CHAPTER III

MATERIALS AND METHODS

1. Subjects

Sixty-four healthy human volunteers of both sexes ranged in age from 17-19 years. They were students at the Thai Red Cross College of Nursing, Chulalongkorn University. No subject had been on antibiotic treatment or consumed yogurt for at least two months prior to fecal sampling. Each individual gave informed consent. Fresh fecal samples were taken by sterile cotton swab and inserted into a modified Cary-Blair transport medium. The samples were processed immediately upon receipt.

2. Culture of Fecal Samples

The fecal samples were suspended in normal saline solution (NSS) and 10-fold serial dilutions were diluted to 10^6 times. Finally 100 µl of each dilution was plated in duplicate by spreading onto deMan-Rogosa-Sharpe (MRS) agar plates (Becton Dickinson, Sparks, MD, USA.). The plates were incubated under anaerobic condition at 37°C for 48-72 hr in an anaerobic chamber (Envimed, England). Bacterial colonies that developed on the plates with different morphologies were individually picked and streaked on new MRS agar plates. After anaerobic incubation, single pure colonies were isolated and subcultured for experimental use.

3. Selection of *Lactobacillus* Isolates

Each of the isolates was presumptively tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase-negative were examined by Gram stain. Subsequently, only those which were gram-positive were tested by vancomycin (VA) susceptibility testing. VA susceptibility test was assessed by a modified Kirby-Bauer disk diffusion test as previously described [5]. Briefly, *Lactobacillus* isolates were suspended in NSS to a 0.5 McFarland standard and swabbed onto MRS agar plates. Vancomycin impregnated disks (5 μg; Becton Dickinson, Sparks, MD, USA) were applied to bacterial cultures, which then grown under anaerobic conditions at 37°C for 24-48 hr. Isolates displaying inhibition zones of greater than 15 mm were considered susceptible. All isolates that were gram-positive, regular rods or short rods, catalase-negative and vancomycin resistant were maintained as frozen cultures in MRS broth with 20% glycerol at -80°C for experimental use.

4. Antagonistic Activity Assay by Using Agar Well Diffusion Method

The frozen cultures of *Lactobacillus* isolates were streaked for isolation on MRS agar. After anaerobic incubation, a single colony was isolated and subcultured on MRS agar for experimental use. The isolates were kept at 4°C and subcultured every 3-5 days. For determination of antimicrobial activities, eight gastrointestinal pathogens including *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311, *Shigella flexneri* DMST 4423, *Vibrio cholerae non* O1 DMST 2873,

enterohemorrhagic *E.coli* (EHEC) O157:H7 DMST 12743, enterotoxigenic *E. coli* (ETEC) DMST 20970, enteropathogenic, *E. coli* (EPEC) DMST 20972 and enterinvasive *E. coli* (EIEC) DMST 20971 were provided by the Department of Medical Science, Ministry of Public Health, Thailand. They were grown on Mueller-Hinton agar (MH: Difco, USA) under aerobic condition at 37°C for 24 hr. Two gastrointestinal pathogens of *Campylobacter jejuni* and *Clostridium difficile* were provided by Division of Bacteriology, Faculty of Medicine, Chulalongkorn University. *C. jejuni* was grown on Brucella agar (Difco, USA) and incubated at 42°C for 48 hr under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) generated by using a gas package (BBL, Becton Dickinson, USA) in anaerobic jars. *C. difficile* was grown on Brucella agar and incubated at 37°C for 48 hr under anaerobic conditions (0% O_2, 5% CO_2, 5% H_2, 90% N_2) in anaerobic chamber.

