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Mechanism of Triglyceride Lowering Action of *Akkermansia muciniphila* and Fenugreek in a Genetic Induced Hyperlipidemia

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**Mechanism of Triglyceride Lowering Action of *Akkermansia
muciniphila* and Fenugreek in a Genetic Induced
Hyperlipidemia**

by

Jing Shen

A THESIS

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Mechanism of Triglyceride Lowering Action of *Akkermansia muciniphila* and Fenugreek
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University of Nebraska, 2016

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Hyperlipidemia is a condition of abnormally elevated levels of lipids and/or lipoproteins in the blood circulation. It is usually accompanied with obesity, type 2 diabetes, insulin resistance, hypertension and non-alcoholic fatty liver diseases (NAFLD) and has become a great threat to human health. New therapeutic strategies are needed for the prevention and treatment of both genetic intervention-induced hyperlipidemia and environmentally induced hyperlipidemia. In this study, we determined the therapeutic effects of a disease-protecting gut bacteria, *Akkermansia muciniphila*, and an annual legume, fenugreek, on hypertriglyceridemia induced by genetic depletion of cAMP responsive binding protein H (CREBH KO) in mice. We found that inoculation of *A. muciniphila* ameliorates both acute and chronic hyperlipidemia in mice. Increased colonization of *A. muciniphila* in CREBH KO mice enhanced the expression of hepatic LDL receptor and facilitated the clearance of triglyceride (TG) rich lipoproteins. Moreover, *A. muciniphila* administration in mice also alleviated hepatic endoplasmic reticulum (ER) stress and metabolic inflammation. Feeding CREBH KO mice with a diet containing fenugreek seed (2%) also attenuated the hypertriglyceridemia caused by depletion of CREBH or induced by a high fat diet (HFD). Fenugreek seed inhibited hepatic apoB100 biosynthesis and suppressed very low density lipoprotein (VLDL) assembly and secretion. It further improved insulin resistance induced by HFD.

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List of Abbreviations

FFA	free fatty acid
FC	free cholesterol
apoB	apolipoprotein B
apoE	apolipoprotein E
VLDL	very low density lipoprotein
CMs	chylomicrons
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LDLR	LDL-receptor
HDL	high density lipoprotein
LPL	lipoprotein lipase
apoA1	apolipoprotein A1
LCAT	Lecithin- cholesterol acyltransferase
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
NAFLD	non-alcoholic fatty liver diseases
CREBH	cyclic AMP-responsive element-binding protein H
PEPCK	phosphoenolpyruvate carboxykinase
NC	negative control
PBS	phosphate buffer saline
BSA	bovine serum albumin
Tris	tris (hydroxymethyl) aminomethane

SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
PVDF	polyvinylidene fluoride
TBST	1x Tris-buffered saline with tween
HRP	horseradish peroxidase
TEMED	N, N, N', N',-tetramethylethylenediamine
TG	triglycerides
CHOL	cholesterol
SCFA	short chain fatty acids
IBD	Inflammatory Bowel Disease
HCL	hydrochloric acid
NaCl	sodium chloride
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
WT	wild type
KO	knockout

Chapter one

General Introduction

1.1 Obesity

Obesity represents abnormal accumulation of body fat. According to NIH (2009-2010), the prevalence of overweight, which means the body mass index [BMI] is 25 to 29.9, and obesity, the BMI is more than 30, was approximately 68.8% in the United States among adults aged 20 or older. Obesity is not strictly a U.S. phenomenon. It has become a worldwide epidemic [1, 2]. Obesity is one of the major risk factors of metabolic diseases such as hypertension, dyslipidemia and insulin resistance and so on[3].

Obesity is induced by calorie accumulation, which means that more calories are absorbed than consumed. The excess calories are usually stored as adipose tissue which can be subcutaneous or abdominal. Genetic and environmental factors such as excess intake of energy-rich foods or even air pollution can all increase the risk of obesity [4, 5]. Studies have revealed that obesity and metabolic syndrome are associated with the changes of bacterial divisions [6].

1.2 Lipids and Lipoprotein metabolism

A major proportion of lipids in human and animals are triglycerides (TGs), cholesterol (CHOL) and phospholipids. Besides these, lipids in the body contain sterols, fat-soluble vitamins (A, D, E and K), mono- and diglycerides, and so on. Lipids are transported within lipoproteins in circulation, which is the only way that these fatty substances could

be dissolved in the blood. Lipoproteins are risk factors for cardiovascular and metabolic disease.

Structurally, lipoproteins consist of two parts: a core of fats containing TGs, cholesterol esters (CHOL linked to fatty acids), and fat-soluble vitamins; and a monolayer membrane consisted of phospholipids and small amounts of free cholesterol. Apolipoproteins penetrate into or through the monolayer membrane acting as cofactors or ligands for the process of lipid transport and metabolism.

According to the density, lipoproteins are divided into five classes: chylomicrons (CMs), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Each lipoprotein contains specific contents of protein, TG, CHOL and cholesterol ester (details showed in table 1) [7, 8].

Table.1. Major contents of human lipoproteins

	chylomicron	VLDL	IDL	LDL	HDL
Protein	1%	10%	10%	20%	50%
Triglyceride	88%	56%	29%	13%	13%
Cholesterol	1%	8%	9%	10%	6%
Cholesterol ester	3%	15%	39%	48%	30%

Adapted from Biochemistry 2nd Ed. 1995 Garrett & Grisham

CMs are the largest lipoproteins which carry fat from the small intestine to the liver, skeletal muscle and adipose tissue. In the enterocytes, TGs, CHOL, fat-soluble nutrients and vitamins are packaged into CMs. The main apolipoprotein contained in CMs is apoB-48, which is 48 percent as long as apoB-100. In the blood circulation, TGs in CMs are hydrolyzed by lipoprotein lipase (LPL) and CMs are converted into CM remnants. The activation of LPL needs a cofactor, apoC-II in CMs. The CM remnants carry CHOL and the remaining TG to the liver. The clearance of CM remnants by liver is mediated by the receptors, one of which is LDL receptor, on the membrane of hepatocytes [9]. The apolipoprotein E (apoE) on the CM remnants, which is exchanged from HDL, is the ligand of LDL receptor and facilitates the uptake of CM remnants by the liver.

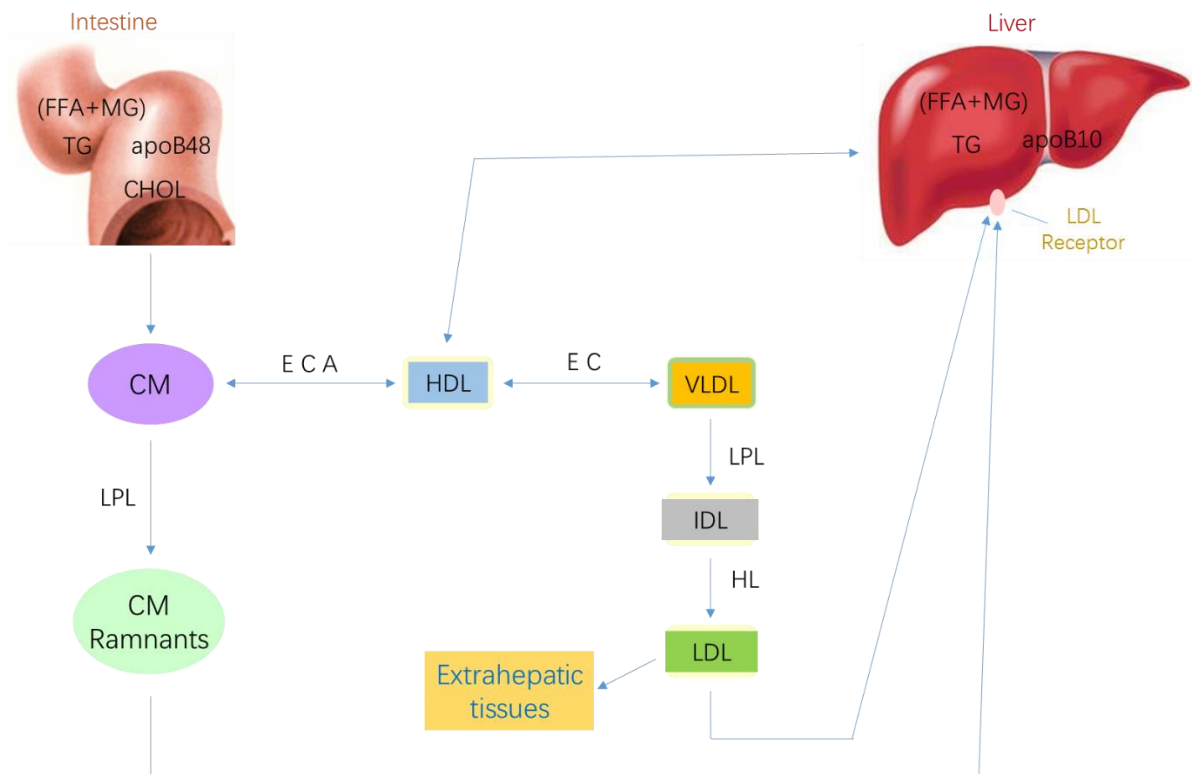
VLDLs are smaller than CMs. It is assembled and secreted by the liver to transport endogenously synthesized TGs to peripheral tissues. The biogenesis and secretion of VLDL by the liver plays an essential role in lipid homeostasis. During the circulation, LPL removes TGs in VLDLs with the assistance of apoC-II. By the removal of TGs and the uptake of CHOL, VLDL is converted to IDL. IDL is further converted to LDL via the hydrolysis of TGs by hepatic lipase.

LDL is smaller than VLDL and IDL and is rich in CHOL. It delivers CHOL to the peripheral tissues by endocytosis. This process is facilitated by the LDL receptors on the surface of cells. When CHOL is required by cells, the cells express LDL receptor for the intake of LDLs. The hepatocytes also express LDL receptors. A proportion of LDLs enter

the liver to be catabolized. The recognition of LDL with LDL receptors is mediated by apoB100, the essential apolipoprotein in LDL.

HDL is the smallest lipoprotein. It is referred to as “good” lipoprotein because it carries CHOL from the peripheral tissues back to the liver. HDL is originated from the liver.

Apolipoprotein A1 is the major apolipoprotein in HDL and plays an important role in the function of HDL. Lecithin-cholesterol acyltransferase (LCAT) in the outer layer of HDL esterifies CHOL to form cholesterol esters (CE). HDLs provides apoE, C and CE to CMs and VLDLs and transports the remaining CHOL to the liver.



