CHAPTER 4

Materials and methods

Animal Study

The animal usage protocols in this study were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee. 200-250 gm male Wistar rats were purchased from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand. All 24 rats were allowed to acclimate for 7 days and housed in 12/12 dark/light cycle room with controlled temperature (25°C) at the Laboratory animal facility, Faculty of Medicine, Chiang Mai University. Diet and water were provided *ad libitum* to the experimental animals during the study. After acclimatization, rats were then divided into 2 groups (12 rats per group) and fed with standard laboratory pelleted diet (standard mouse feed food No. 082, CP company, Bangkok, Thailand) defined as normal diet (ND) and high-fat diet (HFD), respectively for 12 weeks. Normal diet-fed rats (Diet Group#1) which had an energy content of 4.02 kcal/g and obtained 19.77% energy from fat, whereas high-fat diet-fed rats (Diet Group#2) received 59.28% energy from fat. After that, ND and HFD-fed rats were further subdivided into 2 subgroups (6 rats per group) and orally gavaged Diet Group#1.1 and 2.1 with 10^8 colony forming unit (CFU) of probiotic *Lactobacillus paracasei* ST11 (HP4). Vehicle control group (Diet Group#1.2 and 2.2) fed with 1 ml of sterile phosphate buffered saline (PBS) daily for 12 weeks. Probiotic *L. paracasei* ST11 (HP4) used in this study was isolated and prepared by Assistant Professor Chaiyavat Chaiyasut et al., Faculty of Pharmacy, Chiang Mai University. Probiotic *L. paracasei* ST11 (HP4) is a novel non-human origin-isolated strain of lactic acid-producing bacteria which has been approved by the Food and Drug Administration, Thailand.

Table 1 Normal Diet (ND) Composition: Normal diet from Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand

Composition	Amount (g)
Carbohydrate	495.30
Fat	83.70
Protein	269.00
Vitamins	65.40
Fiber	34.30

Table 2 High-fat diet (HFD) composition

High Fat Diet (HFD) Composition	Amount (g/kg diet)
Standard pelleted rat chow from Mouse feed Food No.082, CP, Company, Bangkok, Thailand	365
Lard	310
Casein	250
Cholesterol	10
Vitamins	60
DL-Methionine	3
Yeast powder	1
Sodium chloride	1

Metabolic parameters

Rat metabolic parameters in this study including rat body and visceral fat weights (g), plasma glucose for oral glucose tolerance test (OGTT), plasma lipid profiles as described in the previous study by Apaijai et al. (65). All experiments of this study, rats were anesthetized with isoflurane before blood collection for pain relief.

Rat body and visceral fat weights Rat body weights were recorded weekly during the study and visceral fat was collected and weighed immediately after sacrifice at the end of the study (24^{th} week).

Plasma lipid profiles Rat whole blood was collected from lateral tail vein into EDTA microcentrifuge tubes using aseptic technique after 12 hour fasting and the blood was then centrifuged at 3,000 rpm (round per minute) for 15 minutes. Rat plasma was separated and stored at -20°C until measurement. Plasma lipid profiles including total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL)/very low-density lipoprotein (VLDL) levels which were detected at 12^{th} week after diet-induced period and 24^{th} weeks after probiotic intervention (defined as pre- and post-treatment, respectively).

Plasma total cholesterol Rat frozen plasma was thawed at room temperature (25°C) before measurement. Cholesterol levels were determined using a commercial colorimetric assay kit (Erba Diagnostics Mannheim, Germany) as previously described (65). The reagent was developed on the basis of the formulation of Allain et al. (66) and further modified by Roeschlau et al. (67). Cholesterol ester in plasma samples is enzymatically hydrolyzed by cholesterol esterase (provided in the reagent) and turned into cholesterol and fatty acid. Then, free cholesterol is oxidized by cholesterol oxidase (in the reagent) with O_2, generating cholest-4-en-3-one and hydrogen peroxide (H_2O_2). By product of cholesterol oxidation, H_2O_2 further reacts with 4-aminoantipyrine (4-AAP) and phenol activated by peroxidase enzyme forming a chromophore (quinoneimine dye) which can be detected at 505 nm. The brief procedure includes the following steps: *(1)* prepared of reagent blank (1.0 ml of Reagent 1+ 0.01 ml of distilled water), standard (1.0 ml of Reagent 1+ 0.01 ml of calibrator) and sample (1.0 ml of Reagent 1+ 0.01 ml of rat plasma sample), *(2)* completely mixed and incubated for 10 minutes at 37°C, *(3)*