Agar well diffusion assay as previously described [4, 93, 113] was used to determine antimicrobial activity of the *Lactobacillus* isolates against gastrointestinal pathogens. Approximately 10^9 cells/ml of each *Lactobacillus* isolate were inoculated (10%) into 9 ml of modified MRS broth (MMRS) pH 6.6-6.8 containing only 0.2% glucose [4] and incubated anaerobically at 37°C for 24-48 hr. After incubation, the supernatant was collected by centrifugation at 6,000 x g at 4°C for 10 min and filter-sterilized by using a 0.22 μm pore size filter (Millipore, MA, USA). Supernatants were neutralized with sterile 1 M NaOH to raise their pH to 6.6-6.8 equal to MMRS. Non-neutralized supernatants pH 5.6-5.8 and neutralized supernatants of *Lactobacillus* isolates were tested for antibacterial activities against gastrointestinal pathogens. Approximately 1 x 10^8cells/ml overnight cultures of *E. coli*, *S.* Typhimurium, *Sh. flexneri, V. cholerae,* EHEC, ETEC, EPEC and EIEC in MH and

C. jejuni and *C. difficile* in Brucella were inoculated at a final concentration of $1x10^7$ cells/ml into each tube of 25 ml pre-warmed MH agar and Brucella agar at 45°C respectively. Each mixture was then poured into each plate and allowed to solidify. Circular wells of 10 mm diameter and 4 mm deep were made in the agar using sterile cork borers. Subsequently, 300 µl of the non-neutralized supernatant and neutralized supernatants of each *Lactobacillus* isolate were transferred into a well. Control test material was also prepared using uninoculated MMRS broth bacterial media cultures. The plates were incubated for 24-48 hr at appropriate conditions of pathogens as described above to allow pathogen growth. The inhibition zone, a clear zone around the well showing no growth of pathogens, was measured and recorded in mm from the center of the well. The experiments were performed in duplicate.

5. Reuterin Detection of *Lactobacillus* Isolates

Reuterin detection was used to detect reuterin-producing strains of *L. reuteri* according to the instructions by Biogaia AB (BioGaia AB, Stockholm, Sweden) [158]. A total of 437 *Lactobacillus* isolates were tested for reuterin production. *L. reuteri* SD2112 (ATCC 55730), a reuterin-producing strain (BioGaia AB, Stockholm, Sweden), was used as a positive control and MRS was used as a negative control. Each *Lactobacillus* isolate was inoculated into 200 µl MRS broth in each well of the first 96-well plate and incubated anaerobically at 37°C for 24-48 hr. Each culture of *Lactobacillus* was transferred by using Frogger (DAN-KAR CCRP, MA, USA) into 175 µl MRS broths in each well of second 96-well plate. After incubation under anaerobic conditions at 37°C for 24 hr, each culture was inoculated by using Frogger and spotted onto the surface of brain heart infusion agar (BHI; Difco, USA)

supplemented with 20 mM glucose in a 140 mm large plate. *Lactobacillus* spots were allowed to develop by incubated anaerobic conditions at 37°C for 24 hr. Soft agar (1% agar) solutions were made with 2% glycerol (Fisher, USA) and boiled until agar dissolved, mixed well and kept in a warm water bath at 46°-47°C. Subsequently, 30 ml of soft agar were overlaid over the surface of *Lactobacillus* plate and allowed agar to solidify for 5 min and then incubated the plates straight up anaerobically at 37°C. After incubation for 1 hr, plates were removed and placed on the laid white paper towel on the bench top. The plates were opened and flood the surface with 25 ml of DNPH solution (2-4 dinitrophenylhydrazine: Sigma, USA) and waited for 5 minutes. After discarded the DNPH in a waste container, 25 ml of KOH (potassium hydroxide: Fisher, USA) solution was added to the plate and watched for reddish brown color to develop around the reuterin producing strains which indicated reuterin positive strains appropriately. KOH solution was discarded as a hazardous chemical waste. These experiments were performed two times in duplicate.

6. Antagonistic Activity Assay by Using Agar Spot Method

Four hundred and thirty seven *Lactobacillus* isolates were tested for pathogens inhibition against *Salmonella enterica* and *Vibrio cholerae.* Pathogen inhibitory strain of *L. reuteri* SD2112 was used as positive control and MRS bacterial media culture was used as negative control.

