise the possibility of targeting individual species of the gut microbiota for treatment of atherosclerosis.

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\textbf{Conflict of Interest Disclosures:} None.

\textbf{References:}


15. Wright SD, Burton C, Hernandez M, Hassing H, Montenegro J, Mundt S, Patel S, Card DJ,


Clinical Perspective

Hypercholesterolemia and chronic inflammation are the two important risk factors for atherosclerosis, which is a major cause for fatal cardiovascular complications including coronary heart disease and myocardial infarction. While cholesterol-lowering drugs can effectively reduce the incidence of atherosclerosis, anti-inflammatory therapies against this disease have achieved limited success, partly due to the lack of the understanding on etiological factors that trigger vascular inflammation. Previous clinical studies have reported a close association of bacterial infection and endotoxemia with cardiovascular diseases. However, whether or not altered gut microbiota contributes to the pathogenesis of atherosclerosis remains unknown. Our study showed a markedly reduced abundance of *Akkermansia muciniphila*, which is a strain of commensal bacteria in the gut, in a murine mode with Western-diet induced atherosclerosis. Replenishing this strain of bacteria by oral gavage substantially diminished the Western diet-induced atherosclerotic lesions. The anti-atherogenic effect of *Akkermansia muciniphila* was attributed to its ability in reducing aortic and systemic inflammation by protecting the integrity of gut barrier, thereby leading to alleviation of endotoxemia. These findings suggest that reduced abundance of *Akkermansia muciniphila* contributes to vascular inflammation and atherosclerosis, and manipulation of a single-strain of bacteria in gut microbiota is sufficient to reverse the progression of this disease. Prebiotics, dietary or therapeutic modulations in favor of increasing the abundance of *Akkermansia muciniphila* can be a potential therapeutic option for vascular inflammation in atherosclerosis.
Figure Legends:

**Figure 1.** Western diet-fed Apoe<sup>−/−</sup> mice treated with *A. muciniphila* exhibited reduced atherosclerosis. Eight-week old Apoe<sup>−/−</sup> mice were fed either a normal chow diet (NCD) or Western Diet (WD) for eight weeks. The Western diet-fed mice were further separated into three groups: one receiving daily oral gavage with live *A. muciniphila* (WD+Akk) or heat-killed *A. muciniphila* (WD+hk-Akk), and the other one gavaged with PBS as vehicle control (WD+PBS). (A) The abundance of *A. muciniphila* in feces was examined by quantitative real-time PCR (qPCR). (B) The lipid content of aorta was visualized by staining with Oil Red O, and (C) the sections of aortic roots were analyzed by hematoxylin and eosin staining. Representative images of each group are shown. (D, E) The lesion area in aorta and the size of atherosclerotic lesions in aortic root sections were analyzed by Image J software. Data are presented as mean ± s.e.m; n=8-10. Global significance among four groups was determined by one-way ANOVA, followed by post-hoc pairwise comparisons with Tukey’s HSD.

**Figure 2.** Local inflammation in aortic arch was reduced in Apoe<sup>−/−</sup> mice after oral gavage with *A. muciniphila*. Apoe<sup>−/−</sup> mice were grouped and treated as in Figure 1. The expressions of specific proteins in atherosclerotic lesions were detected with antibodies against (A) MOMA, (B) MCP-1 and (C) ICAM-1, and visualized by immunofluorescent staining using (A, C) Alex Fluor-596 or FITC conjugated secondary antibodies, or (B) by immunohistochemical staining. (D) The positive stained area was quantified by Image J software and calculated as the percentage of total lesion area. Representative images of each group are shown. Data are presented as mean ± s.e.m; n=8-10. Global significance among four groups was determined by one-way ANOVA followed
by post-hoc pairwise comparisons with Tukey’s HSD for MCP-1 and ICAM-1 analysis, or by
Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for
MOMA analysis.

**Figure 3.** Apoe<sup>−/−</sup> mice treated with *A. muciniphila* showed amelioration in both aortic
expressions and systemic levels of inflammatory molecules. Apoe<sup>−/−</sup> mice were grouped and
treated as in Figure 1. (A-D) Total RNA was extracted from the dissected aorta. The mRNA
expressions of F4/80, MCP-1, ICAM-1 and TNFα were quantified by qPCR and normalized
against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (E-H) The circulating levels of
MCP-1, IL-1β, sTNFR II and adiponectin were measured by ELISA. Data are presented as mean
± s.e.m; n=8-10. Global significance among four groups was determined by one-way ANOVA
followed by post-hoc pairwise comparisons with Tukey’s HSD for A, and D to H, or by Welch’s
ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for B and C.

**Figure 4.** Treatment of Apoe<sup>−/−</sup> mice with *A. muciniphila* led to decreased intestinal permeability
and circulating level of LPS. Apoe<sup>−/−</sup> mice were grouped and treated as in Figure 1. (A) *In vivo*
gut permeability was determined by measurement of serum concentrations of DX-4000-FITC at
one hour after oral gavage. (B, C) Total RNA of ileum were extracted and mRNA levels of
occludin and ZO-1 were determined by qPCR. (D, E) The localizations and expressions of (D)
occludin and (E) ZO-1 in intestinal villa were visualized by immunofluorescent staining using
FITC or Alex Fluor-596 conjugated secondary antibodies, respectively. Representative images of
each group are shown. (F, G) Quantitative analysis of images from 4D and 4E was performed.
(H, I) The LPS levels in mesenteric adipose tissue and serum were quantified by LAL assay.
Data are presented as mean ± s.e.m; n=8-10. Global significance among four groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD for C, and F to H, and by Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for A, B and I.

Figure 5. The beneficial effect of *A. muciniphila* against atherosclerosis was abolished by chronic infusion with LPS. Eight-week old *Apoe*−/− mice were fed on Western diet for eight weeks. During this period, either PBS (WD+PBS) or *A. muciniphila* were given orally to *Apoe*−/− mice daily for four weeks, followed by chronic infusion of LPS (250µg/kg/day) (WD+Akk+LPS) or vehicle control (WB+Akk) for another 4 weeks. (A) The LPS level in serum was measured with LAL assay. (B, D) Atherosclerotic lesion areas and (C, E) sizes were evaluated as in Figure 1. Representative images of each group are shown. Data are presented as mean ± s.e.m; n=5-7. Global significance among three groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD.