measured the absorbance of the sample (A_{sam}) and standard (A_{st}) over reagent blank, then calculated the cholesterol concentration using the following formula:

$$\textbf{Total cholesterol concentration (mg/dL)} = \frac{\Delta \textbf{Asam}}{\Delta \textbf{Ast}} \times \textbf{Cst}$$

C_{st} is concentration of standard (calibrator)

Plasma triglyceride Rat plasma was collected as described above and stored at -20°C until measurement. Commercial available colorimetric assay kit (Erba Diagnostics, Mannheim, Germany) (6) was applied to quantify triglyceride levels from rat plasma at the specific time points. Triglycerides are hydrolyzed into glycerol and free acids by enzymatic activity of lipase. The glycerol is phosphorylated by action of glycerol kinase (GK) along with adenosine triphosphate (ATP) to generate glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol phosphate oxidase oxidizes glycerol-3-phosphate to dihydroxy-acetone phosphate (DAP) producing H_2O_2. The produced H_2O_2 further reacts with 4-chlorophenol and 4-AAP catalyzed by peroxidase resulting in a red colored dye production. Briefly, triglyceride detection procedure consists of the following steps: preparation of reagent blank by adding 0.01 ml of distilled water into 1.0 ml of Reagent 1, standard by mixing 1.0 ml of Reagent 1 with 0.01 ml of calibrator and sample by transferring 0.01 ml of rat plasma sample into 1.0 ml of Reagent 1. The absorbance of the sample (A_{sam}) as well as standard (A_{st}) was detected at 500 or 546 nm against reagent blank. The concentration of triglyceride was calculated by the following formula:

$$\textbf{Triglyceride concentration (mg/dL)} = \frac{\Delta \textbf{Asam}}{\Delta \textbf{Ast}} \times \textbf{Cst}$$

C_{st} is concentration of standard

Plasma HDL Rat plasma HDL level was examined using a commercial HDL quantification colorimetric assay kit (Biovision, CA, US) (65). There were 6 main following steps for measuring rat plasma HDL levels including: *(1) Separation of HDL* by adding 100 µl of 2X Precipitation Buffer with 100 µl of sample into microcentrifuge tube. The preparation was incubated at room temperature for 10 minutes and then centrifuged at 5000 rpm for 10 minutes. The supernatant, the HDL fraction, was transferred to a new microcentrifuge tube and the precipitates were the LDL/VLDL.

(2) Standard curve and sample preparations adding 20 µl of the Cholesterol Standard to 140 µl of Cholesterol Assay Buffer for diluting the Cholesterol Standard to 0.25 µg/µl and mixed thoroughly, then added 0, 4, 8, 12, 16, 20 µl into a series of 96-well plate. To generate 0, 1, 2, 3, 4, 5 µl/well of the Cholesterol Standard, volume in each well was adjusted to 50 µl/well. *(3) Reaction Mix Preparation* by preparing a total 50 µl Reaction Mix for each assay containing: 44 µl Cholesterol Assay Buffer, 2 µl Cholesterol Probe, 2 µl Enzyme Mix and 2 µl Cholesterol Esterase. *(4)* 50 µl of the Reaction Mix was added to each well containing the Cholesterol Standard or rat plasma sample, and mixed thoroughly. *(5)* The reaction was incubated at 37°C for 60 minutes (the plate was protected from light) and measured the absorbance at 570 nm by micro-titer plate reader. *(6) Calculation* the standard curve was plotted after subtracting the readings by 0 standard reading and the sample readings were applied to the standard curve to determine plasma HDL amount in each well. HDL concentration was calculated by the formula

$$\textbf{HDL concentration (µg/µL)} = \frac{\textbf{A (µg)}}{\textbf{V (µL)}}$$

below:

A represents the plasma HDL amount from the standard curve (µg)

V represents original sample volume added to the sample reaction well (µL)

Plasma LDL/VLDL Rat plasma LDL/VLDL was calculated from plasma total cholesterol and triglyceride levels along with HDL concentration using the following formula by Friedewald et al. (68).