An agar spot test was used to detect antimicrobial activities of *Lactobacillus* isolates against *Salmonella enterica* and *Vibrio cholerae*. This method was carried out as previously described [159, 160] with modification by Splinler et al. [88]. Briefly, overnight cultures of *Lactobacillus* isolates in each well of 96-well plate were spotted

by using frogger (DAN-KAR CCRP, MA, USA) onto the surface of BHI agar supplemented with 20 mM glucose in 140 mm large plate by using frogger. The spots were developed by incubated anaerobic condition at 37°C for 24 hr. The *Lactobacillus* spots in each plate was overlaid with 25 ml of BHI broth containing 0.7% agar, 2% glycerol and each of overnight culture of *S. enterica* or *V. cholerae* at a final concentration of 1 x 10^6 cells/ml. Plates were allowed to solidify for 5 min and then the plates were incubated anaerobically at 37°C for reuterin production. After incubation for 1 hr, plates were removed and incubated aerobically at 37°C for 24 hr. Inhibition zones were measured after 18-24 hr and a clear zone of equal or more than 1 mm around a spot was scored as positive. These experiments were performed two times in duplicate.

7. Assay for Immunomodulatory Effects of *Lactobacillus* Isolates on TNF-α Production in LPS-Activated THP-1 Monocytic Cells

7.1 Preparation of conditioned media

Forty-six *Lactobacillus* isolates of human fecal samples were randomly selected and recovered from -80°C to test for TNF-α inhibitory activity in THP-1 human monocytic cell line. *Lactobacillus reuteri* MM4-1A (ATCC PTA 6475), TNF-α inhibitory strain was used as positive control and *L. reuteri* SD2112 (ATCC 55730), non-TNF-α inhibitory strain, was used as negative control. These two strains were isolated from human breast milk by Biogaia AB (BioGaia AB, Stockholm, Sweden). The *Lactobacillus* conditioned media (LCM) was prepared as previously described [45]. In brief, 46 *Lactobacillus* isolates, *L. reuteri* MM4-1A and *L. reuteri*

SD2112 were grown anaerobically in MRS broth at 37°C overnight. The overnight cultures of lactobacilli were diluted to an OD_{600} of 0.1 (representing approximately 10^8cells/ml) in MRS broth and grown anaerobically at 37°C for 24 hr. *Lactobacillus* cell-free conditioned media was collected by centrifugation at 4,000 x g for 10 min and filter-sterilized using a 0.22 μm pore size filter (Millipore, Bedford, MA, USA). The conditioned media was then pH adjusted by speed-vacuum drying (Speed vacuum DNA 110; Savant Instruments, USA) and the pellet was re-suspended in an equal volume of cell culture media. All *Lactobacillus*-conditioned media were frozen at -20°C until analysis.

7.2 THP-1 cell culture and bioassay

Cell culture and *in vitro* bioassays were performed as previously described [45]. Briefly, the THP-1 human monocytic cell lines (ATCC TIB 202) were maintained in RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA, USA) in 96-well flat-bottomed tissue culture plates (Falcon; Becton Dickinson) and incubated at 37°C in humidified 5% CO_2 incubator. For bioassays, cells were counted with hemacytometer (Hausser Scientific, USA) under an inverted microscope (Nikon eclipse TS100: Nikon, Japan) and diluted to desired cell densities. Two hundred microliters of THP-1 cells were seeded in 96-well plates at a final density $2.5x10^5$ cells/ml. Cells were incubated with 10 μl of *Lactobacillus* conditioned media (5% v/v) and 5 μl of (final concentration 100 ng/ml) purified lipopolysaccharide (LPS) from *Escherichia coli* serotype O127:B8 (Sigma, St. Louis, MO, USA).

After 3.5 hr at 37°C in a humidified 5% CO_2 incubator, supernatants were collected by centrifugation at 1,000 x g for 9 min in 4°C for TNF-α measurement. Cell viability was assessed by the Trypan-blue (Gibco-Invitrogen, Carlsbad, CA, USA) stain exclusion assay.

Strains with most potent TNF-α inhibitory activity was chosen for determining the optimal condition of TNF-α inhibition. *Lactobacillus* strains were cultivated in MRS and incubated anaerobically at 37°C for 24 hr and 48 hr. *Lactobacillus* conditioned media was prepared as described above.

