Akkermansia muciniphila improves metabolic profiles by reducing inflammation in chow diet-fed mice

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Abstract

Abnormal shifts in the composition of gut microbiota contribute to the pathogenesis of metabolic diseases, including obesity and type 2 diabetes (T2DM). The crosstalk between gut microbes and the host affects the inflammatory status and glucose tolerance of the individuals, but the underlying mechanisms have not been elucidated completely. In this study, we treated the lean chow diet-fed mice with *Akkermansia muciniphila*, which is thought to be inversely correlated with inflammation status and body weight in rodents and humans, and we found that *A. muciniphila* supplementation by daily gavage for five weeks significantly alleviated body weight gain and reduced fat mass. Glucose tolerance and insulin sensitivity were also improved by *A. muciniphila* supplementation compared with the vehicle. Furthermore, *A. muciniphila* supplementation reduced gene expression related to fatty acid synthesis and transport in liver and muscle; meanwhile, endoplasmic reticulum (ER) stress in liver and muscle was also alleviated by *A. muciniphila*. More importantly, *A. muciniphila* supplementation reduced chronic low-grade inflammation, as reflected by decreased plasma levels of lipopolysaccharide (LPS)-binding protein (LBP) and leptin, as well as inactivated LPS/LBP downstream signaling (e.g. decreased phospho-JNK and increased IKBA expression) in liver and muscle. Moreover, metabolomics profiling in plasma also revealed an increase in anti-inflammatory factors such as α -tocopherol, β -sitosterol and a decrease of representative amino acids. In summary, our study demonstrated that *A. muciniphila* supplementation relieved metabolic inflammation, providing underlying mechanisms for the interaction of *A. muciniphila* and host health, pointing to possibilities for metabolic benefits using specific probiotics supplementation in metabolic healthy individuals.

Key Words

- ▶ *Akkermansia muciniphila*
- ▶ glucose tolerance
- ▶ ER stress
- ▶ inflammation
- ▶ LBP

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Introduction

Metabolic diseases such as obesity and type 2 diabetes (T2DM) have increasing prevalence worldwide because of the sedentary lifestyles and easy access to energy-rich

food (Hossain *et al.* 2007). Abundant evidence shows that the development of metabolic diseases is affected by genetics and environmental factors (Ussar *et al.* 2015).

Gut microbiota, acting as an environmental factor, is linked to the development and progression of obesity and T2DM (Cani *et al.* 2012). A metagenome-wide association study of gut microbiota in patients with T2DM depicts intestinal dysbiosis and altered composition of gut microbiota (Qin *et al.* 2012). Chatelier and coworkers revealed that metabolic disorders are associated with a low gene richness in the microbiome (Le Chatelier *et al.* 2013). Many studies demonstrated that gut microbiota maintain energy homeostasis by affecting gut barrier, which is important for metabolic endotoxemia and metabolic disorders (Cani *et al.* 2009). These findings raise the possibility of targeting gut microbiota to prevent metabolic diseases and benefit human health.

Accumulating data uncovered the effects of probiotics or prebiotics on human health, providing a new strategy for treatment of metabolic diseases (Cano *et al.* 2013, Anhe *et al.* 2015). Meanwhile, several lines of evidence pointed to *A. muciniphila* as a potential probiotic. *A. muciniphila* is a mucin degrader residing in the mucus layer of the human intestinal tract (Derrien *et al.* 2008) and maintains the gut barrier (Reunanen *et al.* 2015). *A. muciniphila* is established during the first month of the whole life and develops within a year to a level close to that in healthy adults (Collado *et al.* 2007), accounting for 1–4% of the bacterial population in the colon of healthy subjects (Belzer & de Vos 2012). It is capable of using mucin as a carbon, energy and nitrogen source and is able to release sulfate in a free form from mucin fermentation (Derrien *et al.* 2004). Human and mouse studies repeatedly demonstrate that the abundance of *A. muciniphila* is reduced in subjects who are overweight or obese (Le Chatelier *et al.* 2013), as well as in patients with type 1 diabetes (Hansen *et al.* 2012), except one report showing that *A. muciniphila* is increased in type 2 diabetic subjects (Qin *et al.* 2012). Based on a recent research, anti-diabetic metformin treatment improved glucose homeostasis in association with increased *Akkermansia* spp. population in the gut microbiome of mice (Shin *et al.* 2014). Additionally, it is reported that the integrity of the developing healthy intestinal tract is correlated with an increasing abundance of *A. muciniphila* (Collado *et al.* 2007). A study of rodent models manifested that *A. muciniphila* supplementation contributes to the maintenance of the integrity of gut epithelium and helps to improve metabolic disorders in diet-induced obese mice (Everard *et al.* 2013). These findings together supported that *A. muciniphila* may have beneficial effects on human health especially in the disease models; however, whether it could also benefit

health in normal subjects is still unclear, as are the detailed mechanisms.