LDL/VLDL concentration = [TC] - [HDL] - [TG]/5

Friedewald's formula:

[TC] = total cholesterol concentration

[HDL] = HDL concentration

[TG] = triglyceride concentration

Oral glucose tolerance test (OGTT) The rats had been fasted overnight for 12 hours before OGTT was conducted as described previously (69). Fasting blood was collected in sodium fluoride (NaF)-containing microcentrifuge tube at 0-minute before feeding with 2.0 g/kg body weight of glucose. After glucose administration, rats were blood withdrawn at 15, 30, 60, and 120 minutes. Plasma was then separated and stored at -20°C immediately for plasma glucose level measurement. Plasma glucose concentration was determined using a commercial available colorimetric assay kit (Erba Diagnostics Mannheim, CZ). Glucose in sample is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase (H_2O_2). Then H_2O_2 was further catalyzed by peroxidase in the presence of phenol and 4-AAP to generate red dye. In brief, reagent blank was prepared by adding 10 µl distilled water into 1.0 ml Reagent 1. Standard and samples were prepared by mixing 1.0 ml of Reagent 1 with 10 µl standard (calibrator) or 10 µl sample, respectively. After well-mixing, prepared reagent blank, standard and samples were incubated at 37°C for 10 minutes. Absorbance of the sample and standard was measured against reagent blank at 500 nm. Rat glucose concentrations were calculated by applying to the following formula:

$$\textbf{Glucose concentration (mg/dL)} = \frac{\Delta \textbf{Asam}}{\Delta \textbf{Ast}} \times \textbf{Cst}$$

ΔA_{sam} = absorbance of the sample (A_{sam}) against reagent blank

ΔAst = absorbance of standard (Ast) against reagent blank

C_{st} = concentration of standard

Plasma insulin and the HOMA index Plasma insulin levels were assessed using a commercial ELISA kit (LINCO research, Missouri, US) (65) and the homeostasis model assessment (HOMA) index, a parameter providing more accurate insulin sensitivity evaluation, which was calculated from fasting plasma insulin (FPI) and fasting plasma glucose (FPG) (70) (71). Rat plasma insulin concentration was determined following a Sandwich ELISA assay kit protocol as mentioned above. The HOMA index calculated using a computer-solved model by the following formula:

$$\textbf{HOMA-Insulin resistance} = \frac{\textbf{FPI x FPG}}{\textbf{22.5}}$$

FPI = fasting plasma insulin

FPG = fasting plasma glucose

Metabolic Endotoxemia

Serum lipopolysaccharide (LPS) level was used as an indicator of metabolic endotoxemia (36)(35)(34)(1). Serum LPS levels were measured by colorimetric method using the Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Rat serum was collected at 12th (pre-treatment) and 24th (post-treatment) weeks and then diluted (1:10) with sterile endotoxin-free water, inactivated at 70°C for 15 minutes. The heat-inactivated serum was incubated with limulus amoebocyte lysate (LAL) at 37° for 10 minutes as described previously (34). After adding substrate solution, the development of magenta-colored derivative was detected using the absorbance at 410 nm. The concentrations of serum LPS in the samples were then calculated using the standard curve and reported in EU/mL.