7.3 TNF-α measurements

TNF-α production in THP-1 monocytic cell culture supernatants were measured with cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well microtiter plates (Immunolon 2 HB, Thermo,USA) were coated overnight with 100 µl mouse anti-human TNF-α antibodies as capture antibodies diluted in phosphate-buffered saline (PBS: Sigma, USA) pH 7.4. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST) to remove excess capture antibody. To reduce non-specific binding, wells were blocked with 300 µl of 1% (w/v) bovine serum album (BSA: Sigma, USA) in PBS (reagent diluent) for 2 hr and washed three times with PBST. Recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) was used as standard diluted in reagent diluent at the concentration of 15.625, 31.5, 62.5, 125, 250, 500, and 1,000 pg/ml. Reagent diluent was used for blank. Standard or samples were added (100 µl volume) to appropriate wells and plates were incubated overnight. After

washing three times with PBST, 100 µl per well of biotinylated goat anti-human TNF-α antibodies, (R&D Systems, Minneapolis, MN, USA) diluted in reagent diluent were added as detection antibodies and incubated for 2 hr. The plates were washed three times with PBST and incubated with 100 µl per well of streptavidin-horseradish peroxidase conjugate for 20 min (R&D Systems, Minneapolis, MN, USA). After washing three times, 100 µl per well of TMB substrate (tetrametyl benzidine: BioFX, USA) was added to the plates as color indicator and incubated for 20 min. Stopping reagent consisting of H_2SO_4 was added (50 µl to each well) to stop the reaction. Absorbance was measured at 450 nm using a Spectramax 340PC (Molecular Devices Corporationvale, CA, USA). All procedures were performed at room temperature. Results of cytokine concentration were quantified from standard curve and expressed as pg/ml of culture medium.

7.4 Statistical analysis

These experiments were performed three times in triplicate. Results were reported as means and standard deviations (SD). The statistical differences were evaluated by using a Student's *t* test with a one-tailed distribution. A p value ≤ 0.05 was considered statistically significant, and n represents the number of experiments performed.

8. Effect of Selected *Lactobacillus* Strains on Nuclear Factor kappa B (NF-κB) Activation

8.1 Bioassay for assessment of NF-κB and preparation of nuclear extract

THP-1 monocytic cells suspended in RPMI 1640 medium supplemented with 10% FBS were counted as described above, and one ml of cell suspensions were seeded at the final density of 1.0 x10^6 cells/ml in 12-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA). THP-1 monocytic cells were incubated with 50 µl (5% v/v) *Lactobacillus* conditioned media in the presence or absence of 1 µg/ml of LPS from *Escherichia coli* serotype O127:B8 (Sigma, St. Louis, MO, USA) at 37°C in humidified 5% CO_2 incubator for 30 minutes. The THP-1 cells were collected to 1.5 ml tube by centrifugation and kept on ice before isolation of nuclear extracts with the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, THP-1 1x10^6 cells were washed with ice-cold PBS and suspended in 200 µl of hypotonic buffer (Active Motif, Carlsbad CA, USA) and suspended couple of times by pipetting up and down several times. The tube was then kept on ice 15 min, 25 µl of detergent were added (Active Motif, Carlsbad, CA, USA) and then mixed thoroughly using a vortex mixer 30 sec 2 times at highest setting. The nuclear pellets were collected by centrifugation 10,000 x g at 4°C for 10 min and re-suspended in 20 µl of ice-cold complete lysis buffer containing 10 mM dithiothreitol (DTT) and protease inhibitor by pipetting up and down. The samples were vortexed 8 times for 30 seconds with each pulse at highest setting and subjected to centrifugation at 14,000 x g for 10 min in a microcentrifuge pre-cooled at 4°C. The nuclear extracts were aliquoted and stored at -80°C for further study.

8.2 Protein Content

The protein contents of nuclear extracts were quantitated using the microplate procedure of BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) based on manufacturer's instructions. In brief, bovine serum album (BSA: Sigma, USA) was used as standard diluted in lysis buffer (the same diluent of nuclear extract sample) at the concentration of 250, 500, 1,000, 2,000, and 4,000 μg/ml. Lysis buffer was used for blank. BCA™ Working Reagent (WR) was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, reagent A:B). Each standard or samples were added with 25 μl in replicate into appropriate wells. Working Reagent was added with 200 μl into each well and plates were mixed thoroughly on a plate shaker for 30 seconds. Plates were covered and incubated at 37°C for 30 min, and cooled to room temperature. Absorbance was measured at 560 nm using a Spectramax 340PC (Molecular Devices Corporationvale, CA, USA). Results of protein measurements were quantified from standard curve and expressed as μg/ml.