In this study, we treated chow diet-fed mice with *A. muciniphila* at 2×10^8 colony-forming units (CFUs)/200 μ L by daily gavage for five weeks to reveal the effects of *A. muciniphila* supplementation in normal mice regarding glucose metabolism and inflammation. Our results demonstrated improved adiposity, glucose tolerance and low-grade inflammation in chow diet-fed mice by *A. muciniphila*. Hence, our research provided new evidence for the metabolic benefits of *A. muciniphila* on chow diet-fed mice, pointing out the possible mechanisms underlying the interaction of *A. muciniphila* and the host's metabolism.

Materials and methods

Preparation of *A. muciniphila*

A. muciniphila (ATCC BAA-835) was cultured in sterilized brain heart infusion broth (BD Difco) at 37°C in an airtight pot with AnaeroPack (Thermo Fisher Scientific) for approximately 48 h to reach a late exponential growth phase under strict anaerobic conditions. Cultures were centrifuged at 11,500 *g* for 10 min, washed with sterile PBS for twice, and then the bacterial cells were re-suspended with sterile PBS to 10^8 colony-forming units (CFUs)/200 μ L. It was placed on ice immediately before administering to each mouse by gavage.

Experimental animal

Six-week-old male specific-pathogen-free (SPF)-grade C57BL/6 mice ($n=20$) purchased from Shanghai Laboratory Animal Center were maintained in groups of no more than 5 mice per cage with a stable controlled environment and free access to food and water. The mice were raised for 2 weeks without any intervention to adapt to the new environment and then divided into two groups randomly: (1) a normal chow diet (NCD) with sterile PBS (Gibco, Life Technologies) and (2) a NCD with *A. muciniphila* supplementation. The *A. muciniphila*-treated mice were administered daily with *A. muciniphila* (ATCC BAA-835) by oral gavage at a dose of 2×10^8 CFUs/200 μ L, whereas mice from the first group received sterile PBS as vehicle by daily gavage. The body weight of each mouse was measured once a week during the animal trial, which lasted for 5 weeks. The following experiments were based on these mice except the measurement of food intake and fecal triglyceride content. For measurement of these two

parameters, we conducted a new gavage experiment in which 40 mice were maintained in 20 cages with 2 mice in each cage and divided into two groups as mentioned previously. The measurements of food intake and energy excretion (fecal triglyceride content) were conducted and recorded with each cage as a unit ($n=10$ for each group). The measurement of food intake and the collection of stool were conducted in the second week of this trial. All these procedures were approved by the Animal Care Committee of Shanghai Jiao Tong University School of Medicine.

Intraperitoneal glucose tolerance test

After 14 h of fasting, the basal blood glucose level of each mouse was measured by the blood taken from the tail vein and recorded in mmol/L. After an intraperitoneal injection with glucose at a dose of 2 g/kg body weight, blood glucose concentrations were measured at 15, 30, 60, 90 and 120 min as described previously (Wang *et al.* 2013).

Body composition

The body composition was assessed by an animal whole body composition analyzer (EchoMRI 100H) in accordance with the manufacturer's instructions.

Blood and tissue sample collection

At the end of the trial, the mice were anesthetized with 10% chloral hydrate, and the blood from the orbital plexus was collected in tubes. Blood samples were centrifuged for 15 min at 1000g, 4°C, and plasma was isolated and stored at -80°C for subsequent biochemical testing, and plasma LBP (Abnova, KA4302) and leptin (Millipore, EZML-82K) levels were measured using a commercial ELISA kit. Inguinal fat, epididymal fat and the liver and intestine of each mouse were precisely dissected, and immediately stored at -80°C for further analysis.

Triglyceride measurement

Fecal triglyceride levels were extracted and measured following the manufacturer's instructions using a colorimetric triglyceride assay kit (Bio vision). Briefly, 0.1 g of feces was homogenized in 1 mL solution containing 5% NP-40 in water, it was slowly heated to 80–100°C in a water bath for 5 min, and then cooled down to room temperature. The procedure of heating was repeated

one more time to solubilize all triglycerides, and then the insoluble material was pelleted by centrifugation (16,200g for 2 min). An aliquot of the supernatant was collected for subsequent triglyceride level analysis. 50 µL test samples and triglyceride standard were added to a 96-well plate. 2 µL of lipase was added to each standard, and sample well, samples and lipase were mixed and incubated 20 min at room temperature to convert the triglyceride to glycerol and fatty acid. 50 µL of the reaction mix containing triglyceride probe and triglyceride enzyme were mixed and added to each well, incubating at room temperature for 60 min. The absorbance at 570 nm was measured, and the TG standard curve was plotted to calculate the concentration of triglyceride. For plasma triglyceride measurement, we used a triglyceride assay kit (Kehua Bio-Engineering, Shanghai, China) according to its instructions. In brief, mixed reagents were added to both the plasma sample and the standard sample, incubating in 37°C for 5 min, and then the absorbance at 550 nm was measured and the TG standard curve was plotted to calculate the concentration of triglyceride in plasma.