Figure 6. *A. muciniphila*-mediated amelioration of local and systemic inflammation was reversed by chronic infusion of LPS. *Apoe*−/− mice were grouped and treated as in Figure 5. (A-C) The protein expressions of MOMA, MCP-1 and ICAM-1 in lesions, (D-G) mRNA expression of F4/80, MCP-1, ICAM-1 and TNFα in aortas, and (H-J) the levels of MCP-1, IL-1β and sTNFR II in serum were evaluated as in Figure 2 and 3. Representative images of each group are shown. Data are presented as mean ± s.e.m; n=5-7. Global significance among three groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD for H to J, or by Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for D to G.
Akkermansia Muciniphila Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe^{-/-} Mice
Jin Li, Shaoqiang Lin, Paul M. Vanhoutte, Connie W. Woo and Aimin Xu
SUPPLEMENTAL MATERIAL

Supplemental Tables

Supplemental Table 1. The sequences of primers used in this study1-4

<table>
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<td>F4/80 (mouse)</td>
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Supplemental Figures and Figure Legends

A. NCD, WD+PBS, WD+Akk

B. Global p=0.011

C. Global p=0.036

D. Global p=0.002

E. Global p=0.018

F. Global p=0.030

G. Food intake (gram/mouse/day)

H. Body weight (gram)

I. Fat mass percentage (%)

J. Adipose tissue weight (g)
Supplemental Figure 1. The effects of *A. muciniphila* on the global pattern of gut microbiota and physiological parameters.

*Apoe<sup>-/-</sup>* mice were grouped and treated as in Figure 1. Fecal DNA was extracted and (A) the pattern of gut microbiota was analyzed with denaturing gradient gel electrophoresis. Representative samples are shown. (B-F) The abundance of specific phyla and genera of bacteria was quantified by qPCR using the specific primers. (G) Daily food intake was recorded throughout the experimental period. (H) Body weight, (I) total fat mass and (J) weight of individual adipose depots including inguinal subcutaneous fat (iSAT), epidydimal white adipose tissue (eWAT), mesenteric WAT (mWAT) and interscapular brown adipose tissue (BAT) were measured at the end of the experiments. Data are presented as mean ± s.e.m; n=5-8. Global significance among three groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD for D and F to J, and by Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for B, C and E.
Supplemental Figure 2. *A. muciniphila* did not change lipid and glucose metabolic profiles in *Apoe*<sup>−/−</sup> mice.

*Apoe*<sup>−/−</sup> mice were grouped and treated as in Figure 1. (A-E) Serum total cholesterol, total triglyceride, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and glucose levels were determined after fasting for 12 hours. (F) Glucose tolerance test was performed seven weeks after treatment with *A. muciniphila*. Data are presented as mean ± s.e.m; n=8-10. Global significance among the three groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD for B, D and E, or by Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test for A and C.
Supplemental Figure 3. Treatment with *A. muciniphila* modestly increased the thickness of mucin layer in ileum of *Apoep/-* mice fed the Western diet.

*Apoep/-* mice were grouped and treated as in Figure 1. Inner mucin layer (IM) in ileum was stained with alcian blue and visualized under a Nikon Eclipse Ni-U microscope. The thickness of the inner mucin layer was measured by the SPOT Software 5.0 (SPOT Imaging Inc., Sterling Heights, MI). Data are presented as mean ± s.e.m; n=5-6. Global significance among three groups was determined by Welch’s ANOVA followed by post-hoc pairwise comparisons with Games-Howell test.
Supplemental Figure 4. The secretory products of *A. muciniphila* induced expressions of occludin and ZO-1 in human intestine epithelial cells.

(A, B) Caco-2 cells were treated with plain inoculating medium (Plain IM, 100µL/mL), or inoculating medium from *A. muciniphila* or E. coli culture (Akk-IM or Ecoli-IM, 100µL/mL) for 6 hours. (C, D) Caco-2 cells were treated with PBS vehicle, butyrate (Bu, 2mM), or propionate (Pro, 4mM) for 10 hours⁵ with Akk-IM treatment as a positive control. Total RNA was extracted and mRNA expressions of (A, C) occludin and (B, D) ZO-1 were examined. Data are presented as mean ± s.e.m; n=3 independent experiments. Statistical analysis was performed with the Kruskal-Wallis nonparametric test.
Supplemental Figure 5. *A. muciniphila* has no obvious effect on gut permeability, circulating cytokines and lesion formation in *Apoe-/-* mice fed a normal chow diet. *Apoe-/-* mice on a normal chow diet (NCD) were orally gavaged with PBS vehicle or live *A. muciniphila* (Akk) for 8 weeks. (A) In vivo gut permeability was determined by measurement of serum concentrations of DX-4000-FITC at one hour after oral gavage. (B-D) The circulating levels of MCP-1, IL-1β, and sTNFR II were measured by ELISA. (E) The lipid content of aorta was visualized by staining with Oil Red O, and (F) the sections of aortic roots were analyzed by hematoxylin and eosin staining. Representative images of each group are shown. Data are presented as mean ± s.e.m; n=6. Statistical analysis was performed with Student’s t-test.
Supplemental Figure 6. *A. muciniphila* decreased adipose tissue inflammation, which was reversed by LPS infusion.

*Apoe*<sup>-/-</sup> mice were grouped and treated as in Figure 5. Total RNA was extracted from epididymal adipose tissue. The mRNA expressions of (A) F4/80, (B) IL-1β, (C) MCP-1, and (D) TNFα were quantified by real-time PCR and normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as mean ± s.e.m; n=5. Global significance among the three groups was determined by one-way ANOVA followed by post-hoc pairwise comparisons with Tukey’s HSD.
Supplemental Figure 7. Graphic summary of anti-atherogenic effect of *A. muciniphila*.

(Left panel) Western diet-induced reduction in abundance of *A. muciniphila* in the gut is associated with increased penetration of LPS from the gut lumen into the bloodstream. Elevated circulating LPS can elicit pro-inflammatory response in vascular cells and subsequently aggravate the development of atherosclerosis. (Right panel) Replenishing the *A. muciniphila* in the gut by oral administration increases the expression of tight junction proteins and restores the gut barrier, resulting in lower blood concentration of LPS. The anti-atherogenic effect of *A. muciniphila* is mediated in part by alleviation of metabolic endotoxemia.
Supplemental References


좋은 장내 세균은 동맥경화증을 예방할 수 있다

이 상연 교수 서울대학교병원 순환기내과

초록

배경
장내 세균층의 변화는 비만이나 당뇨병의 발생 및 진행을 조절한다. 하지만 장내 세균층의 변화가 심혈관질환의 발생에 미치는 영향에 대해서는 잘 알려져 있지 않다. 본 연구에서는 비만과 대사질환을 예방하는 것으로 알려진 *A. muciniphila* (*Akkermansia muciniphila*)라는 mucin 분해 세균이 apolipoprotein E 결핍(Apoe⁻) 생쥐에서 동맥경화의 발생에 미치는 영향을 조사하였다.