8.3 NF-κB measurements

NF-κB levels of THP-1 nuclear extracts were measured by NF-κB p65 ELISAs with TransAM™ NF-κB p65 Transcription Factor Assay Kits (Active Motif, Carlsbad CA, USA) according to the manufacturer's instructions. Briefly, 30 μl complete binding buffer (containing 5 μl of 1 M dithiothreitol (DTT)) and 10 μl of protease inhibitors were added to each well. Each well has immobilized oligonucleotides containing the NF-κB consensus site (5′-GGGACTTTCC-3′), and 20 μl of 10 μg nuclear extracts diluted in complete lysis buffer were added to each

well. Twenty microliters of NF-κB p65 which contained 2.5 μg of Jurkat nuclear extracts were added as positive controls and 20 μl of complete lysis buffer were added for blank. Plate was incubated for 1 hr at room temperature with mild agitation (100 rpm on a rocking platform). After washing three times with 200 μl washing buffer (Active Motif, Carlsbad CA, USA), 100 μl per well of diluted NF-κB p65 antibody were added and incubated for 1 hr at room temperature without agitation. Each plate was washed three times with 200 μl washing buffer and incubated with 100 μl of diluted HRP in binding buffer (containing 2 μl of 1 M DTT, and 10 μl of 1 μg/μl herring sperm DNA per ml of binding buffer) for 1 hr at room temperature without agitation. Plate was washed 4 times and 100 μl of developing solution was added and incubated for 5 min at room temperature protected from direct light. One hundred μl of stopping solution was added to each well to stop the reaction. Absorbance was measured at 450 nm using a Spectramax 340PC (Molecular Devices Corporationvale, CA, USA).

9. Phenotypic Characterization of *Lactobacillus* TH58 Strain

9.1 Morphology

Colony morphology of TH58 and TH14 strains were grown on MRS agar and incubated anaerobically for 24-48 hr at 37°C. Microscopic characteristics of these strains were examined by Gram stain and observed by light microscope (Nikon, Japan).

9.2 Growth characteristic of *Lactobacillus* TH58 strain

The most potent TNF-α inhibitory strain was studied for growth characteristics in MRS broth. This *Lactobacillus* isolate was grown anaerobically in MRS broth at 37°C overnight. Overnight cultures of *Lactobacillus* TH58 were diluted to an OD 600 of 0.1 which represented approximately 10^8 cells/ml in MRS broth and grown anaerobically at 37°C. *Lactobacillus* cultures were taken at time point 0, 2, 4, 6, 8, 12, 24, 28, 36, 40, 48, 54 and 57 hrs respectively to measure optimal densities at 600 nm using spectrophotometer (SmartSpec 3000, BioRad Laboratories, Richmond, CA, USA). Growth curves of this candidate strain was plotted between cell numbers of bacteria (cells/ml) versus each time point. The generation time was calculated by the formula as follows.

$$G = t/n$$

G = generation time

t = time interval in hours or minutes

n = number of generations; n = 3.3 log b/B

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

9.3 Acid, bile and aerotolerance

9.3.1 Acid tolerance

MRS broth was adjusted with 1 N HCL to pH 1.2, 2.5, 3.5 and 4.5 respectively. Unadjusted MRS broth was used as control. Overnight cultures of strain TH58 was diluted to an initial bacterial concentration of 10^8cells/ml in each 10 ml of

adjusted pH MRS broth in triplicate and incubated at 37°C for 3 hr under anaerobic condition. *Lactobacillus* cultures were taken and serial 10-fold dilutions (in PBS, pH 7.2) of each sample were prepared ($10\text{-}10^{-6}$). Viable bacterial counts were determined by spreading 100 µl of each serial dilution onto MRS agar plates. The plates were incubated under anaerobic condition at 37°C for 24-48 hr in an anaerobic chamber (Model 1025 S/N, Forma Scientific, Inc., Marietta, Ohio, USA). Viable bacteria were displayed as cell numbers and the log10 of colonies grown on MRS agar compared to the initial bacterial concentration.