Oil Red O staining

Liver and muscle were dissected from each mouse ($n=9$ and 8 mice for each group, respectively) and fixed with 4% paraformaldehyde for more than 24 h, and then embedded in OCT. The tissue was sectioned, and the thickness was approximately 8–10 µm. Before Oil Red O staining, slides were stored at -20°C. Slides were dried for 10 min, washed three times with PBS and stained with Oil Red O solution for 15 min. Rinse the slides for 1 min with water, and the nuclei were counter-stained by immersion for 1 min in hematoxylin solution, and then washed by flow water. After covering the slide, three fields of each mouse were photographed and analyzed.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from tissues using TRIzol Reagent (Invitrogen), and 1 µg of RNA was transcribed following the manufacturer's instruction by using the Reverse Transcription System Kit (Promega), which produced high-quality complementary DNA. A real-time polymerase chain reaction was performed on LightCyclerH 480 (Roche) using SYBR Green II Master (Takara). All mRNA quantification data were normalized to the housekeeping 36B4 gene. All samples were run in duplicate in a single 384-well reaction plate, and data of the reaction were

collected and analyzed according to the $\Delta\Delta C_T$ method. Primers used in this study are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Protein preparation and western blotting

Proteins were extracted from dissected tissues and prepared using radioimmunoprecipitation assay buffer. All these samples were from different mice of the two groups and subjected to concentration determination and immunoblot assay with the indicated antibodies. Primary antibodies phospho-Ser⁴⁷³ AKT (#4060), AKT (#9272), phospho-Ser⁵¹EIF2A (#9721), phospho-PERK Thr980 (#3179), phospho-SAPK/JNK Thr183/Tyr185 (#9251), JNK (#9252) and IKBA (#4814) were obtained from Cell Signaling Technology (CST) to detect the targeted proteins. Heat Shock Protein90 (Hsp90, #4874) was purchased from Cell Signaling Technology (CST), and used as the internal control. The blotting bands were visualized using Image Quant LAS 4000 following the manufacturer's guide.

Statistical analysis

Significant differences between the two groups were assessed by unpaired two-tailed Student's *t*-test. All of these data were tested for the normality before using Student's *t* test. For non-normal distributions, non-parametric statistical analysis such as Mann–Whitney *U* test was used. Numerical data are expressed as mean $\pm$ S.D. In these figures, data with '*' are significantly different at $P < 0.05$, whereas data with '***' indicate a meaningful difference at $P < 0.01$. All the results were considered statistically significant when $P < 0.05$.

Metabolites extraction and sample derivatization

A sample of 20 μ L of thawed plasma sample and 80 μ L of cold methanol was added successively into an Eppendorf tube, successively. The mixture was vortex-mixed for 30 s, and placed at -20°C for 1 h prior to centrifugation at 16,000 *g* and 4°C for 15 min. In total, 70 μ L of supernatant were transferred into a glass vial containing 10 μ L of 0.05 mg/mL dulcitol and 0.02 mg/mL of L-phenylalanine-13C₉,15N as the internal standard. The mixture was dried under a gentle nitrogen stream. The glass vial with dry residue was added with 30 μ L of 20 mg/mL methoxylamine hydrochloride in anhydrous pyridine. The resultant mixture was vortex-mixed vigorously for 30 s

and incubated at 37°C for 90 min. A 30 μ L of BSTFA (with 1% TMCS) was added into the mixture and derivatized at 70°C for 60 min.

Gas chromatography–mass spectrometry analysis

Metabolomics instrumental analysis was performed on an Agilent 7890A gas chromatography system coupled to an Agilent 5975C inert MSD system (Agilent Technologies). A HP-5ms fused-silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m; Agilent J&W Scientific, Folsom, CA, USA) was used to separate the derivatives. Helium (>99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min through the column. Injection volume was 1 μ L by splitless mode, and the solvent delay time was 6 min. The initial oven temperature was held at 70°C for 2 min, ramped to 160°C at a rate of $6^\circ\text{C}/\text{min}$, to 240°C at a rate of $10^\circ\text{C}/\text{min}$, to 300°C at a rate of $20^\circ\text{C}/\text{min}$, and finally held at 300°C for 6 min. The temperatures of the injector, transfer line and electron impact ion source were set to 250°C , 290°C and 230°C , respectively. The electron energy was 70 eV, and data were collected in a full scan mode (*m/z* 50–600).

Data preprocessing and statistical analysis

The extraction, alignment, deconvolution and further processing of raw gas chromatography–mass spectrometry (GC–MS) data were referred to the previous published protocols ([Gao et al. 2010](#)). The mass window was set to 80–600 *m/z*. The final data were exported as a peak table file, including observations (sample name) and variables (rt_mz). The data were normalized against internal standard dulcitol before performing univariate and multivariate statistics.

The peak table (named matrix X) file was imported to Simca-P (version 11.0, Umetrics AB, Umeå, Sweden), where multivariate statistical analysis, such as PCA, PLS-DA and OPLS-DA were performed. All data were mean-centered, and unit variance (UV) scaled prior to multivariate statistical analysis. The quality of the models is described by the R²X or R²Y and Q² values. R²X (PCA) or R²Y (PLS-DA and OPLS-DA) is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit. Q² is defined as the proportion of variance in the data predictable by the model and indicates the predictability of the current model, calculated by cross-validation procedure. To avoid model overfitting, a default 7-round cross-validation

in Simca-P was performed throughout to determine the optimal number of principal components. The values of R2X, R2Y and Q2 were used as indicatives to assess the robustness of a pattern-recognition model.

