**Akkermansia Muciniphila** Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe⁻/⁻ Mice

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**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 endotoxemia. *A. muciniphila*—mediated reduction in circulating endotoxin level could be attributed to the induction of intestinal expression of the tight junction proteins (zona occludens 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 endotoxemia-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

The gut microbiota, a complex community of >100 trillion microbes, plays an important physiological role in modulating host nutrition, metabolism, and immunity.¹²³

Altered composition and function of gut microbiota have been linked to a number of chronic diseases, including colon cancer, irritable bowel syndrome, colitis, obesity, and diabetes mellitus.⁴ 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

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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 have been found in individuals with autoimmune diabetes mellitus compared with healthy control subjects,⁷ and supplementation with butyric acid improves the metabolic profiles in a dietary obese murine model.⁸ A high-fat diet increases gut permeability and enhances the penetration of gut microbiota–derived endotoxins...
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 atherogenic 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, which are without the colonization of gut microbiota, 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 mice. It is possible that modulation rather than elimination of the gut microbiota is a potential therapeutic strategy to prevent or slow the atherosclerotic process.

Akkermansia muciniphila, a mucin-degrading bacterium belonging to the genera 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 LP90 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 cosin 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 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 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 chemotactrant 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 ImageJ software and calculated as the percentage of total area of lesion or villi 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 transcription 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), 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 Hy涿ct 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% PBS 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 test 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 5×10⁶cfu 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 1A 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 1B–1F in the online-only Data Supplement), which is in line with the previously reported data on high fat diet–induced mice.1 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 1A–1F 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.21 In our atherogenic Apoe−/− model, there was no obvious change in food intake after A muciniphila treatment (Figure 1G in the online-only Data Supplement), although there was a slight decrease in body weight and fat mass (Figure 1H–1J in the online-only Data Supplement). Consistent with previous findings,25 the Western diet led to hyperlipidemia in Apoe−/− mice compared with normal chow diet (Figure 1I–1D 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 1E–1H 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.

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 (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.
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*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). Total RNA was extracted from the dissected aorta. The mRNA expression of F4/80, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and tumor necrosis factor-α (TNFα) was quantified by quantitative polymerase chain reaction and normalized against GAPDH. E through H, The circulating levels of MCP-1, interleukin-1β (IL-1β), soluble tumor necrosis factor receptor II (sTNFR II), and adiponectin were measured by ELISA. 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 A and D through H or by the Welch ANOVA followed by post hoc pairwise comparisons with the Games-Howell test for B and C.

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, whereas these Western diet–induced elevations were obviously diminished by daily oral gavage with *A. muciniphila* but not the heat-killed bacteria (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 *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 antiatherosclerotic activity (Figure 3H).
Figure 4. Treatment of Apoe<sup>−/−</sup> mice with Akkermansia muciniphila led to decreased intestinal permeability and circulating level of lipopolysaccharide (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 1 hour after oral gavage. B and C, Total RNA of ileum was extracted, and mRNA levels of occludin and zona occuldens 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 villa 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* treatment partially 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* was 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.\textsuperscript{17,18,34} Bacterial DNA from the gut microbiota has been detected in human atherosclerotic plaques.\textsuperscript{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 \textit{A. muciniphila} in the gut. Replenishment of \textit{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 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. Although the

![Image](https://via.placeholder.com/150)
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. A prebiotics-induced hypocholesterolemic effect has been reported in animals, suggesting a possible role in combating atherosclerosis. Notably, supplementation with prebiotics causes a >100-fold enrichment in gut A muciniphila. Furthermore, the metabolic benefits of dietary polyphenol, cranberry extract, and metformin are 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 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. 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 include mainly platelet aggregation inhibitors, statins, antihypertensives, and thrombolytic agents. However, the risks for fatal cardiovascular complications in these patients remain high because of the unsolved inflammation. 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. In this study, treatment with A muciniphila substantially reduced the expression of several chemokines and the adhesion molecules MCP-1, TNFα, and ICAM-1, along with lower aortic infiltration of macrophages and diminished atherosclerotic lesion in Apoe−/− mice. In contrast, A muciniphila had no effect on the Western diet–induced hypercholesterolemia and alterations in other metabolic profiles, suggesting that the antiatherosclerotic effect of A muciniphila is attributed mainly to its anti-inflammatory activity. This notion is consistent with recent studies showing that supplementation with A muciniphila in obese mice reduced IL-6 and IL-1β expression and increased the percentage of regulatory T cells in visceral adipose tissue.