방법 및 결과
고콜레스테롤 식이(Western diet)나 일반 식이(normal chow diet)를 하면서 *A. muciniphila*를 8주간 셀프(oral gavage) Apoe⁻ 생쥐에서 대동맥의 동맥경화 병변을 평가하였다. Real-time PCR(polymerase chain reaction) 분석 결과, 고콜레스테롤 식이를 하면 대변의 *A. muciniphila*가 유의하게 감소하였고, *A. muciniphila*를 먹인 경우, 고콜레스테롤 식이에 의한 동맥경화증의 진행이 완화되었으나, 혈중 콜레스테롤의 수치에는 변화가 없었다. *A. muciniphila*는 대식세포의 침윤을 줄이고 염증성 사이토카인과 케모카인의 발현을 감소시키며, 고콜레스테롤 식이에 의한 동맥경화반과 혈액 내 염증을 감소시켰다. 이러한 변화는 metabolic endotoxemia의 감소와 동반되었다.

*A. muciniphila*에 의한 혈중 endotoxin의 감소와 함께 장내 tight junction 단백의 발현이 증가하였고, 고콜레스테롤 식이에 의한 장의 투과성(gut permeability) 증가가 억제되었다. Endotoxin을 다시 주입하면 *A. muciniphila*에 의한 동맥경화 감소 효과가 사라졌다.

결론
*A. muciniphila*는 장의 차단기능을 회복시켜 metabolic endotoxemia에 의한 염증을 줄이고, 동맥경화의 진행을 억제한다.
The gut microbiota, a complex community of >100 trillion microbes, plays an important physiological role in modulating host nutrition, metabolism, and immunity.1,2 Altered composition and function of gut microbiota have been linked to a number of chronic diseases, including colon cancer, irritated bowel syndrome, colitis, obesity, and diabetes mellitus.3 For example, decreased diversity within the phylum of Firmicutes in the gut microbiota is commonly found in patients with Crohn disease,4 and supplementation with one of the species in this phylum, *Faecalibacterium prausnitzii*, improves the survival rate of chemical-induced colitis in animals.5 Conversely, higher proportions of Firmicutes and lower levels of Bacteroidetes have been observed in obese individuals, and dietary intervention or bariatric surgery reverses these changes.6

**Background**—Altered composition of the gut microbiota is involved in both the onset and progression of obesity and diabetes mellitus. However, the link between gut microbiota and obesity-related cardiovascular complications has not been explored. The present study was designed to investigate the role of *Akkermansia muciniphila*, a mucin-degrading bacterium with beneficial effects on metabolism, in the pathogenesis of atherosclerosis in apolipoprotein E–deficient (*Apoe*−/−) mice.

**Methods and Results**—*Apoe*−/− mice on normal chow diet or a Western diet were treated with *A muciniphila* by daily oral gavage for 8 weeks, followed by histological evaluations of atherosclerotic lesion in aorta. Real-time polymerase chain reaction analysis demonstrated that the fecal abundance of *A muciniphila* was significantly reduced by Western diet. Replenishment with *A muciniphila* reversed Western diet–induced exacerbation of atherosclerotic lesion formation without affecting hypercholesterolemia. *A muciniphila* prevented Western diet–induced inflammation in both the circulation and local atherosclerotic lesion, as evidenced by reduced macrophage infiltration and expression of proinflammatory cytokines and chemokines. These changes were accompanied by a marked attenuation in metabolic endotoxia. *A muciniphila*–mediated reduction in circulating endotoxin level could be attributed to the induction of intestinal expression of the tight junction proteins (zonula occuldens protein-1 and occludin), thereby reversing Western diet–induced increases in gut permeability. Long-term infusion of endotoxin to *Apoe*−/− mice reversed the protective effect of *A muciniphila* against atherosclerosis.

**Conclusion**—*A muciniphila* attenuates atherosclerotic lesions by ameliorating metabolic endotoxia–induced inflammation through restoration of the gut barrier. *(Circulation. 2016;133:2434-2446.DOI: 10.1161/CIRCULATIONAHA.115.019645.)*

**Key Words:** atherosclerosis • endotoxemia • gut microbiota
into the circulation, resulting in metabolic endotoxemia, and the elevated endotoxins in circulation exacerbate hepatic insulin resistance and promote weight gain. Although associations between alterations in gut microbiota and many chronic diseases have been observed, it remains unclear whether such changes are the cause or the consequence of the pathologies.

Atherosclerosis, the main contributor to cardiovascular mortality, is a chronic inflammatory disease. Bacterial infection has been proposed as one of the triggers of inflammation in atherosclerosis. For example, Chlamydia pneumonia is present in atherosclerotic lesions of patients with previous exposure, and infection with this bacterium exacerbates atherosclerosis in animals. Bacterial DNA has been detected in atherosclerotic lesions, and the pyrosequencing result reveals that the bacteria in lesions are derived from gut and oral cavity, suggesting a possible involvement of gut microbiota in the development of the disease. However, the germ-free athogenic mice lacking apolipoprotein E (ApoE-/-) mice, which are without the colonization of gut microbiota, show a worsening of atherosclerotic lesions after being fed a high-cholesterol diet compared with the conventionally raised mice, and antibiotic therapy fails to elicit any beneficial effect on cardiovascular events in human trials. On the contrary, a metabolomics analysis shows that metabolism of dietary phosphatidylcholine by gut microbiota produces proatherogenic trimethylamine-N-oxide, which can accelerate atherosclerosis in animals. For example, modulation rather than elimination of gut microbiota is a potential therapeutic strategy to prevent or slow the atherosclerotic process.

Akkermansia muciniphila, a mucin-degrading bacterium belonging to the genus of Verrucomicrobia, has recently emerged as an important component of the gut microbial ecosystem. It accounts for 1% to 3% of the microbial community in healthy subjects, and its abundance is inversely correlated with body weight in mice and humans. A smaller amount of A muciniphila in the gut has been observed in obese children and pregnant women. In mice fed a high-fat diet, A muciniphila given by daily oral gavage reverses fat mass gain, adipose tissue inflammation, and insulin resistance, suggesting a beneficial effect in combating obesity-related metabolic disorders. However, its beneficial role against obesity-related cardiovascular diseases remains to be addressed.