Identification and structural validation of differential metabolites

The differential metabolites were determined by the combination of the variable importance in the projection (VIP) value (>1) of OPLS-DA model and the *P* values (<0.05) from two-tailed Student's *t* test on the normalized peak intensities. Fold change was calculated as the binary logarithm of average normalized peak intensity ratio between Group 1 and Group 2, where the positive value means that the average mass response of Group 1 is higher than that of Group 2. The structural identification of differential metabolites was performed as follows. The AMDIS software was applied to deconvolute mass spectra from raw GC-MS data, and the purified mass spectra were automatically matched with an in-house standard library including retention time and mass spectra, Golm Metabolome Database and Agilent Fiehn GC/MS Metabolomics RTL Library.

Results

Effects of *A. muciniphila* supplementation on body weight gain, fat mass, glucose tolerance and insulin signaling in metabolic tissues

In context of the comparable baseline body weight (Supplementary Fig. 1A), supplementation of *A. muciniphila* at a dose of 10⁸ CFUs/200 µL for five weeks alleviated body weight gain (Fig. 1A) and significantly reduced fat mass as well as increased lean mass in mice fed a normal chow diet (Fig. 1B) compared with the vehicle-administered control mice. Moreover, the visceral fat weight that was more linked with the pathogenesis of insulin resistance was decreased more obviously (Fig. 1C). Food intake and fecal triglyceride content were unaltered by *A. muciniphila* supplementation (Supplementary Fig. 1B and C). Plasma triglyceride levels were unaltered (Fig. 1D). Notably, although the fasting glucose level was not changed by *A. muciniphila* (Fig. 1E), which was in agreement with a previous study (Everard *et al.* 2013), we found that glucose tolerance was greatly improved by *A. muciniphila* supplementation in mice fed chow diet, as reflected by intraperitoneal glucose tolerance test (IPGTT) (Fig. 1F) and the corresponding reduced area

under curve (AUC) (Fig. 1G). Although fasting plasma insulin levels were comparable between the two groups (Fig. 1H), we detected a significant increase in phospho-AKT Ser473 levels in liver (Fig. 1I) and muscle (Fig. 1J) in *A. muciniphila* group, suggesting an improved insulin sensitivity in liver and muscle. As insulin inhibits the transcriptional expression level of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) (Lochhead *et al.* 2000), increased insulin sensitivity in the liver consequently resulted in decreased hepatic G6P and PEPCK expression (Fig. 1K). These results together demonstrated that *A. muciniphila* supplementation improved energy homeostasis and glucose tolerance in chow diet-fed mice.

A. muciniphila supplementation improves lipid accumulation in liver and muscle

Given the improved fat mass and glucose tolerance, we next examined lipid metabolism in insulin-responsive tissues. Interestingly, *A. muciniphila* supplementation significantly decreased the gene expression of sterol regulatory element-binding proteins (SREBP1c), fatty acid translocase (CD36) involved in lipogenesis and the transport of fatty acids both in liver (Fig. 2A) and in muscle (Fig. 2B). SREBP1c encodes a transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a decamer flanking the low-density lipoprotein receptor gene and some genes involved in sterol biosynthesis (Capel *et al.* 2013, Kim *et al.* 2015). CD36 contributes to fatty acids translocation (Wilson *et al.* 2016). Meanwhile, the expression of acetyl-CoA carboxylase (ACC) was significantly downregulated by *A. muciniphila* supplementation in muscle (Fig. 2B). The most important function of ACC is to provide the malonyl-CoA substrate for the biosynthesis of fatty acids (Tong 2005). However, we did not observe similar changes in adipose tissue (Fig. 2C). Gene expression of C/EBPA and PPARG involved in adipocyte differentiation as well as in body weight homeostasis was either unchanged in adipose tissues (Supplementary Fig. 2) (Lefterova *et al.* 2008). Oil Red O staining was further used for fat deposition measurement showing reduced fat accumulation in *A. muciniphila* supplemented group than the control group both in liver and in muscle (Fig. 2D and E). The results showed that *A. muciniphila* supplementation strikingly attenuated fat deposition in hepatic tissue and muscle, suggesting that *A. muciniphila* supplementation may reduce lipid accumulation in liver and muscle thereby improving insulin sensitivity.

Figure 1

A. muciniphila supplementation alleviated body weight gain, reduced fat mass, improved glucose homeostasis. (A) Average body weight gain (%) of *A. muciniphila*-supplemented mice and their counterparts after five-week treatment ($n=9-8$ for each group, data were shown as median $\pm$ interquartile range, Mann–Whitney test, $*P<0.05$, $**P<0.01$). (B) Total fat mass and lean mass measured using Echo MRI ($n=10-8$ for each group, data shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (C) Weight of IWAT, EWAT and LIVER normalized to body weight in *A. muciniphila*-supplemented mice and their counterparts ($n=10-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (D) Plasma TG level between *A. muciniphila*-treated mice and their counterparts ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (E) Fasting blood glucose between two groups ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (F) Glucose tolerance test between two groups ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (G) Mean area under the curve (AUC) measured between 0 and 120 min after glucose injection. (H) Plasma insulin level between *A. muciniphila*-treated mice and their counterparts ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (I and J) Protein levels of insulin signaling in liver (I) and muscle (J) of PBS-treated vehicle and *A. muciniphila*-supplemented mice. The levels of phospho-Akt were normalized to the levels of total Akt. HSP90 was used as the loading control. Quantitation of p-AKT was performed, all these samples were from 3 different mice from each of the two groups. (K) Relative mRNA levels of G6PC and PEPC in liver ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$).