Adapted from Durrington, P. et al. 2014, Metabolism of Human Diseases: Organ Physiology and Pathophysiology.

ApoB can be divided into two forms according to its molecular size, apoB100 and apoB48. In human, ApoB100 is produced by the liver and is the essential structure protein of VLDL and LDL, while apoB48 is synthesized in the small intestine and is the main protein in CMs. In mice, however, the expression level of apoB48 is much higher than that of apoB100 in the liver. ApoB cannot be exchanged from VLDL to other lipoproteins, making it an ideal marker of VLDL [10]. Both apoB100 and apoE are capable of binding with LDL receptor and thus mediate the clearance of LDL and CM remnants respectively. It has been reported that increased level of lipids in the liver can lead to apoB accumulation and ER stress [11-13].

1.3 Hyperlipidemia

Hyperlipidemia represents an abnormal increase of lipids and/or lipoproteins in blood [2]. There are many conditions that could be covered in hyperlipidemia. But the most common form is high levels of LDL [14]. It has been widely accepted that hyperlipidemia is one of the main comorbidities of obesity, since obese people are more likely to develop hyperlipidemia [15]. The excess lipids in the arteries, the main symptom of hyperlipidemia, can lead to the development of atherosclerosis and coronary heart disease [16]. Hyperlipidemia usually complicates with obesity, type 2 diabetes, insulin resistance, hypertension and non-alcoholic fatty liver diseases (NAFLD) [17, 18]. The prevalence in the United States is more than thirty percent of the adult population [10]. Hyperlipidemia has become a great threat to the public health [15, 19, 20]. Treatment of

hyperlipidemia includes dietary and drug treatment. Weight loss is beneficial for hyperlipidemic patients with obesity. In the diets of patients, saturated fat and CHOL should be avoided in order to prevent the increase of LDL CHOL. Even in some cases, all fat should be restricted to prevent the formation of CMs. Drug treatment generally focused on reducing LDL CHOL [21]. There are some drugs that could be used in treating hyperlipidemia, such as statins, ezetimibe and so on. However, side effects and low efficiency remains. Therefore, it is required to find more therapeutic options, especially for severe and genetic hyperlipidemia. In this study, we determined the therapeutic effects of *Akkermansia muciniphila*, a disease protecting gut bacteria and fenugreek, an annual legume, on hypertriglyceridemia induced by depletion of Cyclic AMP-responsive element-binding protein H (CREBH) in mice.

1.4 CREBH

CREBH (encoded by the gene CREB3L3) belongs to the CREB/ATF family, and was identified as a hepatocyte-specific endoplasmic reticulum (ER) –bound bZIP transcription factor [22]. CREBH is expressed only in the liver and the small intestine [23, 24]. The expression of CREBH is dependent on hepatocyte nuclear factor 4 α [25, 26]. It is activated from precursor protein by cleavage of protease S1P and S2P in Golgi [27]. Mature N-terminal portion was liberated and localized into the nucleus as transcriptional regulator [27, 28]. In the nucleus, CREBH activates the transcription of genes which is driven by CRE-containing promoter [29].

CREBH expression can be induced by fatty acids (FAs) and fasting [30, 31]. And it could be activated by ER stress. The promoter of CREBH gene contains a peroxisome proliferator responsive element (PPRE) for PPAR α transactivation [32]. PPAR α regulates the genes related with FA oxidation in peroxisomes and mitochondria and thus plays a crucial role in fasting response [33]. During fasting or exposure to synthetic agonists such as fibrates, adipose tissue would secrete increased concentrations of FFA, which further activates PPAR α . Studies has reported that FFA induces CREBH transcription in hepatocytes [32], possibly by activating PPAR α . Furthermore, it has been revealed that CREBH is involved in the acute phase response [25] and hepatic gluconeogenesis [34]. Recently, it has been revealed that CREBH plays a role in lipid metabolism. TG concentration in the plasma of CREBH knockout (KO) mice after fasting was higher compared with that of wild type (WT) mice [26] The TG content was specifically increased in the VLDL fraction. These mice have reduced mRNA expression levels of several genes involved in TG metabolism in the liver. The increase of the plasma TG appears to be induced by impaired TG clearance [26]. On the other hand, overexpression of CREBH in mice reduced the plasma TG levels [26]. Interestingly, there are some nonsynonymous and insertional mutations within the CREBH gene in some individuals with hypertriglyceridemia, but not in normolipidemic controls [26].

1.5 Microbiota and *Akkermansia muciniphila*

1.5.1 Microbiota

Microbiota is the general term indicating all the microorganisms in the body of animals or humans. In the GI tract of human, there are as many as 10 to 100 trillion

microorganisms. It is approximately 10 times more than the amount of human body cells [35]. The component of human microbiota is complex. The major part of microbiota is prokaryotes, most of which are bacteria [36, 37]. The other small part consists of eukaryotes and viruses [37]. Most of the bacteria in human are located in ileum, the terminal part of small intestine, and the large intestine. The major energy source of microbiota is undigested components from the diet of human. Carbohydrates, most of which are oligosaccharides, such as fibers, resistant starch and non-starch polysaccharides, are the main substrates that would be metabolized by microbiota [20, 38]. Moreover, microbiota could also ferment some metabolites of the host, like glycosylated protein mucins, immunoglobulins and lipid derivatives [39, 40]. Numerous of products of the fermentation include gases and short chain fatty acids (SCFA). Some metabolic products of the microbiota are beneficial for the host. For instance, the fermentation of resistant starch would produce butyrate, which is a kind of energy source of host epithelial cells and could stimulate cell proliferation [41].

Louis Pasteur in 1897 first discovered that the microbiota was crucial for the host [42]. Studies on germ-free animals investigated the importance of microbiota. Germ free animals are animals that have no bacteria in their body, which are important model to study the function of microbiota. It was showed that the life span of germ free animals were short [43]. Microbiota is very important in the metabolism and physiology of animals [35]. It has been reported that microbiota could impact the glucose metabolism [44-48] and energy homeostasis [6, 45, 49, 50]. It was also associated with obesity, low-grade inflammation, diabetes and cancers [50]. Studies have confirmed that conventionally raised mice with microbiota in their bodies developed more fat mass,

which is about 40% more, than germ-free mice [49]. Compared with gut microbiota from lean mice, transplanting isolated gut microbiota from obese mice to germ-free mice would stimulate the mice to get more body fat [6]. Another study showed that high fat diet was not able to induce obviously increased body weight in germ-free mice, suggesting a connection between gut microbiota and weight gain [51]. Gut microbiota was strongly influenced by high fat diet feeding, and further induced inflammatory responds and insulin resistance [45, 52-54]. A high fat diet, however, could not induce germ-free mice to develop inflammation or insulin resistance [55, 56]. Inoculation of strain *Enterobacter cloacae* B29, which is a kind of gut bacteria in an obese human, into germ-free mice triggered obesity with a high fat diet [56]. Beneficial bacteria were found to be reduced in metabolic complex animal models. The composition modulation of gut microbiota affected by high fat diet has been reported. Hildebrandt et al. confirmed that Firmicutes and Proteobacteria would increase while Bacteroidetes would decrease in mice treated with high fat diet [57]. Murphy et al. also found abundant Firmicutes and reduced Bacteroidetes in similar condition [58]. It has been widely accepted that gut microbiota play a role in the metabolism of the host. However, the exact mechanism of the interaction between gut microbiota and the host is still unclear [59].

1.5.2 Akkermansia muciniphila

A. muciniphila was first isolated from the fecal sample of a Caucasian female [60, 61]. So far, it has been discovered in the intestines of different animals, which includes rodents, rabbits, donkeys, pigs, horses and so on [62-66]. *A. muciniphila* is the first kind of bacterium isolated from human which belongs to the phylum Verrucomicrobia [67]. The medium used to isolate *A. muciniphila* contained only one carbon source, mucin [61]. *A.*

A. muciniphila is localized in the mucus layer of the intestine [67]. One evidence was that *A. muciniphila* could metabolize mucin [61]. Another study showed that *A. muciniphila* was one of the first prior utilizers of the mucus in the mice intestine [68]. It has been reported that *A. muciniphila* is more abundant in colon than in the ileum [69, 70]. It makes up more than 1% of the whole microbiota in human feces [67].

Interestingly, it has been revealed that the relative abundance of *A. muciniphila* increased under the conditions of caloric restriction in humans, mice, hamsters and snakes [67]. Mucosal analysis also showed that *A. muciniphila* was abundant in healthy subjects whereas it was reduced in inflammatory bowel disease (IBD) patients. In contrast, the amounts of *Ruminococcus gnavus* in IBD patients increased [71]. Different nutritional interventions and pharmaceutical treatments could affect the level of *A. muciniphila*. [67]. Some studies reported that the administration of some special dietary components, including complex polyphenols, oligofructose, dietary resistant starch, whole grain barley and so on, which improved the health of the host also increased the abundance of *A. muciniphila* [72-74]. High fat diet, however, would decrease *A. muciniphila* population [59, 75, 76]. A kind of antidiabetic drug, metformin, was investigated to be able to improve the proportion of *A. muciniphila* in mice and stimulate the growth of *A. muciniphila* in vitro [76, 77]. Flos Lonicera and ganoderma lucidum that are used as medicine in Asia, also enhanced the population of *A. muciniphila* [78-80]. Several studies revealed the association between the reduction of *A. muciniphila* abundance and various metabolic disorders and diseases, including obesity and related disorders and intestinal diseases [67]. Studies focusing on mouse models which developed obesity or other symptoms of metabolic disorder induced by genetic depletion, for example, leptin

deficiency [59, 81], or diet (high fat diet) [82, 83] showed that the abundance of intestinal *A. muciniphila* decreased in these models [67]. Remarkably, it has been reported that daily gavage of *A. muciniphila* improved the metabolic parameters of mice fed with high fat diet. It prevented weight gain, restored glucose tolerance and epithelial integrity, and counteracted metabolic endotoxemia [59, 76]. Another study also proved that *A. muciniphila* administration significantly decreased body fat and insulin resistance of a mouse strain which is prone to obesity (AxB19) [84]. These studies suggested that *A. muciniphila* played a role in reducing body fat accumulation and improving insulin resistance. However, there are also conflicting data. Rat studies reported increased abundance of *A. muciniphila* in rats fed with high fat diet [85-87]. Further studies should be conducted to demonstrate the relationship between *A. muciniphila* and the metabolism of the host. In this study, we investigated the effect of *A. muciniphila* on hypertriglyceridemia induced by genetic depletion of CREBH in mice and tried to figure out the underlying mechanism.