In this study, we explored the relationship between the abundance of A muciniphila in the gut and the severity of atherosclerosis in ApoE-/- mice. Our results showed that Western diet–induced aggravation of atherosclerotic lesion was accompanied by a marked reduction of A muciniphila, whereas restoration of A muciniphila by daily oral gavage substantially reduced atherosclerotic lesions in ApoE-/- mice. Therefore, we further investigated the mechanisms underlying this protective effect of gut-residing A muciniphila.

Methods

Animal Model
ApoE-/- mice on a C57BL background were purchased from The Jackson Laboratory (Bar Harbor, MA). Eight-week-old male ApoE-/- mice were fed either a normal chow diet or a Western diet (catalog no. D12079B, Research Diet, New Brunswick, NJ) ad libitum for 8 weeks. Total fat mass was measured by the Minispec LF90 Body Composition Analyzer (Bruker, Billerica, MA). The fat mass in inguinal subcutaneous adipose tissue, epididymal and mesenteric white adipose tissue, and interscapular brown adipose tissue was determined by measuring the wet weight of each adipose depot after the mice were euthanized. All the animal experiments were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

Culture and Administration of A muciniphila
A muciniphila (catalog no. BAA-835, American Type Culture Collection, Manassas, VA) were cultured anaerobically in BHI (brain-heart-infusion) broth (BD Bioscience, San Jose, CA) supplemented with 0.5% porcine mucin (Sigma-Aldrich, St. Louis, MO) and 0.05% cysteine (Sigma-Aldrich). The concentration of bacteria was calculated by measuring the absorbance at the wavelength of 600 nm. Then, 5×10⁹ cfu of A muciniphila in 200 µL PBS was orally gavaged daily to ApoE-/- mice. In 1 group of experiment, A muciniphila were heat killed at 121°C under 225-kPa pressure for 15 minutes.

Quantitative Analysis of Atherosclerotic Lesions
For analysis of lesion area, Oil Red O staining of the area from the aorta arch to thoracic aorta was performed, whereas for analysis of atherosclerotic lesion in aortic sinus, the proximal aorta attached to heart was harvested and fixed in 4% paraformaldehyde. Serial 6-µm-thick paraffin-embedded sections from the middle portion of the ventricle to the aortic arch were collected. Sections of aorta were stained with hematoxylin and eosin for analysis of morphometric lesion. The quantification of lesion area and size was performed with ImageJ software (National Institutes of Health, Baltimore, MD).

Immunofluorescent and Immunohistochemical Staining
For immunofluorescent staining, aortic sinus sections were incubated with anti-monocyte and –macrophage-2 antibody (Abcam, Cambridge, UK) or anti-intercellular adhesion molecule-1 (ICAM-1; Santa Cruz Biotechnology, Dallas, TX) antibodies, and sections of ileums were treated with anti–zona occuldens protein-1 (ZO-1) or occludin (Abcam) antibodies, followed by incubation with Alex Fluor 596– or FITC-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and counterstaining with DAPI. For immunohistochemistry staining, after the endogenous peroxidase activity had been inhibited by hydrogen peroxide (H₂O₂) for 20 minutes, sections were incubated overnight with anti–monocyte chemoattractant protein-1 (MCP-1) antibody (Santa Cruz Biotechnology), followed by staining with horseradish peroxidase–conjugated secondary antibody. The target proteins were visualized with 3,3-diaminobenzidine in the presence of H₂O₂, and sections were counterstained with Harris hematoxylin. For quantitative analysis of images, 5 random fields were captured from different areas of a single section, and the intensity of positive staining was analyzed by Image J software and calculated as the percentage of total area of lesion or villa in each field.

RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction Analysis
Total RNA was extracted from aorta or ileum with TRIzol reagent (Life Technologies), followed by reverse transcription into cDNA with an ImProm-II reverse transcript kit (Promega, Madison, WI). Quantitative real-time polymerase chain reaction (PCR) was performed with Applied Biosystems StepOne Plus Real-Time PCR Systems (Life Technologies). The primers for each specific gene are listed in Table I in the online-only Data Supplement.

In Vivo Gut Permeability Assays
Mice were orally gavaged with FITC-labeled dextran (DX-4000-FITC, 500 mg/kg body weight, Sigma-Aldrich) after fasting for 6 hours, followed by collection of serum samples via the tail vein. The concentration of DX-4000-FITC in serum was measured by a
fluorescence spectrophotometer (Synergy H1, BioTek, Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Denaturing Gradient Gel Electrophoresis**

Fecal DNA was extracted with the QIAamp DNA Stool Mini Kit (51504, Qiagen, Venlo, the Netherlands) and subjected to PCR amplification targeting the V3 region of the 16S rRNA gene with the universal primers (Table I in the online-only Data Supplement). The PCR products were further separated by electrophoresis with a gradient gel from 27% to 52% using the Dcode System apparatus (Bio-Rad, Hercules, CA). Gels were stained with SYBR Green I (Life Technologies) for 30 minutes for visualization under ultraviolet transillumination.

**Biochemical and Immunological Assays**

Serum levels of total cholesterol, total triglyceride, low-density lipoprotein, high-density lipoprotein, and glucose were measured with commercially available kits (Stanbio Laboratory, Boerne, TX). Inflammatory molecules in serum, including MCP-1, interleukin-1β (IL-1β), and soluble tumor necrosis factor receptor II (sTNFR II) levels, were determined by immunoassays from R&D Systems (Minneapolis, MN). Serum adiponectin level was determined by immunoassay from Antibody and Immunoassay Services at the University of Hong Kong. Lipopolysaccharide levels in mesenteric adipose tissue and serum were measured by LAL assay from Hycult Biotechnology (Uden, the Netherlands).

**Glucose Tolerance Test**

Apoe−/− mice were fasted overnight, and glucose (2 g/kg body weight) was injected intraperitoneally. Blood glucose level was determined at various time points with a glucometer (Accu-Check Performa, Roche Diagnostics, Basel, Switzerland) after the initial injection of glucose.

**Chronic Infusion of Lipopolysaccharide**

Osmotic pumps (model 1004, Alzet, Cupertino, CA) filled with either lipopolysaccharide (catalog No. L6386, Sigma-Aldrich) or vehicle (PBS) were subcutaneously implanted into Apoe−/− mice at the fourth week of Western diet treatment to deliver lipopolysaccharide at a constant rate (250 µg/kg body weight per day) for another 4 weeks.

**Culture of Intestinal Epithelial Cells**

Human intestinal epithelial cells, Caco-2 cells, were purchased from American Type Culture Collection and cultured in Minimum Essential Medium (Life Technologies) with 10% FBS and antibiotics (100 U penicillin, 0.1 mg streptomycin, and 0.25 µg/mL amphotericin B).