Figure 2

A. muciniphila supplementation reduced fat deposition in liver and muscle. (A) Relative mRNA levels of SREBP1C, ACC and CD36 in liver between *A. muciniphila*-supplemented mice and their PBS-treated counterparts. (B) Relative mRNA levels of SREBP1C, ACC and CD36 in muscle, as indicated in B. (C) Relative mRNA levels of SREBP1C, ACC and CD36 in epididymal adipose tissue ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed *t*-test, * $P<0.05$, ** $P<0.01$). (D and E) Representative Oil Red O staining for fat deposition measurement in liver (D) and muscle (E) between *A. muciniphila*-supplemented mice and the control group.

A. muciniphila supplementation attenuates ER stress in metabolic tissues

Recent evidence recapitulates the interaction among fat deposition, inappropriate activation of ER stress and insulin resistance (Goodpaster *et al.* 2001, Ozcan *et al.* 2004). Therefore, we hypothesized that the improvement of glucose tolerance by *A. muciniphila* supplementation may be partly dependent on the reduced lipotoxicity and ER stress. We further measured the mRNA expression of markers related to ER stress, and in line with the changes in lipid accumulation in liver and muscle, we detected the downregulation of unfolded protein response signals representing ER stress such as immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 (BiP/GRP78) and PKR-like ER kinase (PERK) in the liver (Fig. 3A) in the *A. muciniphila* group. These proteins could facilitate diverse functions in the endoplasmic reticulum such as folding and assembly of newly synthesized proteins and initiation of unfolded protein response (Shi *et al.* 1998). The reduced expression levels of BiP, PERK protein disulfide isomerase (PDI) and XBP1 were also observed in the muscle of mice treated with *A. muciniphila* (Fig. 3B). However, similar changes were not detected in the adipose tissues (Fig. 3C). Protein levels of ER stress indicators such

as phosphorylated EIF2A and phosphorylated PERK were assessed in both hepatic and muscle tissues. Consistent with the result of gene expression, levels of p-EIF2A and p-PERK were lower in liver and muscle of *A. muciniphila*-treated mice compared with those of controls (Fig. 3D and E). These results supported our hypothesis that the improvement of glucose tolerance by *A. muciniphila* supplementation is associated with the alleviation of ectopic intracellular lipid accumulation and ER stress.

A. muciniphila supplementation improves chronic low-grade inflammation

The interactions between inflammation and insulin resistance are widely accepted nowadays (Shoelson *et al.* 2006). Moreover, it is reported that *A. muciniphila* administration could restore gut barrier function, decrease intestinal permeability and may prevent the development of high-fat diet-induced metabolic endotoxemia, as revealed by decreased circulating LPS levels (Everard *et al.* 2013). Here, we detected a significantly reduced level of plasma LBP (Fig. 4A), which is the indicator of circulating LPS (Romani *et al.* 2013), in *A. muciniphila*-treated chow diet-fed mice. Meanwhile, inflammation factors such as leptin were also decreased by *A. muciniphila* (Fig. 4B).

Figure 3

A. muciniphila supplementation influences the expression of ER stress-related genes in liver and muscle. (A) Real-time PCR results of CHOP, BIP, PDI, PERK and XBP1 in liver. (B) Real-time PCR results of CHOP, BIP, PDI, PERK and XBP1 in muscle between *A. muciniphila*-administered group and PBS-treated vehicle. (C) Real-time PCR results of CHOP, BIP, PDI, PERK and XBP1 in epididymal adipose tissue ($n=9-8$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t-test, $*P<0.05$, $**P<0.01$). (D and E) The levels of p-EIF2A and p-PERK involved in the unfolded protein response were detected both in hepatic tissue (D) and in muscle (E) of *A. muciniphila*-supplemented mice and their counterparts, as well as the quantitation of representative proteins. All these samples were from 3 different mice from each of the two groups.

It was evidenced that the attenuated ER stress of the intestine is associated with inflammation and the gut barrier function (Ma 2008). We also detected a decrease in the mRNA expression levels of ER stress-related genes, CHOP and TRIB3 in colon (Supplementary Fig. 3A) and a decrease in PDI mRNA levels in jejunum (Supplementary Fig. 3B). It is because CHOP, TRIB3 and PDI are ER stress transducers, leading to the activation of unfolded protein response (Cnop *et al.* 2012). These results suggested that *A. muciniphila* supplementation alleviated ER stress in the intestine and chronic endotoxemia.