1.6 Fenugreek

Fenugreek is an annual legume which belongs to Fabaceae family. It is one of the oldest Fabaceae family plant used as medicine in central Asia around 4000 BC [88]. It is now being commercially grown in various countries distributed in South Asia, Middle East, Europe, Africa and America [89]. Fenugreek seed is the most valuable part of the plant. It is rich in fiber, proteins, neural lipids, vitamins and minerals. The fiber in fenugreek seeds is mainly non-starch polysaccharides [88]. Fenugreek fiber plays a role in

moderating glucose metabolism in human. It helps to reduce glucose absorption, regulate sugar level and facilitates insulin action [88]. For instance, a study showed that galactomannans, a main soluble fiber in fenugreek seeds, lowered glucose absorption [88, 90]. Moreover, the special materials, mucilage, tannins, pectin and hemicellulose, in the seeds inhibit the absorption of bile salt in the colon and hence reduce the LDL level in the blood [88, 91]. Proteins in fenugreek seeds mainly include albumin, globulin and lecithin [88]. It contains a large amount of free amino acids, especially histidine and 4-hydroxyisoleucine. These amino acids may play a role in improving insulin activity [92]. Fenugreek is also rich in alkaloids, flavonoids, saponins and other antioxidants, which makes fenugreek possess a powerful antioxidant property [88]. All these compounds could help to ameliorate hypercholesterolemia, diabetes and even cancer [90, 91, 93]. In many Asian and African civilizations, fenugreek seeds were used as part of the medicine to treat diabetes [92, 94]. Fenugreek has various pharmacological properties [95]. The polyphenolics in fenugreek could inhibit peroxidation and attenuate oxidative hemolysis of erythrocytes in humans [88]. Studies have reported that the optimal consumption may decrease the TG and CHOL level in the blood (Afef et al., 2000) [96], prevent cancer [97] and control diabetes mellitus [98]. It has also been investigated that the ethyl acetate extract of fenugreek seeds reduced TG and CHOL in LDL while increased CHOL contained in HDL [99]. Due to its unique properties, here we studied whether fenugreek seeds were capable of alleviating genetic hyperlipidemia in mice and explored the mechanism of the regulatory effect of fenugreek seeds on the mouse lipid homeostasis.

1.7 Working Hypothesis

We hypothesized that increased colonization of *A. muciniphila* could protect the host from hyperlipidemia by enhancing the uptake of lipoproteins and alleviating hepatic ER stress and the inflammatory response in CREBH-null mice and that fenugreek seeds could improve hyperlipidemia by inhibiting the biosynthesis of lipoproteins and ameliorating insulin resistance.

1.8 Study Aims

Specific Aim 1:

Determine the impact and mechanism of *A. muciniphila* on hyperlipidemia. Firstly, we will explore the impact of *A. muciniphila* on acute (olive oil gavage) and chronic (CREBH depletion) hyperlipidemia in mice. To figure out the mechanism of the regulatory effect of *A. muciniphila* on the host, we will subject different mice models with vehicle (as control), heat-inactive *A. muciniphila* and active *A. muciniphila*. Lipid content and lipoprotein levels in the plasma and liver of the mice will be measured to demonstrate if they are impaired by *A. muciniphila* inoculation. LDL receptor KO mice will be treated with *A. muciniphila* to verify whether LDL receptor plays an important role in the interaction of *A. muciniphila* and the host. And also, the mRNA and protein levels of LPL co-factors and the biomarkers of ER stress and inflammation factors in the liver of the mice will be analyzed to investigate whether *A. muciniphila* administration is able to alleviate hepatic stress and inflammation.

Specific Aim 2:

Determine the impact and mechanism of fenugreek hyperlipidemia induced by CREBH depletion.

Firstly, we will feed CREBH KO mice with fenugreek seeds rich diet to investigate the impact of fenugreek seeds on the hyperlipidemia of the mice. And hepatic expression level of apoB and lipid content will be measured to study the effect of fenugreek on VLDL assembly. Impact of fenugreek seeds on insulin resistance induced by high fat diet will also be explored.

Chapter Two

Materials and Methods

2.1 Animals and animal experimental protocols

Three kinds of mice models were used during the experiments. Wide type (C57BL/6J) mice and LDLR KO mice (B6.129S7-Ldlr^{tm1Her}/J) were got from the Jackson Laboratory, Maine, US. Heterozygous CREBH KO mice were kindly provided by Dr. Zhang Kezhong's lab. Animals were housed in a 12h light and dark cycle with free access to food and water. A standard chow diet was provided to the mice which contains 62.1% carbohydrate, 24.6% protein, and 13.2% fat (kcal/100 kcal) (Dyets Inc., USA). Both male and female mice were used during the experiment. And the mice used were 12 to 14 weeks old.

For *A. muciniphila* treatment experiments, CREBH KO mice were divided into 3 groups, which are control group, heat-inactivated *A. muciniphila* treatment group, and active *A. muciniphila* treatment group (n=6-12/per group). Both WT mice and LDLR KO mice were divided into 2 groups, which are control group and active *A. muciniphila* treatment group. In control group, the mice were administrated by oral gavage with 200 μ L PBS that contained 25% (vol/vol) glycerol, which was the dissolving solution of *A. muciniphila* bacteria. In the heat-inactivated *A. muciniphila* treatment group, the mice were administrated by oral gavage with 200 μ L heat-inactivated *A. muciniphila* with a final concentration of 10^9 CFU/mL. In the active *A. muciniphila* group, the mice were administrated with 200 μ L (10^9 CFU/mL) *A. muciniphila* by oral gavage. The administration was conducted every 2 days for two weeks. During the treatment, blood

samples was collected from submandibular vein after the mice were fasted overnight for 12 hours and the TG and CHOL concentrations of the plasma were monitored every week.

For fenugreek treatment experiments, both WT mice and CREBH KO mice were divided into control group and fenugreek group. WT mice were fed with high fat diet, which contains 60% fat and 20% carbohydrate (kcal/100g) (Dyets Inc., USA). CREBH KO mice were fed with standard chow diet. In fenugreek group, fenugreek seeds were added into the diet according to a ratio of 2% (g/g). Fenugreek seeds were kindly provided by Dr. Dipak Santa. All the mice were fed for 7 weeks. Weight of the mice and the food intake amount were monitored every week.

Mice were euthanized by isoflurane (3% mixed with oxygen). Blood was obtained from the heart after the mice were anesthetized by isoflurane, and was collected in lithium heparin coated capillary tubes (Bd Vacutainer Labware Medical, USA). Blood samples was then centrifuged at 6000 rpm for 10 min and plasma was collected and stored at -80 °C for further analysis. Tissues, including liver, duodenum, jejunum, ileum, adipose tissue and muscle, were snap frozen in liquid nitrogen and stored at -80 °C for further analysis. All the animal care, treatment and experiments were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

2.2 Preparation of *A. muciniphila* bacterial stocks

The bacteria *A. muciniphila* *MucT* (ATTC BAA-835) were purchased from American Type Culture Collection (ATCC). The bacteria were cultured in brain heart infusion agar (BD 211065) or broth (BD 237500) medium. And then the bacteria were washed and re-

suspended in anaerobic PBS which included 25% (vol/vol) glycerol. The stocks were frozen and stored at -80 °C immediately.

Plate counting was used to determine the concentration (CFU/mL) of the bacteria in the stocks. Three representative samples were gradient diluted into 4 concentrations respectively and inoculated in plates with mucin media containing 1% agarose. The average amount of the bacterial colonies were used to calculate the bacteria concentration in the stocks. Before used to administrate the mice, the bacteria were diluted to a final concentration of 10^9 CFU/mL with anaerobic PBS that contained 25% (vol/vol) glycerol. All the culture and preparation procedures were under strict anaerobic conditions.

2.3 *In vivo* chylomicron collection assay

Mice were fasted overnight for 12 hours and then administrated with 200 μ L virgin olive oil (Great Value, Walmart) by oral gavage. 20 min after olive oil gavage, the mice were treated with poloxamer 407 (Pluronic F-127, Sigma) by intraperitoneal (IP) injection according to an amount of 500 mg/kg. Poloxamer was mixed into saline to make a 20% solution one day before treatment and given 2.5 μ L/g body weight by IP. The mice were euthanized at 2 hours after poloxamer treatment. Before olive oil administration, baseline blood (0h) was collected from submandibular vein for the baseline lipid and protein contents determination. One hour after poloxamer treatment, blood samples were collected via submandibular vein. When sacrifice, final blood (2h) was collected via cardiac puncture. All the blood samples were centrifuged at 6000 rpm for 10 min for the plasma separation. Plasma and tissues were collected and stored at -80°C for further experiments.

2.4 Vitamin A excursion assay

Vitamin A excursion assay was used as a method to measure the clearance of CMs derived from dietary fats [100]. Mice were fasted for 4 hours and then administrated with mixture of olive oil and [11, 12-3H]-retinol by oral gavage. 1 mL olive oil was mixed with 27 μ Ci of [11, 12-3H]-retinol (PerkinElmer) in ethanol. Submandibular vein blood was sampled at 0h (baseline), 2h, 3.5h, 7.5h and 11h for determining the radioactivity in the blood. Baseline blood was collected before gavage. 10 μ L of serum was used to detect the 3H level by scintillation counting. The measurement was conducted in triplicate. At 11 hours after fat administration, mice were euthanized. Cardiac puncture was conducted to collect the blood samples. Plasma and tissues were stored at -80°C for further experiments.

2.5 Lipoprotein Characterization

Gel filtration fast protein liquid chromatography (FPLC) was used to separate the lipoproteins subclasses in the plasma [101]. 150 μ L plasma samples were injected onto a Superose 6 10/200 GL column (Amersham Pharmacia Biotechnology, Piscataway, NJ). The flow rate of the whole process was 0.5 ml/min. Specific eluent was used to elute the different fractions from the column. And the TG and CHOL concentrations of each fractions were measured. The receipt of the eluent is: 10 mM Tris, 150 mM NaCl, 2 mM CaCl_2 , 100 μ M DTPA, 0.02% NaN_3 , pH 7.4.

2.6 Immunoblot analysis

Tissue Lysate Preparation: 100mg tissue (liver or jejunum) was homogenized in 200 μ L RIPA buffer in 1.5mL microfuge tube using tissue homogenization mixer (VWR

International, Radnor, PA) till there was no fragment left. Then add 800 μ L RIPA buffer and mix completely by vortex. Sonicate for 3×10 seconds. And keep the samples in ice for 10 min. After centrifuging at 12000 rpm for 10 min at 4°C in a Heraeus Biofuge Pico centrifuge (Thermo Electron Corporation Canada, Gormley, ON), carefully transfer the protein lysate under the fat cake to a new 1.5mL microfuge tube. The lysates were then mixed with $4 \times$ SDS Loading Buffer by vortex and heated at 100°C for 6 to 10 min. The samples could be stored at -80°C before further analysis.