**Statistical Analysis**

Statistical analyses were performed with Statistical Package for Social Sciences version 23.00 (SPSS, Chicago, IL). Data were presented as mean±SEM. One-way ANOVA was applied for comparisons between multiple experimental groups, followed by post hoc analysis with the Tukey honest significant difference for data with equal variance or Games-Howell for data with unequal variance. Data with small sample size were analyzed with the Kruskal-Wallis test, a nonparametric 1-way ANOVA. An unpaired Student t test was applied for comparison of 2 groups with normal distribution. Values of P<0.05 were accepted to indicate statistically significant differences.

**Results**

**Oral Gavage With A muciniphila Protected Against Western Diet–Induced Atherosclerotic Lesion Formation in Apoe−/− Mice**

To explore the possible role of *A muciniphila* in atherosclerosis, Apoe−/− mice fed the Western diet were treated with live *A muciniphila*, heat-killed *A muciniphila*, or vehicle (PBS) by oral gavage daily for a period of 8 weeks, followed by the assessment of atherosclerotic lesions. Real-time PCR analysis showed a significantly reduced amount of *A muciniphila* in the feces of Western diet–fed Apoe−/− mice compared with the mice fed a normal chow diet (Figure 1A), whereas the fecal amounts of this bacteria were comparable between wild-type and Apoe−/− mice fed a normal chow diet (7.31±0.51 versus 7.04±0.69 log10 bacteria/g feces in wild-type versus Apoe−/−). Daily oral gavage with 5×10^9cfu live *A muciniphila* was sufficient to restore the diminished level caused by the Western diet (Figure 1A).

The Western diet induced formation of atherosclerotic lesions, as demonstrated by Oil Red O staining of longitudinally opened aortas and hematoxylin and eosin staining of aortic root regions (Figure 1B and 1C). Lipids accumulated in the aortic arch and at the roots of branchiocephalic, left common carotid, and left subclavian arteries after 8 weeks of Western diet (Figure 1B). The Western diet resulted in a 3.7-fold increase in lesion area and a 2.9-fold increase in lesion size in Apoe−/− mice compared with normal chow diet (Figure 1D and 1E). Treatment with *A muciniphila* substantially reduced the lesion area and size by 31% and 48%, respectively, in Apoe−/− mice fed a Western diet (Figure 1D and 1E). Administration of the same dose of heat-killed *A muciniphila* did not show any improvement in lesion area or size in Apoe−/− mice (Figure 1B–1E), indicating that the protective effect of this bacteria was dependent on their viability.

We next investigated whether oral gavage with *A muciniphila* altered the ecosystem and changed the composition of the gut microbiota using denaturing gradient gel electrophoresis and real time PCR analyses. The Western diet substantially altered the gut microbiota pattern compared with normal chow diet (Figure IA in the online-only Data Supplement). This pattern change was characterized by increased amounts of Proteobacteria and Firmicutes and a decreased quantity of Bacteroidetes, Fusobacteria, and Tenericutes (Figure IB–IF in the online-only Data Supplement), which is in line with the previously reported data on high fat diet–induced mice. However, the daily oral gavage with *A muciniphila* did not affect the overall pattern of gut microbiota and abundance of the above-mentioned bacterial species (Figure IA–IF in the online-only Data Supplement).

**Treatment With A muciniphila Did Not Alter Lipid Metabolism in Apoe−/− Mice**

A previous study demonstrated that treatment with *A muciniphila* reduced body weight and fat mass and improved metabolic functions during obesity. In our atherogenic Apoe−/− model, there was no obvious change in food intake after *A muciniphila* treatment (Figure IG in the online-only Data Supplement), although there was a slight decrease in body weight and fat mass (Figure IH–IJ in the online-only Data Supplement). Consistent with previous findings, the Western diet led to hyperlipidemia in Apoe−/− mice compared with normal chow diet (Figure IIA–IID in the online-only Data Supplement). However, the daily oral gavage with *A muciniphila* did not alter serum levels of total cholesterol, total triglyceride, low-density lipoprotein, and high-density lipoprotein (Figure IIA–IID in the online-only Data Supplement).
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In addition, *A muciniphila* treatment did not significantly affect fasting blood glucose level or glucose tolerance in *Apoe−/−* mice (Figure IIE and IIF in the online-only Data Supplement), implying that the beneficial role of *A muciniphila* against atherosclerosis was not attributed to altered lipid or glucose metabolism.

**Figure 1.** Western diet–fed *Apoe−/−* mice treated with *Akkermansia muciniphila* exhibited reduced atherosclerosis. Eight-week-old *Apoe−/−* mice were fed either a normal chow diet (NCD) or Western diet (WD) for 8 weeks. The Western diet–fed mice were further separated into 3 groups: a group receiving daily oral gavage with live *A muciniphila* (WD+Akk), a group receiving daily oral gavage with heat-killed *A muciniphila* (WD+hk-Akk), and a third gavaged with PBS as vehicle control (WD+PBS). **A**, The abundance of *A muciniphila* in feces was examined by quantitative real-time polymerase chain reaction. **B**, The lipid content of aorta was visualized by staining with Oil Red O, and **C** the sections of aortic roots were analyzed by hematoxylin and eosin staining. Representative images of each group are shown. **D** and **E**, The lesion area in aorta and the size of atherosclerotic lesions in aortic root sections were analyzed by Image J software. Data are presented as mean±SEM; n=8 to 10. Global significance among 4 groups was determined by 1-way ANOVA, followed by post hoc pairwise comparisons with the Tukey honest significant difference.

*A muciniphila* Ameliorated Both Aortic and Systemic Inflammation in Western Diet–Fed *Apoe−/−* Mice

Increased local inflammation is one of the hallmarks of the progression of atherosclerosis. Macrophages, the major immune cells in atherosclerotic plaques, play a key role in...
promoting atherosclerosis. The number of macrophages in atherosclerotic lesions increased after Western diet feeding, and daily oral gavage with *Akkermansia muciniphila* significantly reduced the amount, as shown by immunofluorescent staining of the macrophage marker *monocytes/macrophages antigen* (MOMA) (Figure 2A and 2D). Likewise, treatment with *A muciniphila* inhibited the Western diet–induced protein expression of ICAM-1 and MCP-1, which are the major chemokines involved in promoting the adhesion of macrophages onto endothelium and subsequent transmigration into intima, respectively (Figure 2B–2D). A similar effect of *A muciniphila* on suppression of the Western diet–induced mRNA expression of F4/80 (another macrophage marker), MCP-1, ICAM-1, and tumor necrosis factor-α (TNFα) was observed in the aortas of *Apoe*−/− mice (Figure 3A–3D). In contrast, treatment with heat-killed *A muciniphila*...
muciniphila did not have a significant effect on either macrophage accumulation or expression of MCP-1, ICAM-1 and TNFα in the local aortic tissues (Figure 2A–2D).