LPS, known as an endotoxin, could enter the circulation through the impaired intestinal mucosa, and then bind to LBP, which can facilitate the interaction of LPS and its receptors, thereby triggering inflammatory cascades including nuclear factor-KB (NF-KB) and JNK activation (Zweigner *et al.* 2006, Tsaousidou *et al.* 2014). Correspondingly, we found that *A. muciniphila* supplementation significantly reduced phospho-JNK levels and increased the NF-KB inhibitor protein and IKBA protein levels in the liver (Fig. 4C), suggesting an inactivation of these two pathways in the *A. muciniphila* group. Similar changes were observed in muscle (Fig. 4D). These results demonstrated that *A. muciniphila*

supplementation improved metabolic endotoxemia and the following local inflammation cascades, which might mediate the beneficial metabolic effects.

Impact of *A. muciniphila* supplementation on the metabolomics profile of plasma

Taken together, we demonstrated the beneficial effects of *A. muciniphila* administration on host metabolic profiles, metabolic endotoxemia and inflammation. In a recent study, it is well established that gut microbiota could ferment the non-digestible carbohydrates in the colon and produce metabolites that have profound effects on health (De Vadder *et al.* 2014). Therefore, we further investigated the metabolomics profile in the plasma of both the *A. muciniphila*-treated group and their counterparts to clarify whether *A. muciniphila* treatment resulted in a more favorable metabolomics profile (Table 1). Interestingly, among these metabolites, the levels of citrulline and ornithine were significantly lowered by *A. muciniphila* supplementation. Previous studies observed that these two amino acids were higher in obese and diabetic mice and also in humans with obesity (Mochida *et al.* 2011, Verdam *et al.* 2011, Sailer *et al.* 2013). These findings may indicate the possible involvement of these amino

Figure 4

A. muciniphila supplementation is beneficial to improve the low-grade inflammation. (A) Plasma LBP levels ($n=9-6$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (B) Plasma leptin levels ($n=8-6$ for each group, data were shown as mean $\pm$ s.d., unpaired two-tailed t -test, $*P<0.05$, $**P<0.01$). (C and D) Immunoblotting analysis with antibodies against phospho-JNK, total JNK, IKBA and HSP90 of liver (C) and muscle (D), the levels of phospho-Jnk were normalized to the levels of total Jnk, and other protein levels were normalized to Hsp90. Relative gray level value of these proteins were illustrated in figure (C) and (D).

acids in metabolism. Besides, we detected an increase in A-tocopherol concentrations, which acted as an essential antioxidant and anti-inflammatory factor (Gren 2013), as well as an increased levels of B-sitosterol that boosts the immune system and exhibits an anti-inflammatory activity in endothelial cells including intestinal endothelial cells (Loizou *et al.* 2010). These results further support the anti-inflammatory benefits by *A. muciniphila* supplementation.

Discussion

Growing evidence supports the crosstalk between gut microbiota and host health, and alterations in the composition of gut microbiota are involved in the development of metabolic disorders such as obesity and T2DM (Hartstra *et al.* 2015). Thus, elucidating the effects of candidate bacterial species on host metabolism will provide new methods for preventing and treating metabolic diseases. In this study, we examined the

metabolic benefits of *A. muciniphila* in mice fed chow diet. Interestingly, we found that *A. muciniphila* daily gavage for five weeks significantly improved glucose tolerance and insulin sensitivity in liver and muscle. Correspondingly, fat deposition and ER stress were alleviated in liver and muscle by *A. muciniphila*. More importantly, *A. muciniphila* supplementation reduced metabolic endotoxemia and inflammation signaling in liver and muscle. These results firstly identified the metabolic benefits of *A. muciniphila* in metabolic healthy mice, providing the possibilities to prevent the metabolic disturbances using probiotics in the general population.

The mucin-degrading bacterium *A. muciniphila* is established during the first month of human life and resides in the mucosa of healthy adults (Collado *et al.* 2007). Abundant evidence has demonstrated that the abundance of *A. muciniphila* is reduced in obese patients and mice (Everard *et al.* 2013, Le Chatelier *et al.* 2013),

Table 1 Significantly different plasma metabolites between *Akkermansia muciniphila*-intervened mice and the control.

No.	Metabolites ^a	RT (min)	m/z	VIP value ^b (OPLS-DA)	P value ^c (Student's <i>t</i> -test)	Fold-change ^d (log ₂)
1	AMP (adenosine monophosphate)	30	315	1.9	0	-1
2	Hydrocaffeic acid	23	398	1.7	0	-0.6
3	5-aminovaleric acid	19	174	1.7	0	-0.6
4	2-ketoglutaric acid	18	198	1.6	0	-0.6
5	Pipecolinic acid	14	156	2	0	-0.3
6	Ornithine	19	142	1.6	0	-0.2
7	Citrulline	22	157	1.9	0	-0.2
8	Pyrophosphate	19	451	1.7	0	-0.1
9	Acetoacetate	8.8	188	1.6	0	0.1
10	Malonic acid	10	233	1.6	0	0.4
11	Fucose	20	117	1.8	0	0.2
12	4-hydroxyproline	17	230	1.5	0	0.3
13	Uric acid	25	441	2.1	0	0.4
14	3,4-dihydroxybutyric acid	15	233	2.2	0	0.4
15	α-tocopherol	31	502	2	0	0.9
16	β-sitosterol	33	129	1.8	0	0.2
17	Cholesterol	31	329	1.8	0	0.3

^aThe metabolites were structurally identified by reference standards. ^bVariable importance in the projection (VIP) was obtained from the OPLS-DA model.