Plasma Lysate Preparation: 2 μ L plasma was mixed with 98 μ L RIPA buffer. Then add 100 μ L 2 x SDS loading buffer and mix the lysate by vortex. After that, the lysates were boiled at 100°C for 6 to 10 min and stored at -80°C for further analysis.

The receipt of RIPA buffer is: 150 mM sodium chloride (NaCl) (Fisher Scientific, Waltham, MA), 10 mM tris (hydroxymethyl) aminomethane (Tris) (pH 7.4) (Fisher Scientific, Waltham, MA), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Canada Ltd., Oakville, ON), 1% Nonidet P-40 (Sigma-Aldrich Canada Ltd., Oakville, ON), and protease inhibitor cocktail tablet (Roche Applied Science, Laval, QC.).

The receipt of 4x SDS loading buffer is: 200mM Tris-HCl, pH6.8 (Fisher Scientific, Waltham, MA), 8% SDS (Fisher Scientific, Waltham, MA), 40% Glycerol (Fisher Scientific, Waltham, MA) and 0.002% Bromophenol Blue (Sigma-Aldrich, St. Louis, MO). Before use, add 200mM DTT immediately from a 1M DTT stock. The buffer could be stored at -20°C for up to 6 months.

Western Blotting Analysis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for protein detection. 6% acrylamide gels were used for apoB detection, 8% acrylamide gels were for Toll-like Receptor 4 (TLR4), and 10% for all other proteins. Running buffer contained 25 mM Tris, 192 mM glycine (Fisher Scientific, Waltham, MA) and 0.1% SDS. And a mini-PROTEAN electrophoresis system (Bio-Rad Laboratories Ltd., Mississauga, ON) was used as the power supply for electrophoresis and transfer. Proteins in gels were transferred onto polyvinylidene fluoride (PVDF) membranes (PerkinElmer, Woodbridge, ON) under a voltage of 40V at 4°C overnight using a wet transfer apparatus (Bio-Rad Laboratories Ltd., Mississauga, ON). Transfer buffer contained 190 mM glycine, 25 mM Tris and 10% methanol which was freshly prepared.

Membranes were then blocked in 5% milk (Bio-Rad Laboratories Ltd., Mississauga, ON) in 1x TBST for at least 2 hours at room temperature on an orbital shaker. The receipt of TBST is 10 mM Tris, 150 mM NaCl and 0.05% triton x 100 (Fisher Scientific, Waltham, MA). After blocking, membranes were washed in 1x TBST 5 times for 5 min using 1x TBST. Then the membranes were incubated with primary antibodies overnight at 4°C. The antibodies were diluted according to the datasheet provided by manufacturers. The antibodies used are as follows: anti-apoB, anti-apoE and anti-albumin (Midland Bioproducts, Boone, IA); anti- β -actin (Sigma-Aldrich, St. Louis, MO); anti-TLR4, anti-JNK-p, anti-Bip (Cell Signaling, Danvers, MA); and anti-eIF2 α (Invitrogen, Carlsbad, CA). Membranes were then washed 5 times for 5 minutes using 1x TBST and then incubated with secondary antibody for 1 hour at room temperature. There are three kinds of secondary antibodies used in the project, including donkey anti-rabbit IgG-HRP (GE

Healthcare, Buckinghamshire, UK), sheep anti-mouse IgG-HRP (GE Healthcare, Buckinghamshire, UK) or donkey anti-goat IgG-HRP (Santa Cruz, Dallas, TX). The anti-rabbit and anti-mouse antibodies were diluted in 5% milk for 1:2000. And the anti-goat antibody was diluted in 5% milk for 1:3333. Membranes were washed 5 times for 5 minutes in 1x TBST. Enhanced chemiluminescence reagents (Amersham Biosciences, Pittsburgh, PA) was used to expose the protein bands on membranes to signals in autoradiography film (Denville Scientific Inc., Metuchen, NJ) in exposure cassettes (Eastman Kodak Company, Rochester, NY). Bands were quantified by densitometry using Image J.

2.7 mRNA analysis by real-time PCR

Total RNA was extracted from liver of mice using TRIzol (Invitrogen, Carlsbad, CA) according to the protocol that was provided with the reagent by the manufacturer. The RNA was reversed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). The mRNA expression levels of target genes were tested by quantitative real-time PCR using a SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA). The qPCR was conducted with a Prism 7300 Sequence Detecting System (Applied Biosystems, Carlsbad, CA).

2.8 Lipid extraction and measurement

Lipids were extracted from tissue according to the method mentioned by Folch, J [102]. 100 mg of tissues (liver or jejunum) were completely homogenized in 1ml triton lysis buffer. The homogenate was then put in ice for 30 min for lipid extraction. After adding 2 ml of Folch/BHT reagent, the samples were oscillated by vortex for 3 x 10 sec. Then the

samples were centrifuged at 2000 rpm for 10 min at RT in a Heraeus Biofuge Pico centrifuge (Thermo Electron Corporation Canada, Gormley, ON). The lower phase was moved to new glass reaction tubes and was dried under N₂ to about 100 ul. 1ml of CHCl₃ (Fisher Scientific, Waltham, MA) and 1% Triton x 100 in CHCl₃ was used to wash the samples successively. And the reagents were all dried by N₂. Samples was finally diluted in 600ul distilled water and stored at 4°C for further analysis.

2 µL of plasma or the extracted lipid aliquots were used to measure the CHOL and TG contents using an enzymatic/GPO TG and CHOL assay kit (Pointe Scientific, Canton, MI) according to the instruction provided by the manufacturer with the kit.

The receipt of Folch/BHT reagent is: 280 ml chloroform(Fisher Scientific, Waltham, MA), 140 ml methanol(Fisher Scientific, Waltham, MA) and 42mg BHT (to 100ug/ml)(Acros Organics, New Jersey, USA)

The receipt of triton lysis buffer is: 80 mM NaCl (Fisher Scientific, Waltham, MA), 50 mM Tris (pH 8.0) (Fisher Scientific, Waltham, MA), 2 mM CaCl₂ (Fisher Scientific, Waltham, MA), and 1% Triton x 100 (Fisher Scientific, Waltham, MA).

2.9 Glucose tolerance test and insulin tolerance test

For glucose tolerance test, the mice were fasted for 12h overnight. The baseline blood glucose concentration was tested. Then the mice were treated with glucose by IP injection or oral gavage according to an amount of 2 µg/g body weight. Blood glucose concentrations of the mice were then tested at 15, 30, 45, 60 and 120 min after glucose treatment.

For insulin tolerance test, the mice were fasted for 4h early in the morning. The baseline blood glucose levels was tested before insulin treatment. Mice was gave insulin (0.5 U/kg) (Eli Lilly, Indianapolis, IN) via IP injection. And then glucose levels in the blood of the mice were measured at 15, 30, 45, 60 and 120 min after insulin treatment.

2.10 Statistical analysis

Digital data were evaluated using GraphPad Prism 5 (La Jolla, CA, USA). In all the groups throughout the project, at least three subjects were analyzed. T-tests were used to compare parallel experiments. Results were presented as means \pm standard error of the mean. P values which were less than 0.05 were considered statistically significant and were marked with a single asterisk (*). P values less than 0.01 were marked with two asterisks (**).

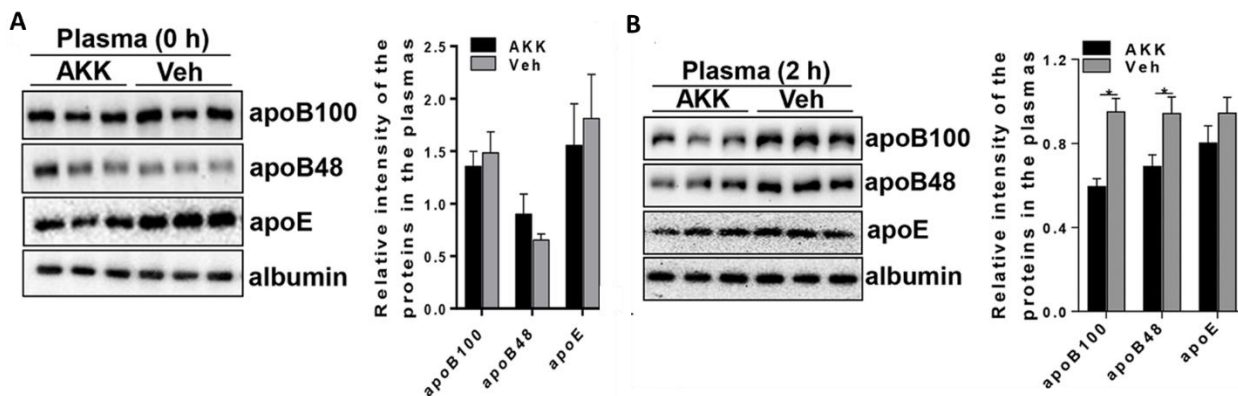
Chapter Three

Results

Part 1

Determine the beneficial property of *A. muciniphila* on genetic induced hyperlipidemia and the underlying metabolism

Previous study in our lab has investigated that after administrating 200 μL *A. muciniphila* (2×10^8 cfu/0.2 mL) to 12-week old WT (C57BL/6J) mice every two days for 2 weeks, the plasma lipid contents (TG and CHOL) of the mice treated with *A. muciniphila* were comparable with that of control group, which was treated with vehicle (PBS containing 25% glycerol) for 2 weeks. To further explore the effects of *A. muciniphila* on the host's acute lipid absorption, after 2 weeks of *A. muciniphila* administration, the mice were fasted for 12 hours, and then treated with 200 μL olive oil by oral gavage. 500 mg/kg poloxamer was further treated by IP injection at 20 min after oil administration. Poloxamer could restrain the activity of LPL and suppress the hydrolysis of lipoproteins in the blood. Protein levels of plasma apoB48, apoB100 and apoE, which were the key proteins in VLDL and CMs, were analyzed. Protein levels before poloxamer treatment were comparable between the two groups (Figure 1 A. Data from Xuedong Tong). 2 hours after poloxamer injection, the expression of apoB48 and apoB100 in the plasma of vehicle group was significantly higher than that of *A. muciniphila* group (Figure 1 B). These data were consistent with the result of plasma lipid concentration analysis. The apoE expression was a little higher in vehicle group. But the difference was not significant (Figure 1 B). This indicates that the secretion of CMs and VLDL was increased, or their clearance was decreased in vehicle group.