Rat Fecal and Colon Content Bacterial Cultures

Fresh rat feces at 12th week and colon content at 24th week were collected for viable bacterial cultures using 1 ml of sterile PBS in 1.5 ml sterile microcentrifuge tube. Feces collecting microcentrifuge tubes were weighed before and after sample collection (approximately 0.1-0.2 g feces or colon content) and processed immediately. Fresh fecal pellets and colon contents were re-suspended and diluted in sterile PBS in ten-fold serial dilution for bacterial counting. Then, 100 µl of the diluted bacterial solutions were plated and enumerated on the selective agars, including MacConkey agar (Himedia®, M008S) for Enterobacteriaceae, Lactobacillus MRS agar (Himedia®, M641) for *Lactobacillus* and Bifidobacterium (BL) agar (Nissui Pharm, Japan) for *Bifidobacterium* (72)(41)(34) . MacConkey agar plates were incubated aerobically at 37°C for 24 hours whereas Lactobacillus MRS agar plates required a microaerophilic condition by pouring sterile melting Lactobacillus MRS agar on the surface of inoculated Lactobacillus MRS medium and incubated at 37°C for 48 hours. BL agar plates were anaerobically incubated using anaerobic gas pack (GasPack-Kit, Schuett-Biotec, DE)-containing anaerobic jar at 37°C

for 72 hours. Pink and colorless colonies on MacConkey agar were represented the Enterobacteriaceae members, white colonies on Lactobacillus MRS media were classified as the Lactobacilli and the Bifidobacteria showed as brown to dark brown colonies on BL agar (73).

Rat Tissue Pro-Inflammatory Cytokine Gene Expressions

Transcriptional levels of pro-inflammatory cytokine genes, including *Il1b and Il6*, were determined as previously described by Peinnequin et al. (74). In brief, the frozen rat ileum or the section of the ileum in RNA preservative solution was homogenized by using 1 mm sterile zirconia/silica bead (Biospec Products, Bartlesville, US) and Minibeadbeater (Biospec Products, Bartlesville, US) and extracted tissue RNA using TRI reagent (TRIzol® Reagent, Ambion, Life Technologies, CA, US) according to the recommendations of the manufacturer. Then, a DNase treatment was performed by adding the DNA removal and inactivation kit (Ambion, Life Technologies, CA, US). The extracted tissue RNA was converted to complementary DNA (cDNA) using reverse transcription reagents (Tetro cDNA synthesis kit, Bioline, US). SYBR-Green (SensiFAST SYBR Lo-ROX kit, Bioline, US) based real-time quantitative PCR was conducted using the primers (final concentration 400 nM) in Table 3 and further analyzed by comparative Ct method. The mRNA expression levels of target genes (*Il1b* and *Il6*) were normalized with the housekeeping gene *Gapdh* mRNA levels.

Table 3 Primer pairs for quantitative real-time RT-PCR of rat tissue

Organism	Target gene	Primers	References
Rattus norvegicus	*Gapdh*	5'-GTATTGGGCGCCTGGTCACC-3' 5'-CGCTCCTGGAAGATGGTGATGG-3'	Peinnequin et al. 2004 (72)
Rattus norvegicus	*Il1b*	5'-CACCTCTCAAGCAGAGCACAG-3' 5'-GGGTTCCATGGTGAAGTCAAC-3'	Peinnequin et al. 2004 (72)
Rattus norvegicus	*Il6*	5'-TCCTACCCCAACTTCCAATGCTC-3' 5'-TTGGATGGTCTTGGTCCTTAGCC-3'	Peinnequin et al. 2004 (72)

Statistical Analysis

Rat body weights, visceral fat weights, plasma total cholesterol, plasma triglyceride, plasma HDL, plasma LDL/VLDL, plasma glucose, and serum LPS were expressed as a mean and standard error of mean (SEM). Bacterial numbers were displayed as a geometric mean. All data were analyzed using GraphPad Prism 6. A one-way analysis of variance (ANOVA) and a Student's *t*-test were performed and P-value ≤ 0.05 determined statistically significant difference between the tested groups. Bacterial numbers and changes in pro-inflammatory cytokine mRNA levels were logarithmically transformed prior to statistical analysis using a one-way ANOVA and a Student's *t*-test.