^cThe *P* value was calculated from Student's *t*-test. ^dFold-change was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between *A. muciniphila* group vs PBS group, where a positive value means that the average mass response of the metabolite in the *A. muciniphila* group is larger than that in the PBS group.

whereas its abundance is increased in obese patients after weight loss treatment (Liou *et al.* 2013) or in diabetic mice after lowering glucose by metformin (Shin *et al.* 2014). There are also studies revealing the anti-diabetic effects of *A. muciniphila* in high-fat diet (HFD)-induced diabetic mice (Everard *et al.* 2013, Shin *et al.* 2014). We firstly observed that *A. muciniphila* supplementation significantly improved body weight gain, fat mass and glucose tolerance in chow diet-fed mice. The reduction in fat mass accompanied an increase in lean mass indicating a body composition change by *A. muciniphila*. Severely reduced fat mass (such as lipodystrophy) is related to insulin resistance (Shay *et al.* 2011). In our study, *A. muciniphila* supplementation improved glucose tolerance and insulin sensitivity. These results overall suggested that the decreased fat mass and increased lean mass by *A. muciniphila* may also metabolically benefit the relatively metabolic healthy subjects. We noticed that a previous study reported by Everard and coworkers did not find similar changes in chow diet-fed mice (Everard *et al.* 2013). The discrepancy might be due to the differences in the length of *A. muciniphila* treatment or the diet content. Both studies used C57BL/6 mice. However, we treated mice with *A. muciniphila* for five weeks in a chow diet containing 9.7% water, 20.5% protein, 4.62% fat, 4.35% fiber and 52.5% nitrogen-free extract, whereas Everard and coworkers treated mice for four weeks in a control diet (AIN93Mi, Research Diet) containing 6.8% water,

12.5% protein, 4% fat and 72.7% carbohydrate, suggesting that *A. muciniphila*'s effect may be diet dependent. It is previously reported that *A. muciniphila* affected intestine length and energy absorption during cold (Chevalier *et al.* 2015). We found that *A. muciniphila* did not affect the intake or energy excretion from the gut at least in ambient temperature. The detailed mechanism of *A. muciniphila* in energy homeostasis remains to be clarified in future study.

Everard and coworkers have demonstrated that *A. muciniphila* treatment alleviated fat storage in adipose tissue in HFD-induced obese mice (Everard *et al.* 2013). In our study, we did not observe the changed expression of genes related to lipid synthesis or transport in fat tissues in the chow diet-fed *A. muciniphila* group compared with the control group, suggesting that *A. muciniphila*'s effects on fat storage may be affected by diet status. However, interestingly, we found a significant reduction in the expression levels of genes involved in fatty acid synthesis and transport in non-fat insulin-responsive tissues such as liver and muscle in *A. muciniphila*-treated mice. The immunoblotting analysis with antibodies against phospho-JNK, total JNK, IKBA and HSP90 of liver and muscle and relative gray level value of these proteins were also illustrated, indicating a reduced lipid accumulation by *A. muciniphila*, which may be one reason for the elevated insulin signaling activation in liver and muscle in *A. muciniphila* group, as it is well established that lipid accumulation in liver and muscle triggers pathways

that impair insulin signaling thereby insulin resistance (Samuel & Shulman 2016).

We found that *A. muciniphila* daily gavage for 5 weeks significantly improved glucose tolerance and insulin sensitivity in liver and muscle. Key gluconeogenic enzymes, PEPCK and G6PC (Lochhead *et al.* 2000), were decreased after *A. muciniphila* supplementation. However, we did not see a reduction in fasting blood glucose, consistent to previous study (Everard *et al.* 2013) indicating that other regulators such as liver glycogenolysis, gluconeogenesis in the kidneys or intestine (Mutel *et al.* 2011, Al-Gayyar *et al.* 2015) may also be involved in fasting glucose regulation by *A. muciniphila*.

ER plays a central role in the embellishment of lipid and protein. The protein synthesized in ER would fail to acquire correct decoration, and misfolded proteins would accumulate in ER under pathological conditions, causing ER stress and activating unfolded protein response signal (Ozcan *et al.* 2004). Murine studies have revealed that ER stress is one of the possible mechanisms responsible for triggering innate immune inflammatory pathways and insulin resistance (Hotamisligil 2010). Besides, ER membranes also contain key lipogenic enzymes affecting the biosynthesis of lipid, which further impair insulin signaling (Lee *et al.* 2008). Our work showed that ER stress in liver and muscle was significantly inhibited by

A. muciniphila treatment, in accordance with the reduced lipid accumulation and activated insulin signaling in these two organs.