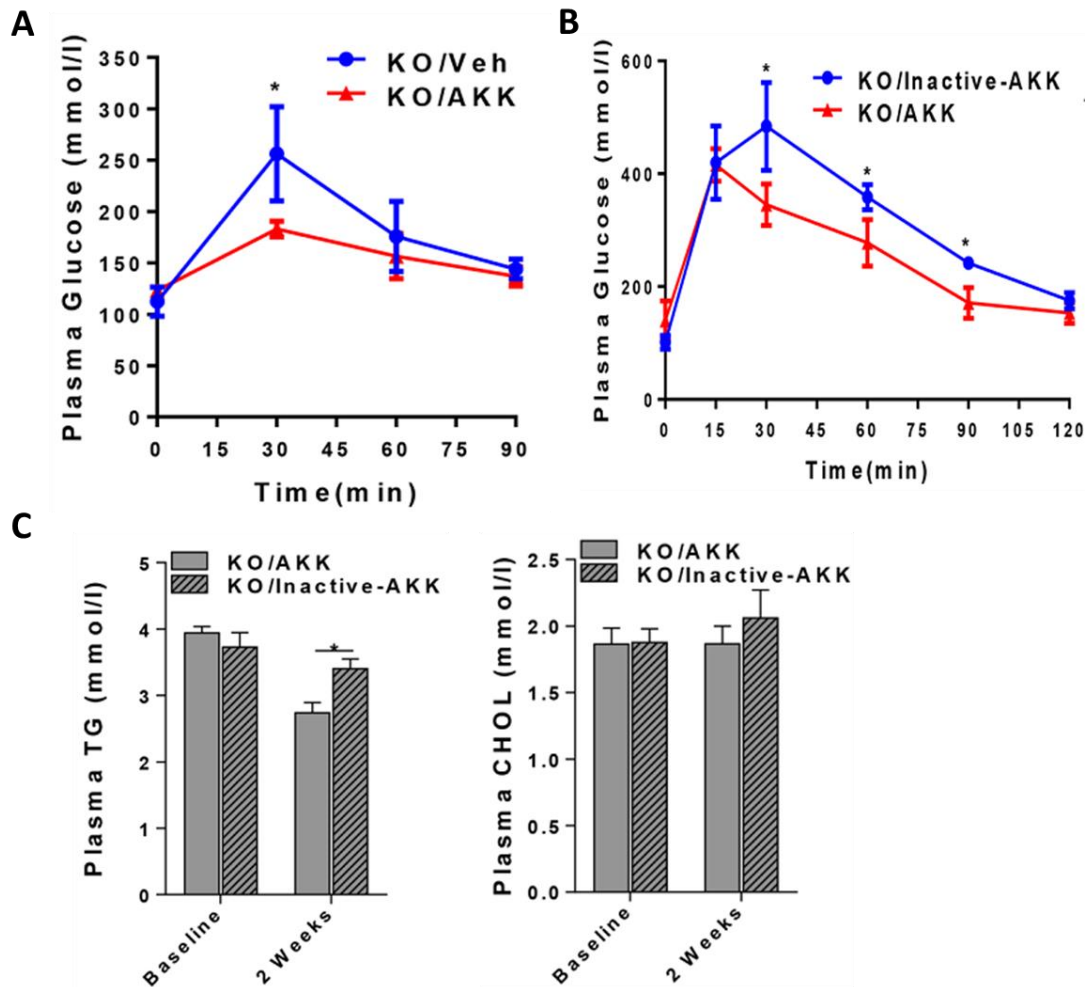
(Shen et al. ATVB 2016)

Figure 1. The impact of *A. muciniphila* on the host's acute lipid absorption. 12-week old WT (C57BL/6J) mice was administrated with 200 μ L vehicle or *A. muciniphila* (2×10^8 cfu/0.2 mL) every two days for 2 weeks. Then the mice were fasted for 12 hours, and then treated with 200 μ L olive oil by oral gavage. 500 mg/kg poloxamer was further treated by IP injection 20 min after oil administration. Protein levels of plasma apoB48, apoB100 and apoE were analyzed, before and 2 hours after poloxamer treatment. A) The protein levels before poloxamer treatment were comparable between the two groups (Data from Xuedong Tong). B) After poloxamer injection, apoB48 and apoB100 levels in the plasma of vehicle group was significantly higher than that of *A. muciniphila* group. Results are shown as means \pm SD for two experiments that were performed in triplicate.

* $P < 0.05$, ** $P < 0.01$.

To investigate the effect of *A. muciniphila* colonization on chronic hyperlipidemia induced by gene depletion, CREBH KO mice were used. The depletion of CREBH of mice could induce hypertriglyceridemia. 200 μ L of vehicle or *A. muciniphila* were respectively administrated by gavage to two groups of CREBH KO mice for 2 weeks. Previous study in the lab indicated that plasma TG level of the mouse group treated with *A. muciniphila* was significantly lower than that of control group after treated for 2 weeks. To find out if the colonization of *A. muciniphila* could ameliorate insulin resistance induced by CREBH depletion, we subjected the two groups of mice to an oral glucose tolerance test. After glucose delivery, the growth of blood glucose level of *A. muciniphila* group was slower than vehicle treatment group (Figure 2 A), indicating that *A. muciniphila* could improve glucose intolerance in CREBH KO mice.

To further explore if it is necessary that *A. muciniphila* is alive to regulate the metabolism of the host, two groups of CREBH KO mice were subjected with active *A. muciniphila* and heat-inactive *A. muciniphila* every two days for two weeks. The plasma TG level of heat-inactive *A. muciniphila* group didn't decrease after two weeks of administration, while the plasma TG level of active *A. muciniphila* group was significantly decreased at the end of two weeks (Figure 2 C). Oral glucose tolerance test also showed that inactive *A. muciniphila* could not improve the glucose tolerance of CREBH KO mice (Figure 2 B).



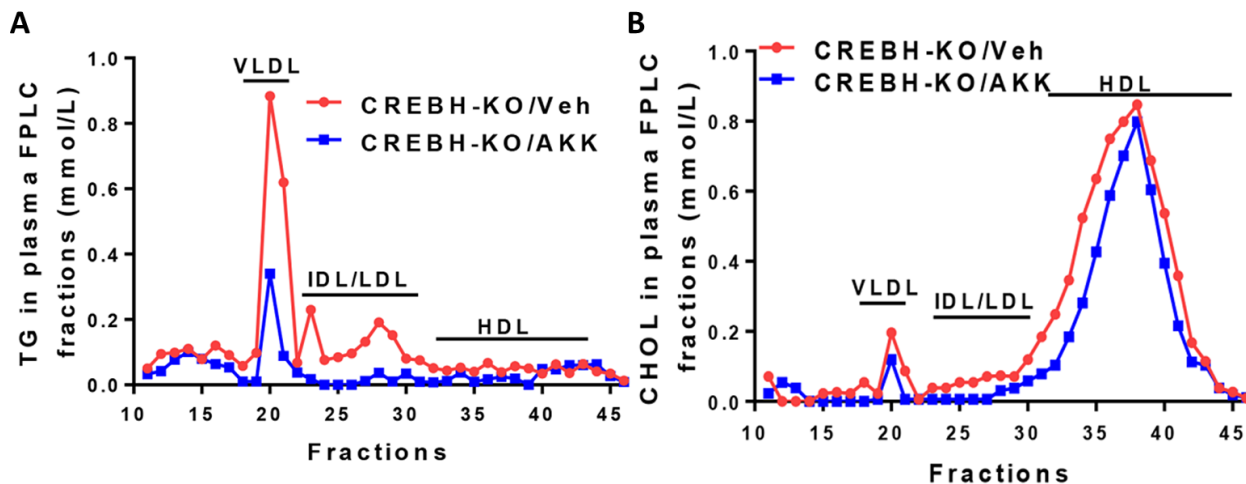
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Figure 2. The impact of *A. muciniphila* colonization on chronic hyperlipidemia induced by gene depletion. A) 200 μ L of vehicle or *A. muciniphila* were respectively administrated by gavage to two groups of CREBH KO mice for 2 weeks. At the end of week 2, the mice were subjected to an oral glucose tolerance test. The alteration of glucose concentration in the mice blood after glucose treatment was shown in the graph. B) CREBH KO mice were subjected with active *A. muciniphila* and heat-inactive *A. muciniphila* every two days for two weeks. Oral glucose tolerance test was conducted after 2-week oral gavage. C) The plasma TG and CHOL levels of heat-inactive *A.*

muciniphila group and active *A. muciniphila* group was compared before and after *A. muciniphila* administration. Results are shown as means \pm SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.

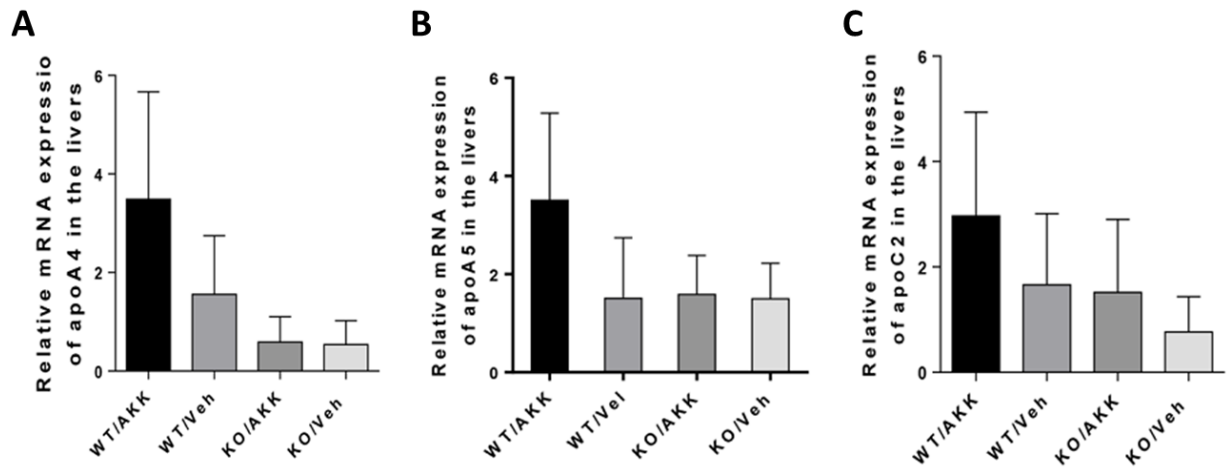
To investigate the mechanism of *A. muciniphila* ameliorating the hyperlipidemia in CREBH KO mice, we tested the mRNA expression levels of LPL co-factors, apoC2, apoA4, apoA5, by real-time PCR in vehicle and *A. muciniphila* treated WT and CREBH KO mice. It has been reported that the depletion of CREBH in mice would depress the expression of LPL co-factors, inducing inefficient hydrolysis of lipoprotein TGs by LPL and thus leading to hypertriglyceridemia. However, there were no significant differences in the mRNA expression of apoA4, apoA5 or apoC2 between vehicle and *A. muciniphila* groups (Figure 4 A B C). We had investigated that the expression of LDL receptor in the liver of CREBH KO mice treated with *A. muciniphila* was significantly higher than that of control group. To further confirm whether LDL receptor signaling could be upregulated by *A. muciniphila* colonization to help clearing VLDL remnants, we used gel filtration fast-phase liquid chromatography (FPLC) to analyze the plasma lipoprotein content. The TG level in the VLDL, IDL and LDL particles in *A. muciniphila* group was significantly decreased, suggesting that the clearance of IDL and LDL was increased by *A. muciniphila* colonization (Figure 3 A). The CHOL level in VLDL, IDL, LDL and large HDL was also slightly decreased in *A. muciniphila* group (Figure 3 B). This shows that *A. muciniphila* treatment may upregulate the clearance of VLDL remnants by LDL receptor pathway. To confirm the specificity of LDL receptor in the effect of *A. muciniphila* on the host, we administrated *A. muciniphila* and vehicle to two groups of LDL receptor KO mice every other day for two weeks respectively. *A. muciniphila* administration didn't ameliorate the hypercholesterolemia induced by LDL receptor depletion (Figure 5 B). Oral glucose tolerance test also revealed that *A. muciniphila* failed to improve insulin sensitivity of LDL receptor KO mice (Figure 5 A), indicating that