Circulating proinflammatory cytokines such as MCP-1 and IL-1β participate in the development of atherosclerosis. The serum levels of these 2 proinflammatory factors were significantly increased in Western diet–fed Apoe−/− mice compared with the mice on normal chow diet (Figure 3E and 3F). Similarly, the Western diet–induced increase in circulating level of sTNFR II, a prognostic marker of fatal complications of atherosclerosis, including congestive heart failure, was significantly attenuated by the administration of live *Akkermansia muciniphila* (Figure 3G). However, daily treatment with *A muciniphila* had no obvious effect on the circulating level of adiponectin, an adipocyte-derived anti-inflammatory adipokine with antiatherosclerotic activity (Figure 3H).
Figure 4. Treatment of Apoe−/− mice with Akkermansia muciniphila led to decreased intestinal permeability and circulating level of lipopolysaccharide (LPS). Apoe−/− mice were grouped and treated as in Figure 1. A. In vivo gut permeability was determined by measurement of serum concentrations of DX-4000-FITC at 1 hour after oral gavage. B and C. Total RNA of ileum was extracted, and mRNA levels of occludin and zona occludens protein-1 (ZO-1) were determined by quantitative polymerase chain reaction. D and E. The localizations and expression of (D) occludin and (E) ZO-1 in intestinal villi were visualized by immunofluorescent staining with FITC- or Alex Fluor-596–conjugated secondary antibodies, respectively. Representative images of each group are shown. F and G. Quantitative analysis of images from D and E was performed. H and I. The LPS levels in mesenteric adipose tissue and serum were quantified by LAL assay. Data are presented as mean±SEM; n=8 to 10. Global significance among the 4 groups was determined by 1-way ANOVA followed by post hoc pairwise comparisons with the Tukey honest significant difference for C and F through H and by the Welch ANOVA followed by post hoc pairwise comparisons with the Games-Howell test for A, B, and I.
**A muciniphila Decreased Intestinal Permeability and Reduced the Penetration of Gut-Derived Lipopolysaccharide Into Circulation in Western Diet–Fed Apoe<sup>−/−</sup> Mice**

Metabolic endotoxemia is a key mediator of obesity-induced chronic inflammatory diseases and has been suggested to be caused by the increased penetration of lipopolysaccharide from gut into circulation. The in vivo gut permeability, as determined by oral administration of fluorescent labeled dextran (DX-4000-FITC) followed by measurement of its circulating concentration, was significantly higher in Western diet–fed Apoe<sup>−/−</sup> mice compared with the normal chow diet–fed controls, whereas the Western diet–induced increase of gut permeability was largely blocked by the treatment with live *A muciniphila* (Figure 4A). Gut permeability is regulated by the mucus layer and tight junctions of intestine. The former serves as the first defense preventing the adhesion of bacteria, whereas the latter further blocks the intrusion of pathogens and bacterial products. Therefore, we next evaluated the effect of *A muciniphila* on the mucin layer thickness and expression levels of the major tight junction proteins in the intestine. In line with a previous report, Western diet–fed Apoe<sup>−/−</sup> mice showed a significant decrease in the inner mucin layer thickness of ileum, and *A muciniphila* treatment partially restored the thickness of this layer (Figure III in the online-only Data Supplement). On the other hand, the expression of the epithelial tight junction protein occludin was significantly reduced by Western diet feeding but was upregulated by treatment with live *A muciniphila* (Figure 4B, 4D, and 4F). *A muciniphila* treatment also increased the expression of another tight junction protein, ZO-1 (Figure 4C, 4E, and 4G). Furthermore, the restoration of the gut barrier by *A muciniphila* treatment led to a significant reduction in the Western diet–induced elevation of lipopolysaccharide levels in both mesenteric adipose tissue and circulation (Figure 4H and 4I), suggesting that *A muciniphila* blocked lipopolysaccharide penetration by preserving the gut barrier.

Because viability of *A muciniphila* is indispensable for its antiatherosclerotic effects, we postulated that molecules or metabolites secreted by *A muciniphila* might affect the expression of tight junction proteins. Treatment with the inoculating medium of *A muciniphila* culture (100 μL/mL) in Caco-2 cells, a human intestine epithelial cell line, significantly upregulated the expression of occludin and ZO-1 compared with those treated with the plain inoculating medium. This *A muciniphila*–induced upregulation of the tight junction proteins was independent of its characteristic as a Gram-negative bacterium because treatment with inoculating medium of *Escherichia coli* did not show any effect (Figure IVA and IVB in the online-only Data Supplement). Because the short-chain fatty acids butyrate and propionate have been shown to stimulate the proliferation of intestinal epithelial cells, we next tested their effects on the expression of tight junction proteins. However, treatment with propionate or butyrate in Caco-2 cells failed to increase the expression of either occludin or ZO-1 (Figure IV C and IVD in the online-only Data Supplement).

In Apoe<sup>−/−</sup> mice on normal chow diet, treatment with *A muciniphila* has no obvious effect on gut permeability (Figure VA in the online-only Data Supplement), circulating levels of inflammatory markers (MCP-1, IL-1β, and sTNFR II; Figure VB–VD in the online-only Data Supplement), and atherosclerotic plaque formation (Figure VE and VF in the online-only Data Supplement), suggesting that the amount of intestinal *A muciniphila* under normal diet is sufficient to maintain the integrity of the gut barrier, thereby preventing endotoxinemia-induced systemic inflammation and atherosclerosis.