We have demonstrated the beneficial effects of *A. muciniphila* supplementation on host metabolism. The next question is how these effects were achieved by oral administration of *A. muciniphila*. As *A. muciniphila* is well demonstrated to maintain the intestinal barrier (Everard *et al.* 2013), which is important to prevent the pathogenic microbial products from entering into circulation, whereas LPS, a known endotoxin, is a crucial component of gram-negative bacteria cell walls, which could enter the circulation through an impaired gut barrier. Recent studies demonstrate that a high-fat-containing diet alters the diversity of gut microbiota and intestinal wall permeability, elevating enterobacterial production and translocation of LPS into the systemic circulation, as referred to metabolic endotoxemia (Cani *et al.* 2007). In circulation, LBP takes up and binds to LPS with high affinity, facilitates LPS activation and transfers it to the TLR4 receptor complex, initiating downstream inflammation signaling that further influences the insulin sensitivity and glucose disposal (Wright *et al.* 1990). We detected that plasma LBP levels were significantly reduced by *A. muciniphila* treatment. As LBP is an indicator of circulating LPS, our result suggested that the reduced metabolic endotoxemia in

Figure 5

Graphical abstract. Functional interaction between *A. muciniphila* and host metabolism. *A. muciniphila* residing in the mucus layer of intestine and maintaining gut barrier inhibits bacteria-derived LPS penetrating into circulation (metabolic endotoxemia), thereby alleviating LPS/LBP/TLR4-initiated inflammation cascade and ER stress in liver and muscle cells, and the inhibited ER stress further inactivated inflammatory pathways and fat deposition, which altogether led to activated insulin signaling in liver and muscle and improved glucose tolerance. ER, endoplasmic reticulum; LBP, LPS-binding protein; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.

A. muciniphila-treated chow diet-fed mice. This effect mainly depends on the gut barrier maintenance ability of *A. muciniphila* (Everard *et al.* 2013). Furthermore, the LPS/LBP downstream signaling such as JNK and NF- κ B (Wright *et al.* 1990) was also inhibited by *A. muciniphila* treatment. These results demonstrated the improved endotoxemia and chronic inflammation by *A. muciniphila* supplementation. It is well testified that JNK can bind to IRS1 and lead to Ser307 phosphorylation, resulting in a decrease of phosphorylated AKT and inactivation of insulin signaling (Hirosumi *et al.* 2002); and activated IKK-NF- κ B pathway also plays a central role in the development of insulin resistance and metabolic abnormalities (Hirosumi *et al.* 2002). There is also evidence demonstrating that additional LPS could induce ER stress in insulin-responsive cells (Wolfe *et al.* 2013). Moreover, circulating LBP levels are significantly associated with insulin resistance and various inflammatory markers (Moreno-Navarrete *et al.* 2012). These findings suggested that *A. muciniphila* supplementation initially alleviated metabolic endotoxemia from the gut, thereby inhibiting inflammation cascade and ER stress, and the inhibited ER stress further inactivated inflammatory pathways (Ozcan *et al.* 2004), which altogether led to activated insulin signaling in liver and muscle and improved global glucose tolerance (Fig. 5).

To further examine the interactions between *A. muciniphila* consumption and metabolic profiles, plasma metabolomics analysis was performed as it has been used to predict the progress of T2DM in advance of onset in human beings (Wang *et al.* 2011). Our results showed *A. muciniphila* treatment decreased the circulating levels of citrulline and ornithine as well as increased the circulating levels of α -tocopherol and β -sitosterol. Amino acids are emerging as a new class of effective molecules in the etiology of obesity and diabetes mellitus (Schwenk & Haymond 1987, Newgard *et al.* 2009). Previous studies observed that these two amino acids were higher in obese and diabetic mice and also in humans with obesity (Mochida *et al.* 2011, Verdam *et al.* 2011, Sailer *et al.* 2013), which may predict the development of metabolic syndromes. Moreover, *A. muciniphila* supplementation increased circulating levels of α -tocopherol and β -sitosterol which are reported to boost the immune system and reduce systemic low-grade inflammation (Bendich 1988, Fraile *et al.* 2012). Ample evidence has demonstrated that α -tocopherol can attenuate LPS-induced inflammatory cytokines secretion (Zhang *et al.* 2010, Lira *et al.* 2011). β -sitosterol also has beneficial effects directly on endothelial and monocytic cells (Bustos *et al.* 2008), alleviating HFD-induced intestinal inflammation by

inhibiting the binding of LPS to toll-like receptor 4 in the NF- κ B pathway (Kim *et al.* 2014). These results further supported the anti-inflammation effects and protective effects on the gut barrier function of *A. muciniphila*.

In summary, for the first time, we demonstrated that *A. muciniphila* supplementation in mice fed a chow diet decreased metabolic endotoxemia, resulting in a more favorable metabolomics profiles in circulation, as well as the inhibited pro-inflammatory pathways, ER stress and lipogenesis in insulin-responsive tissues, leading to improved insulin action and glucose tolerance. Our study illustrated the crosstalk between *A. muciniphila* and host metabolism, providing the possible mechanisms of *A. muciniphila*'s metabolic benefits such as glucose tolerance improvement. These results added new evidence for the supplementation of probiotics to metabolic healthy population and also suggested a new strategy to prevent the development of metabolic diseases such as T2DM at an even earlier stage.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-16-0054>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R L and J H conceived the project and designed the experiments. S Z and W L carried out most of the experiments. J W, R L and S Z wrote the paper. Y S contributed with the mice breeding and animal experiments. J S assisted with statistical analysis. W W and G N contributed comments and advice on the manuscript. All authors were involved in editing the manuscript.

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