LDL receptor was crucial in the regulatory role of *A. muciniphila*.



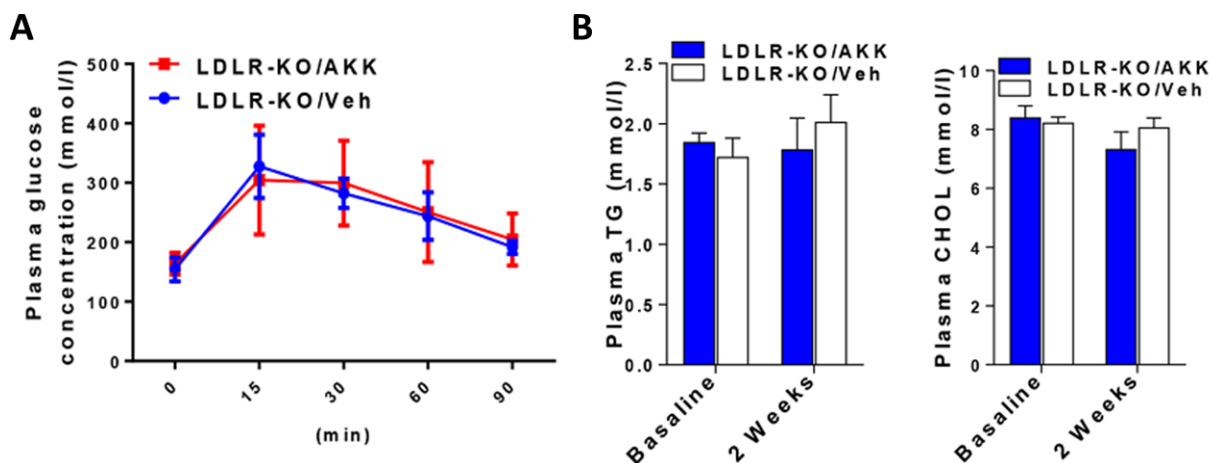
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Figure 3. CREBH KO mice were treated with 200 μ L of vehicle or *A. muciniphila* by gavage for 2 weeks. Gel filtration fast-phase liquid chromatography (FPLC) was used to analyze the plasma lipoprotein content in mice. A) The TG level in the VLDL, IDL and LDL particles in *A. muciniphila* group was significantly decreased. B) The CHOL level in VLDL, IDL, LDL and large HDL was slightly decreased in *A. muciniphila* group.



(Shen et al. ATVB 2016)

Figure 4. Impact of *A. muciniphila* on the expression of LPL co-factors. WT and CREBH KO mice were separately subjected with 200 μ L vehicle or *A. muciniphila* every two days for 2 weeks. The mRNA expression levels of apoA4, apoA5 or apoC2 were analyzed. Results are shown as means \pm SD for two experiments that were performed in triplicate. * $P < 0.05$, ** $P < 0.01$.



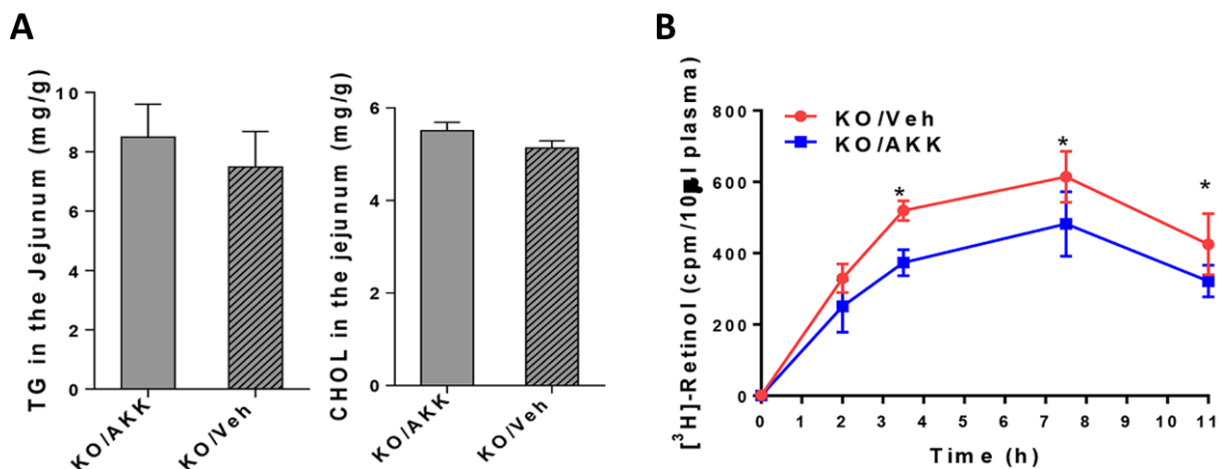
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Figure 5. Effect of *A. muciniphila* on LDL receptor KO mice. *A. muciniphila* and vehicle were administered to two groups of LDL receptor KO mice every other day for two weeks respectively. A) Oral glucose tolerance test was conducted after *A. muciniphila* administration. The result revealed that *A. muciniphila* failed to improve insulin sensitivity of LDL receptor KO mice. B) Plasma lipid concentration analysis showed that *A. muciniphila* administration didn't ameliorate the hypercholesterolemia induced by LDL receptor depletion. Results are shown as means \pm SD for two experiments that were performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

To determine whether *A. muciniphila* inoculation impacts the CM assembly and secretion in the intestinal enterocytes, we treated CREBH KO mice with vehicle or *A. muciniphila* for two weeks followed by an *in vivo* CM collection assay which is described in Materials and Methods. Lipid was extracted from the jejunum of both groups which was collected 2 hours after olive oil gavage. Measurement of lipid contents showed that the TG and CHOL contents in the jejunum of both groups were the same (Figure 6 A). This meant that *A. muciniphila* colonization didn't impair the fat absorption or CM biosynthesis in the intestine. To test the clearance rate of CMs in the circulation, a vitamin A excursion assay was conducted as described in Materials and Methods. The [³H]-retinol which was mixed with olive oil treated by gavage was used to synthesize fatty acid esters in the enterocytes. This kind of [³H]-labeled fatty acid esters was incorporated into CMs, secreted into the blood and removed by the liver [100]. At 3.5, 7.5 and 11 hours after oil gavage, the [³H] level in the plasma of *A. muciniphila* treated CREBH KO mice was significantly lower than that of vehicle treated mice (Figure 6 B), suggesting a faster clearance of CMs by the liver in *A. muciniphila* treated mice. This increased clearance may be mediated by the increased expression of LDL receptors in the liver of *A. muciniphila* group.

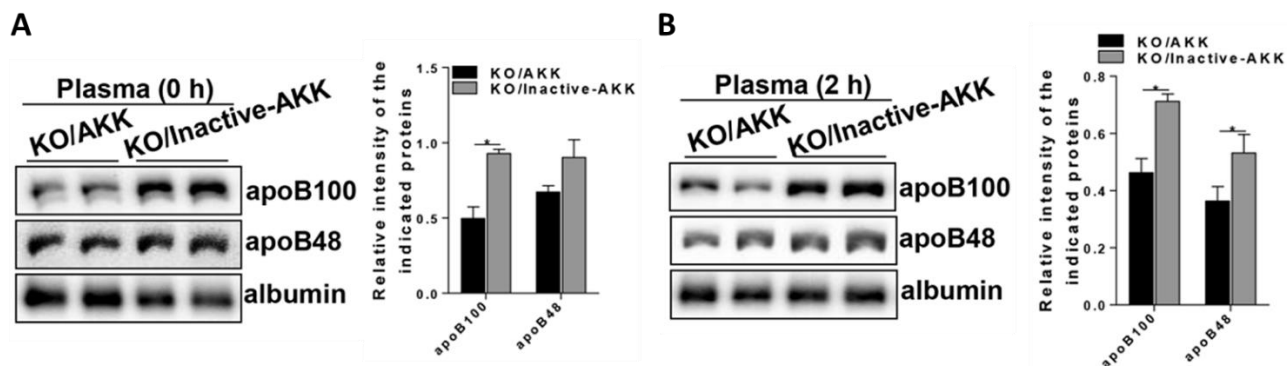
To determine whether inactive *A. muciniphila* could also regulate the CM metabolism of the host, we subjected two groups of CREBH KO mice to CM collection assay which had already been administrated with alive *A. muciniphila* or heat-inactive *A. muciniphila* for two weeks. The protein level of apoB in the plasma of heat-inactive *A. muciniphila* group was significantly higher than that of *A. muciniphila* group before and at 2 hours after

olive oil and poloxamer treatment (Figure 7 A B), indicating that heat-inactive *A. muciniphila* was not able to play a regulatory role in CM clearance.