**Chronic Infusion of Lipopolysaccharide Abolished the Protective Effect of *A muciniphila* Against Western Diet–Induced Atherosclerosis in Apoe<sup>−/−</sup> Mice**

Long-term exposure to lipopolysaccharide can accelerate atherosclerosis via different signaling pathways, including an increase in adhesion molecules and chemotactic and proinflammatory cytokines. Therefore, we hypothesized that the protective effect of *A muciniphila* against atherosclerosis was partially mediated by the decreasing serum level of lipopolysaccharide. To this end, *A muciniphila* was orally gavaged daily to Apoe<sup>−/−</sup> mice for a total of 8 weeks, and during the last 4 weeks, either lipopolysaccharide (250 μg/kg body weight per day) or PBS (vehicle control) was infused long term via subcutaneously implanted osmotic pumps (model 1004, Alzet) into these mice. Long-term infusion of lipopolysaccharide restored the serum level of lipopolysaccharide in *A muciniphila*–treated mice to a level similar to that of the Western diet–fed mice without the bacterial treatment (Figure 5A). Notably, this increased level of lipopolysaccharide was able to reverse the protective effect of *A muciniphila* treatment against atherosclerosis, as demonstrated by increased lesion area (Figure 5B and 5D) and size (Figure 5C and 5E) in Apoe<sup>−/−</sup> mice compared with the *A muciniphila*–treated mice without infusion of lipopolysaccharide. Lipopolysaccharide infusion also augmented the number of infiltrated macrophages and the expression of the inflammatory molecules MCP-1 and ICAM-1 in the atherosclerotic lesions of mice treated with *A muciniphila* (Figure 6A–6C). Real-time PCR analysis further demonstrated that the suppressive effects of *A muciniphila* on mRNA expression of F4/80, MCP-1, ICAM-1, and TNFα were reversed by lipopolysaccharide treatment (Figure 6D–6G).

Similar to the changes in the aortas, Western diet–induced inflammation in visceral adipose tissues, as determined by real-time PCR analysis of the abundance of the macrophage marker F4/80 and the expression of the proinflammatory factors (IL-1β, MCP-1 and TNFα), was attenuated by treatment of Apoe<sup>−/−</sup> mice with *A muciniphila*, whereas such effects of *A muciniphila* were abrogated by infusion with lipopolysaccharide (Figure VI in the online-only Data Supplement), suggesting that amelioration of adipose tissue inflammation by *A muciniphila* was also attributed to its ability to reduce endotoxemia. Notably, the amelioration of systemic inflammation by *A muciniphila*, as determined by circulating levels of MCP-1, IL-1β and sTNFR II, was also abolished by long-term infusion of lipopolysaccharide (Figure 6H–6J).

**Discussion**

Several recent studies have identified trimethylamine N-oxide, a metabolite of the gut microbiota, as an independent risk...
factor for cardiovascular disease. Bacterial DNA from the gut microbiota has been detected in human atherosclerotic plaques. However, direct evidence for the causative role of altered gut microbiota (dysbiosis) in the pathogenesis of atherosclerosis is still lacking. The present study demonstrates that the Western diet–induced deterioration of atherosclerosis in ApoE−/− mice is associated with an increased level of Firmicutes, a decreased amount of Bacteroidetes, and especially a marked reduction in A. muciniphila in the gut. Replenishment of A. muciniphila by daily oral gavage reduced...
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The size of atherosclerotic plaques in Western diet–fed *Apoe*−/− mice. The findings suggest a causal role of the reduction in gut *A muciniphila* in the Western diet–induced exacerbation of atherosclerosis.

Prebiotics such as oligofructose have been used as a food supplement to support the growth of commensal bacteria to maintain the general health of body. Several clinical trials have reported its therapeutic effectiveness. Though the...
mechanism is unknown, it is generally believed that enrichment of gut microbiota by prebiotics plays an important role because lower diversity and abundance of gut microbiota is associated with certain diseases.\textsuperscript{35} Prebiotics-induced hypocholesterolemic effect has been reported in animals, suggesting a possible role in combating atherosclerosis.\textsuperscript{36} Notably, supplementation with prebiotics causes a >100-fold enrichment in gut \textit{A. muciniphila}.\textsuperscript{21} Furthermore, the benefits of dietary polyphenol, cranberry extract, and metformin are associated with increased amount of this bacterial species in the gut.\textsuperscript{24,37,38} In this connection, our result showed that daily oral gavage with the monospecific genus \textit{A. muciniphila}, without an obvious change in the composition of gut microbiota, is sufficient to reduce atherosclerosis, suggesting that therapeutic interventions targeting this single genus/species in gut microbiota may represent a promising strategy for the treatment and prevention of both metabolic and cardiovascular disorders.

Inflammation and hypercholesterolemia are the 2 key etiological factors for atherosclerosis.\textsuperscript{10,39} In the initial stage of atherosclerosis, injured or inflamed endothelium secretes adhesion molecules and chemokines to facilitate the recruitment and transmigration of leukocytes into the intima.\textsuperscript{10} The unresolved inflammation stimulates the expression of macrophage scavenger receptors and promotes the uptake of modified lipoproteins forming lipid-laden macrophages, which drives the inflammatory loop for further migration and proliferation of leukocytes and smooth muscle cells in the lesion area.\textsuperscript{10} Current therapeutic options for treating or preventing atherosclerosis include mainly platelet aggregation inhibitors, statins, anti hypertensives, and thrombolytic agents.\textsuperscript{40} However, the risks for fatal cardiovascular complications in these patients remain high because of the unresolved inflammation.\textsuperscript{30} Animal-based studies have reported the effectiveness of several anti-inflammatory interventions in alleviating atherosclerosis, including the CD40-TNF receptor–associated factor 6–specific blockade and IL-1 neutralization, but clinical implications of these findings remain to be confirmed.\textsuperscript{39} In this study, treatment with \textit{A. muciniphila} substantially reduced the expression of several chemokines and the adhesion molecules MCF-1, TNFα, and ICAM-1, along with lower aortic infiltration of macrophages and diminished atherosclerotic lesion in \textit{Apoe}\textsuperscript{−/−} mice. In contrast, \textit{A. muciniphila} had no effect on the Western diet–induced hypercholesterolemia and alterations in other metabolic profiles, suggesting that the antiatherosclerotic effect of \textit{A. muciniphila} is attributed mainly to its anti-inflammatory activity. This notion is consistent with recent studies showing that supplementation with \textit{A. muciniphila} in obese mice reduced IL-6 and IL-1β expression and increased the percentage of regulatory T cells in visceral adipose tissue.\textsuperscript{21,24}