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. Lipopolysaccharide, a ligand of Toll-like receptor 4, is a potent stimulus of inflammation. 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. 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. 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. 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. Furthermore, lipopolysaccharide–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 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 Apoe−/− and low-density lipoprotein receptor–deficient (Ldlr−/−) 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 long-term infusion of lipopolysaccharide reversed the effect of A muciniphila on alleviation of the Western diet–induced local and systemic inflammation, indicating that the antiatherogenic effect of 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. Administration of A muciniphila has been shown to prevent the thinning of mucus layer in mice with diet-induced obesity. The mucus layer is enriched with various mucins that form 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 seal 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 expression of the 2 tight junction proteins, occludin and ZO-1, was increased in the ileum of Apoe−/− mice after administration of A muciniphila, and treatment with inoculating medium of 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 A muciniphila. However, how gut-residing 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
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**


A muciniphila can be a potential therapeutic option for vascular inflammation in atherosclerosis.

The progression of this disease. Prebiotics and dietary or therapeutic modulations in favor of increasing the abundance of A muciniphila substantially diminished the Western diet–induced atherosclerotic lesions. The antiatherogenic effect of A muciniphila is 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 in the gut microbiota of mice.

Our study showed a markedly reduced abundance of A 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 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.
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### Supplemental Material

**Supplemental Tables**

#### Supplemental Table 1. The sequences of primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80 (mouse)</td>
<td>forward: CTTTGGCTATGGGCTTCAGTC</td>
</tr>
<tr>
<td></td>
<td>reverse: GCAAAGGAGACAGAGTTATCGTG</td>
</tr>
<tr>
<td>GAPDH (mouse)</td>
<td>forward: CTCATGACCACACCATCCAGTCA</td>
</tr>
<tr>
<td></td>
<td>reverse: CCAATTGGGGTTAGGAACAC</td>
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<tr>
<td>GAPDH (human)</td>
<td>forward: GGAGCGAGATCCCTCCAAAAT</td>
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<tr>
<td></td>
<td>reverse: GGTGTGGTCATCTTCTCTATGG</td>
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<tr>
<td>MCP-1 (mouse)</td>
<td>forward: CCACCTACCTGCTGCTACTCA</td>
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<tr>
<td></td>
<td>reverse: TGGTGATCCTTGTAGCTCTCC</td>
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<tr>
<td>Occludin (mouse)</td>
<td>forward: ATGTCCGGCGAGATGCTTC</td>
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<tr>
<td></td>
<td>reverse: TTTGCTGGCTCTTGGGTCTGTAT</td>
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<tr>
<td>Occludin (human)</td>
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<tr>
<td></td>
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<tr>
<td>TNFα (mouse)</td>
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<tr>
<td></td>
<td>reverse: AGATAGCAATTCCGTGACG</td>
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<td>ZO-1 (mouse)</td>
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<tr>
<td></td>
<td>reverse: TGCTGACAGAGTCAAGTAC</td>
</tr>
<tr>
<td>ZO-1 (human)</td>
<td>forward: CAACATACAGTGACCTTCACA</td>
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<tr>
<td></td>
<td>reverse: CACTATGACGGTTCCTCCACTC</td>
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<tr>
<td>16s rRNA</td>
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<td></td>
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<tr>
<td></td>
<td>ACCGGGACAGGGGCAGGGCCTAGGCAGAGCAGGCAG</td>
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<td>Total bacteria</td>
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<td></td>
<td>reverse: ATACCCGGCTGCTGG</td>
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<tr>
<td>Akkermansia muciniphila</td>
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<tr>
<td></td>
<td>reverse: CAGCACCTGAAAGGTGGAC</td>
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<td>Bacteroidetes</td>
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<tr>
<td></td>
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<tr>
<td>Firmicutes</td>
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<td>Tenericutes</td>
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<td>α-Proteobacteria</td>
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<td></td>
<td>reverse: TCTACGARATTCACCYCTAC</td>
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<td>Fusobacteria</td>
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<tr>
<td></td>
<td>reverse: GCCGTGCTCAGTTCCCT</td>
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</table>
Supplemental Figures and Figure Legends
Supplemental Figure 1. The effects of *A. muciniphila* on the global pattern of gut microbiota and physiological parameters.

*Apoe*−/− 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 Apoe-/- mice fed the Western diet.

*Apoe-/-* 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 hours 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