(Shen et al. ATVB 2016)

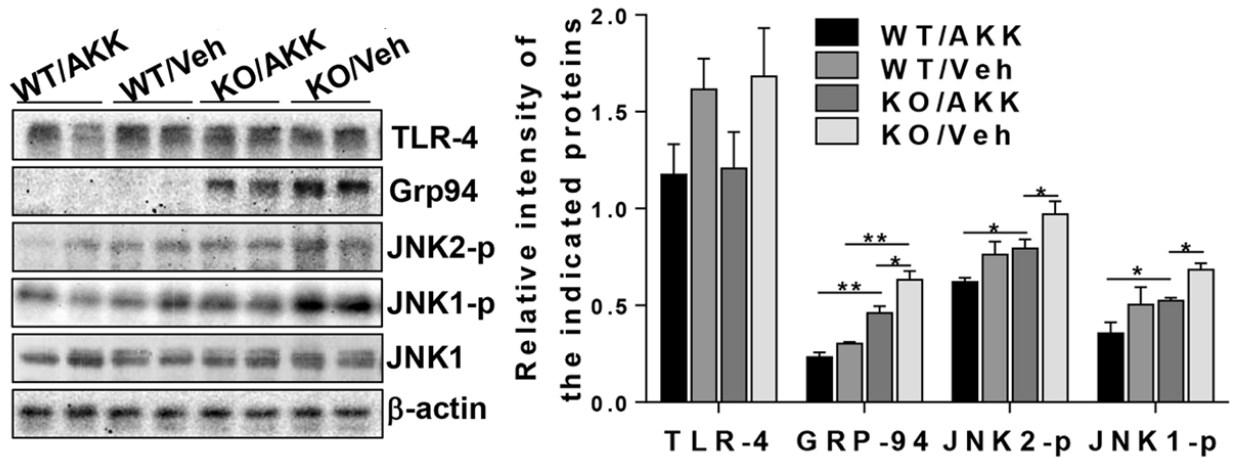
Figure 6. Impact of *A. muciniphila* inoculation on the CM metabolism. CREBH KO mice was administrated with vehicle or *A. muciniphila* every two days for two weeks followed by an *in vivo* CM collection assay. A) Lipid was extracted from the jejunum of both groups of mice which was collected 2 hours after olive oil gavage. The TG and CHOL contents in the jejunum of both groups were the same. B) A vitamin A excursion assay was conducted to test the clearance rate of CMs in the circulation. The [³H]-retinol was mixed with olive oil and subjected to the mice by gavage. At 3.5, 7.5 and 11 hours after oil gavage, the [³H] levels in the plasma of both groups of mice were analyzed. Results are shown as means \pm SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.



(Shen et al. ATVB 2016)

Figure 7. Two groups of CREBH KO mice were subjected to CM collection assay which had already been administrated with alive *A. muciniphila* or heat-inactive *A. muciniphila* for two weeks. The protein levels of apoB48 and apoB100 in the plasma of heat-inactive *A. muciniphila* group and *A. muciniphila* group before (A) and at 2 hours after (B) olive oil and poloxamer treatment were compared. Results are shown as means \pm SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.

Since hyperlipidemia is closely related with hepatic inflammation and ER stress, we hypothesize that increased colonization of *A. muciniphila* could alleviate the ER stress and inflammation induced by CREBH depletion. Both WT and CREBH KO mice were subjected with vehicle or *A. muciniphila* administration by oral gavage every two days for two weeks. Protein levels of biomarkers of ER stress and inflammation in the hepatocytes of the mice were measured by immunoblotting analysis. It was found that the expression of TLR-4, an important proinflammatory factor associated with circulating lipopolysaccharide (LPS) level, was not affected by *A. muciniphila* inoculation (Figure 8). The phosphorylation of JNK1 and JNK2, the inflammation transcription factors which was stimulated by CREBH depletion, was significantly decreased by *A. muciniphila* administration in CREBH KO mice (Figure 8). In WT mice, the colonization of *A. muciniphila* also inhibited the phosphorylation of JNK1 and JNK2 (Figure 8). The expression level of ER stress biomarker, GRP94, was also increased by the depletion of CREBH, and was reduced by *A. muciniphila* inoculation (Figure 8). However, in the liver of WT mice, the GRP94 level was not significantly affected by *A. muciniphila* treatment (Figure 8). These results demonstrate that increased colonization of *A. muciniphila* in CREBH KO mice may ameliorate the inflammation and ER stress in the hepatocytes which were induced by CREBH depletion.



(Shen et al. ATVB 2016)

Figure 8. Impact of increased colonization of *A. muciniphila* on ER stress and inflammation induced by CREBH depletion. Both WT and CREBH KO mice were subjected with vehicle or *A. muciniphila* administration by oral gavage every two days for two weeks. Protein levels of biomarkers of ER stress and inflammation in the hepatocytes of the mice were measured by immunoblotting analysis. The expression of ER stress biomarker, GRP94, and inflammation transcription factors, JNK1 and JNK2 phosphorylation, were stimulated by the depletion of CREBH, and reduced by *A. muciniphila* inoculation. Results are shown as means \pm SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.

Part 2

Determine the beneficial property of Fenugreek on genetic induced hyperlipidemia

To investigate the effect of fenugreek seeds on genetic induced hyperlipidemia, two groups of CREBH KO mice were respectively fed with control chow diet and chow diet mixed with 2% of fenugreek seeds for 7 weeks. CREBH depletion in mice induced a significantly higher TG level in the plasma compared with that of WT mice. At the end of the 7-week feeding, the mice were fasted for 12 hours and the plasma lipid contents of the mice were measured. The TG level of the fenugreek fed CREBH KO mice was significantly lower than that of control group, suggesting that fenugreek helped to alleviate the hypertriglyceridemia in CREBH KO mice (Figure 9 A). The CHOL level in the plasma of the mice was not affected by fenugreek treatment (Figure 9 B).

Hyperlipidemia was closely related with insulin resistance. To verify whether fenugreek seeds were able to regulate the glucose sensitivity, we fed two groups of C57BL/6J (WT) mice with high fat diet to induce insulin resistance, and added 2% fenugreek seeds into the diet of one group. The mice were fed the special diets for 7 weeks and then subjected to glucose tolerance test and insulin tolerance test. After 12-hour fasting, a dose of glucose or insulin was injected into enterocoelia of the mice and glucose concentration in the blood was measured. The blood glucose level of the mice fed with fenugreek decreased faster than that of high fat diet fed mice in both glucose and insulin tolerance tests (Figure 10 A B). This indicates that the uptake of the glucose by adipose tissue and liver was enhanced and insulin resistance was ameliorated by fenugreek seed feeding.

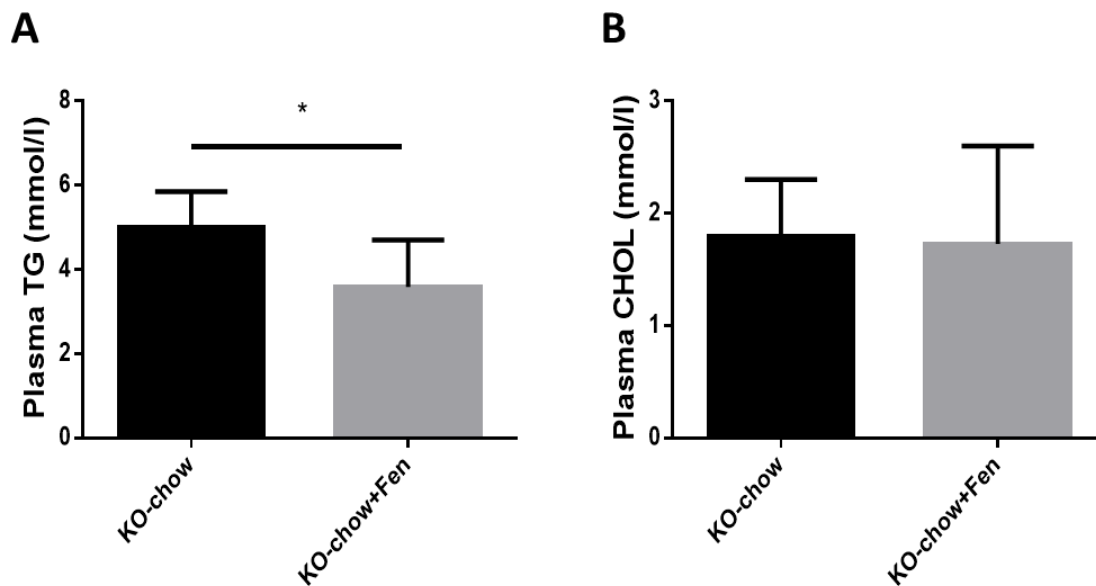


Figure 9. Fenugreek seeds were able to alleviate the hypertriglyceridemia induced by CREBH depletion. Two groups of CREBH KO mice were respectively fed with control chow diet and chow diet mixed with 2% of fenugreek seeds for 7 weeks. After the 7-week feeding, the mice were fasted for 12 hours and the plasma lipid contents, TG (A) and CHOL (B), of the mice were measured. Results are shown as means \pm SD for two experiments that were performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

To demonstrate whether the low TG level in the plasma of fenugreek seed feeding CREBH KO mice was induced by decreased assembly and secretion of VLDL in the liver, we analyzed the expression level of apoB100 in the liver of two groups of CREBH KO mice which were fed chow diet or chow diet mixed with 2% fenugreek seeds for 7 weeks by immunoblotting analysis. The result showed that the protein level of apoB100 in the liver of fenugreek group was significantly lower compared with that of chow diet group (Figure 11 A), indicating that fenugreek seeds treatment inhibited the biosynthesis of apoB100. Lipid was then extracted from the liver and TG and CHOL contents were measured. TG content in the liver of chow diet group was much higher than that of fenugreek group, while CHOL content was comparable in the liver of both groups (Figure 11 B). The results suggests than fenugreek seeds suppressed the apoB100 biosynthesis and inhibited the assembly of VLDL in the liver of CREBH KO mice. This may further lead to the decreased secretion of VLDL into the circulation and helped to attenuate the hypertriglyceridemia induced by depletion of CREBH.