Metabolic endotoxemia, defined as a 2- to 3-fold elevation of the circulating endotoxin/lipopolysaccharide level, has been proposed as an initiating factor of obesity-related cardiometabolic dysfunction.\textsuperscript{9} Lipopolysaccharide, a ligand of Toll-like receptor 4, is a potent stimulus of inflammation.\textsuperscript{33} A clinical study revealed that subclinical endotoxemia is a strong risk factor for carotid atherosclerosis and that weekly injection of lipopolysaccharide worsens the formation of atherosclerosis in animal models.\textsuperscript{41,42} A positive correlation between serum lipopolysaccharide-binding protein level and carotid intima thickness and an association of Toll-like receptor 4 polymorphisms with decreased atherosclerosis have been found in humans.\textsuperscript{43,44} Toll-like receptor 4 is expressed in various vascular cells, including endothelial cells, focal leukocytes, and macrophages. Its activation by lipopolysaccharide not only stimulates the release of proinflammatory molecules from these cells but also inhibits cholesterol efflux from macrophages and thus facilitates foam cell formation. In obesity, inflammatory factors in adipose tissue are the major contributors to systemic inflammation.\textsuperscript{45} Lipopolysaccharide can also act on Toll-like receptor 4 in adipocytes to stimulate the production of proinflammatory adipokines and to further reinforce the systemic inflammation.\textsuperscript{46} Furthermore, lipopolysaccharide-induced inflammatory cytokines in peri vascular adipose tissue, which surrounds almost all the blood vessels, can act in a paracrine manner to exacerbate vascular inflammation and atherosclerosis. Inactivation of the lipopolysaccharide pathway by deletion of Toll-like receptor 4 or the downstream cytosolic adaptor myeloid differentiation factor-88 or the prevention of its transport by inhibiting lipopolysaccharide-binding protein reduces aortic lesions in \textit{Apoe}\textsuperscript{−/−} and low-density lipoprotein receptor–deficient (\textit{Ldlr}\textsuperscript{−/−}) mice.\textsuperscript{46,47} Notably, all these models showed a reduction of lesion area and lesional lipid content without any significant alteration of plasma cholesterol levels, which is similar to our findings of \textit{A. muciniphila} treatment.\textsuperscript{36,48} Here, we show that long-term infusion of lipopolysaccharide reversed the effect of \textit{A. muciniphila} on alleviation of the Western diet–induced local and systemic inflammation, indicating that the antiatherogenic effect of \textit{A. muciniphila} is mediated by limiting the lipopolysaccharide level in the bloodstream and ameliorating metabolic endotoxemia.

Penetration of lipopolysaccharide into the bloodstream is controlled by the integrity of the gut barrier.\textsuperscript{48} Administration of \textit{A. muciniphila} has been shown to prevent the thinning of mucus layer in mice with diet-induced obesity.\textsuperscript{21} The mucus layer is enriched with various mucins that form a hydrated gel layer covering the mucosal surface to prevent adhesion of harmful bacteria.\textsuperscript{49} However, the primary control of the gut barrier relies on an intact epithelium where tight junctions sealing the space between individual epithelial cells maintain the epithelial integrity.\textsuperscript{50} Tight junction is a multiple protein complex including occludin, claudins, and ZOs.\textsuperscript{50} Loss of occludin leads to an increase in gut permeability, whereas deficiency of ZO-1 can interrupt the assembly of tight junction by inhibiting the recruitment of other components.\textsuperscript{50} The expression of the 2 tight junction proteins, occludin and ZO-1, was increased in the ileum of \textit{Apoe}\textsuperscript{−/−} mice after administration of \textit{A. muciniphila}, and treatment with inoculating medium of \textit{A. muciniphila} directly stimulated the expression of these tight junction proteins in intestinal epithelial cells. These findings suggest an additional mechanism of preserving gut barrier by \textit{A. muciniphila}. However, how gut-residing \textit{A. muciniphila} increases the expression levels of these tight junction proteins remains to be determined.

**Conclusions**

Our study uncovered a key link among gut microbiota, gut permeability, and vascular system (Figure VII in the online-only
Data Supplement). The Western diet–induced atherosclerosis is caused partly by a reduction of *A. muciniphila* in gut, resulting in compromised gut barrier and increased endotoxemia, which in turn exacerbate vascular inflammation. Our findings raise the possibility of targeting individual species of the gut microbiota for the treatment of atherosclerosis.

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Disclosures

None.

References


Hypercholesterolemia and chronic inflammation are the 2 important risk factors for atherosclerosis, which is a major cause of fatal cardiovascular complications, including coronary heart disease and myocardial infarction. Although cholesterol-lowering drugs can effectively reduce the incidence of atherosclerosis, anti-inflammatory therapies against this disease have had limited success, partly because of the lack of the understanding of etiological factors that trigger vascular inflammation. Previous clinical studies have reported a close association of bacterial infection and endotoxemia with cardiovascular diseases. However, whether altered gut microbiota contributes to the pathogenesis of atherosclerosis remains unknown. Our study showed a markedly reduced abundance of Akkermansia muciniphila, which is a strain of commensal bacteria in the gut, in a murine model of Western diet–induced atherosclerosis. Replenishing this strain of bacteria by oral gavage substantially diminished the Western diet–induced atherosclerotic lesions. The antiatherogenic effect of A. muciniphila was attributed to its ability to reduce aortic and systemic inflammation by protecting the integrity of gut barrier, thereby leading to alleviation of endotoxemia. These findings suggest that reduced abundance of A. muciniphila contributes to vascular inflammation and atherosclerosis and that manipulation of a single strain of bacteria in gut microbiota is sufficient to reverse the progression of this disease. Prebiotics and dietary or therapeutic modulations in favor of increasing the abundance of A. muciniphila can be a potential therapeutic option for vascular inflammation in atherosclerosis.

**CLINICAL PERSPECTIVE**

Hypercholesterolemia and chronic inflammation are the 2 important risk factors for atherosclerosis, which is a major cause of fatal cardiovascular complications, including coronary heart disease and myocardial infarction. Although cholesterol-lowering drugs can effectively reduce the incidence of atherosclerosis, anti-inflammatory therapies against this disease have had limited success, partly because of the lack of the understanding of etiological factors that trigger vascular inflammation. Previous clinical studies have reported a close association of bacterial infection and endotoxemia with cardiovascular diseases. However, whether altered gut microbiota contributes to the pathogenesis of atherosclerosis remains unknown. Our study showed a markedly reduced abundance of Akkermansia muciniphila, which is a strain of commensal bacteria in the gut, in a murine model of Western diet–induced atherosclerosis. Replenishing this strain of bacteria by oral gavage substantially diminished the Western diet–induced atherosclerotic lesions. The antiatherogenic effect of A. muciniphila was attributed to its ability to reduce aortic and systemic inflammation by protecting the integrity of gut barrier, thereby leading to alleviation of endotoxemia. These findings suggest that reduced abundance of A. muciniphila contributes to vascular inflammation and atherosclerosis and that manipulation of a single strain of bacteria in gut microbiota is sufficient to reverse the progression of this disease. Prebiotics and dietary or therapeutic modulations in favor of increasing the abundance of A. muciniphila can be a potential therapeutic option for vascular inflammation in atherosclerosis.