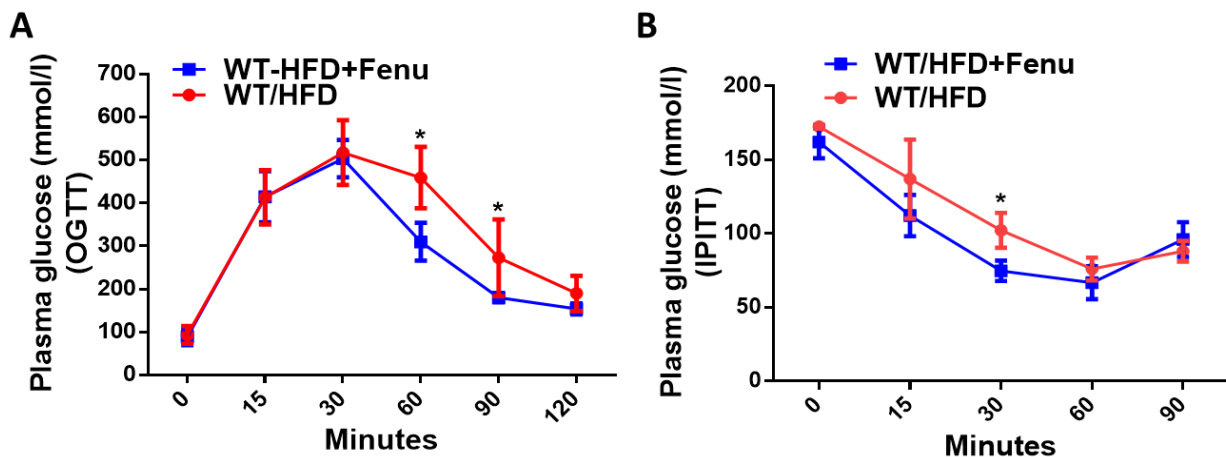


Figure 10. Effect of fenugreek seeds on insulin sensitivity of mice. Two groups of C57BL/6J (WT) mice were fed with high fat diet to induce insulin resistance, and 2% fenugreek seeds were added into the diet of one group. The mice were fed the special diets for 7 weeks and then subjected to glucose tolerance test and insulin tolerance test. After 12-hour fasting, a dose of glucose or insulin was injected into enterocoelia of the mice and glucose concentration in the blood was measured. The results of glucose tolerance test (A) and insulin tolerance test (B) were shown. Results are shown as means \pm SD for two experiments that were performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

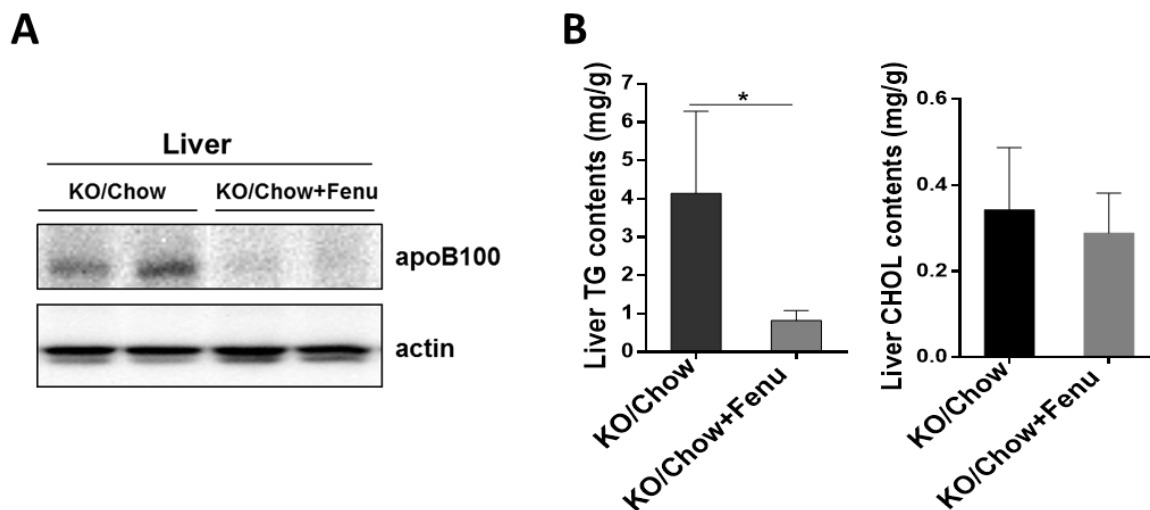


Figure 11. Impact of fenugreek seeds on VLDL assembly. Two groups of CREBH KO mice were fed with chow diet or chow diet mixed with 2% fenugreek seeds for 7 weeks. A) The protein level of apoB100 in the liver of two groups of mice were measured by immunoblotting analysis. Fenugreek seeds treatment significantly reduced the expression of apoB100 in the liver of the mice. B) Lipid was extracted from the liver and TG and CHOL contents were measured. TG content in the liver of chow diet group was much higher than that of fenugreek group, while CHOL content was comparable in the liver of both groups. Results are shown as means \pm SD for two experiments that were performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

Chapter Four

Discussion

Part 1

***A. muciniphila* ameliorates hyperlipidemia by enhancing the expression of hepatic LDL receptors to facilitate the clearance of TG rich lipoproteins and alleviating hepatic ER stress and metabolic inflammation**

Pervious study in our lab has revealed that colonization of *A. muciniphila* didn't affect the lipid content in the plasma of WT mice, whose plasma lipid concentration was within normal levels. However, the inoculation of *A. muciniphila* in CREBH KO mice, which suffered from hypertriglyceridemia because of CREBH depletion, significantly reduced the plasma TG level of the mice after 2-week treatment. We further explored the impact of *A. muciniphila* on lipid metabolism of the host, and found that after acute lipid treatment, the protein concentrations of apoB48 and apoB100 in *A. muciniphila*-WT mice were significantly lower than that of vehicle-WT mice, indicating lower CMs and VLDL content in the plasma of *A. muciniphila* group. This shows that *A. muciniphila* was able to protect the mice from instantaneous fat load. FPLC analysis showed that the TG content of VLDL, IDL and LDL particles in the plasma of CREBH KO mice was decreased by the inoculation of *A. muciniphila*. At the same time, heat-inactive *A. muciniphila* failed to ameliorate the extremely high level of TG in the plasma of CREBH KO mice or improve its insulin sensitivity, suggesting that *A. muciniphila* had to be active to play regulatory role in the metabolism of the host. These data imply that the effect of *A. muciniphila* on

the host may only be exerted when the host is within abnormal conditions, like hyperlipidemia or metabolic complications.

Previous study in our lab also investigated that the hepatic expression of LDL receptors in CREBH KO mice was remarkably increased by *A. muciniphila* administration. LDL receptor is important in mediating the clearance of VLDL remnants, IDL particles into the liver from circulation. Endogenously synthesized lipids are secreted from the liver via VLDL particles and are partially cleared via LDL receptor pathway. TGs in VLDLs are hydrolyzed by LPL in the blood to form IDLs. A part of the IDLs are rapidly endocytosed into hepatocytes mediated by the interaction of LDL receptor in the membrane of hepatocytes and apoE, a major ligand in IDLs. The other portion of IDL particles are converted to LDL by hepatic lipase. Administration of *A. muciniphila* to LDL receptor KO mice proved that *A. muciniphila* was not able to alter the abnormal lipid content in the plasma of the mice. In addition, the glucose sensitivity of LDL receptor KO mice was not improved by *A. muciniphila* treatment. These results suggested that *A. muciniphila* inoculation alleviated the hyperlipidemia by stimulating the expression of LDL receptors and promoting the uptake of lipoprotein particles. FPLC analysis also confirmed that the CHOL contents of VLDL, IDL, LDL and larger HDL particles in the plasma of CREBH KO mice are slightly reduced by increased colonization of *A. muciniphila*, consisting with the enhanced expression of hepatic LDL receptors. One of the important factors that causes hypertriglyceridemia in CREBH KO mice is impaired expression of apoA4, apoA5 and apoC2, which are the coactivators of LPL [56]. This impairment suppressed the LPL activity and inhibited the hydrolysis of TGs in lipoproteins [26]. Our study revealed that *A. muciniphila* treatment didn't alter the

mRNA level of apoA4, apoA5 or apoC2, the expression of which are defected by CREBH depletion. This indicates that the regulatory role of *A. muciniphila* is not mediated by restoring the ineffective LPL activity.

Hyperlipidemia is closely related with hepatic stress, insulin resistance and inflammation responds [103-105]. By analyzing the expression of the biomarkers of hepatic ER stress and metabolic inflammation signaling in *A. muciniphila* treated CREBH KO mice, we determined that *A. muciniphila* colonization helped to attenuate ER stress and inflammation stimulated by CREBH depletion. Moreover, the insulin sensitivity of the mice was improved by *A. muciniphila* treatment.

In summary, the inoculation of *A. muciniphila* improves the hyperlipidemia, induced by genetic depletion, for instance, of the host mice. The underlying mechanisms of the process would be enhancing the expression of hepatic LDL receptors to facilitate the clearance of TG rich lipoproteins and alleviating hepatic ER stress and metabolic inflammation, which could further ameliorate the host's insulin sensitivity. Further studies should be conducted to explore the exact regulatory impact of *A. muciniphila* and the detailed mechanism of the process.

Part 2

Fenugreek seed alleviates hyperlipidemia by inhibiting the assembly of VLDL particles and attenuating insulin resistance

In this study, we investigated the impact of fenugreek seeds on the hyperlipidemia of CREBH KO mice. 7-week treatment of fenugreek rich diet (2%) apparently reduced the

TG level in the plasma of the mice, suggesting the anti-hyperlipidemia property of fenugreek seeds. Fenugreek feeding on another mice model, WT mice fed with high fat diet, revealed that fenugreek ameliorated the impaired insulin sensitivity and enhanced the uptake of glucose into liver and adipose tissue. This further may exert beneficial effect on improving hyperlipidemia of mice. More studies should be conducted to explore the underlying mechanism that fenugreek seeds improve insulin sensitivity.

A major part of the TGs that caused hypertriglyceridemia in CREBH KO mice is in the VLDL particles in the blood of the mice [106]. Our study demonstrated that fenugreek seeds rich diet inhibited the expression of apoB100 in the liver of CREBH KO mice, and reduced the hepatic TG level. This indicated that fenugreek seeds are able to suppress the biosynthesis of apoB100 and the assembly and secretion of VLDL in the liver of CREBH KO mice, which induced the decrease of the TG contents in the circulation. Further researches are needed to find out whether fenugreek seeds could accelerate the clearance of lipoproteins in the blood of the mice.

In summary, fenugreek seeds have a beneficial property to alleviate hyperlipidemia induced by CREBH depletion in mice. Fenugreek seeds rich diet also help to attenuate insulin resistance. A potential mechanism of the anti-hyperlipidemia property of fenugreek seeds is that they could inhibit the biosynthesis of apoB100 and suppress the assembly of VLDL particles. Further studies are needed to investigate the mechanism of how fenugreek seeds impact lipoprotein metabolism and the effect of fenugreek seeds on hepatic stress and inflammatory responds of the mice.

Chapter Five

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