9.3.2 Bile tolerance

The most potent TNF-α inhibitory strain was tested for bile tolerance in order to evaluate the relative survivability of candidate probiotics in the intestine. *Lactobacillus* was inoculated in 10 ml MRS broth supplemented with 1%, 2%, 3%, 4% and 5% bovine bile (Sigma, USA) and MRS control cultures without bovine bile. The initial bacterial concentration was 10^8cells/ml. All samples were incubated for 3 hr at 37°C under anaerobic conditions. After incubation, *Lactobacillus* cultures were taken and 10-fold serial dilutions of each sample were prepared ($10\text{-}10^{-6}$). Viable cell counts were determined by spreading 100 µl of each dilution onto MRS agar plates. The plates were incubated under anaerobic conditions at 37°C for 24-48 hr in an anaerobic chamber (Model 1025 S/N, Forma Scientific, Inc., Marietta, Ohio, USA). Viable bacteria were displayed as cell numbers and the log 10 of colonies grown on MRS agar compared to the initial bacterial concentration.

9.3.3 Aerotolerance

Overnight culture of most potent TNF-α inhibitory strain was inoculated into culture flask of 25 ml MRS broth with initial bacterial concentration of 10^8 cells/ml. *Lactobacillus* was grown and incubated at 37°C under aerobic conditions and shaken at 100 rpm, and grown under anaerobic conditions at 37°C. Bacterial survival in both conditions were measured by reading the optical density (OD) at 600 nm using a spectrophotometer SmartSpec 3000 (Biorad, USA) and determined by plate counting on MRS agar after 24-48 hr.

10. Phenotypic Characterization of Selected *Lactobacillus* Strains by API 50 CHL

Carbohydrate fermentation patterns of selected *Lactobacillus* strains were determined by using the API 50 CH rapid fermentation test strips (BioMerieux, France) and the *Lactobacillus* identification (API 50 CHL) medium (BioMerieux, France) according to the manufacturer's instructions. Briefly, the cell pellets of *Lactobacillus* overnight cultures collected by centrifugation at 20,000 x g for 5 min were resuspended in NSS and diluted to a 2 McFarland standard in API 50 CHL medium. The diluted cultures were then loaded to the API 50 CH test strips and covered with mineral oil, and the test strips were incubated at 37°C for up to 48 hours. The test strip information was read and recorded at 24 and 48 hr. Carbohydrate fermentation profiling was performed in duplicate. Species were determined by characterizing their abilities to ferment 49 carbohydrates, and comparing profiles with

known carbohydrate fermentation profiles of lactobacilli in the API 50 CHL version 5.1 database (http://apiweb.biomerieux.com).

11. Genotypic Characterization of Selected *Lactobacillus* Strains

11.1 DNA extraction

DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation Madison, WI, USA) following the manufacturer's instructions. Briefly, cell pellets in 5 ml overnight culture of *Lactobacillus* in MRS were collected by centrifugation and re-suspended in 2 ml of 50 mM EDTA and 10 µl of 5 U/ µl of mutanolysin. The sample was incubated at 37°C for 2 hr and centrifuged for 2 min at 13,000 x g and the supernatant was then removed. The sample was gently resuspended in 600 µl of nuclear lysis solution and incubated at 80°C for 5 min to lyse the cells, then cooled to room temperature. Three microliters of RNase solution were added and incubated at 37°C for 60 min. Sample was cooled to room temperature and then 200 µl of protein precipitation solution was added and vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. After incubation on ice for 5 min, sample was centrifuged at 13,000 × g for 5 min. Supernatants containing DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. Tube was gently mixed by inversion until the thread-like strands of DNA formed a visible mass followed by and centrifugation at 13,000 x g for 2 min. The supernatants were poured off carefully and drained the tube on clean absorbent paper. The DNA pellet was washed by adding 600 µl of room temperature 70% ethanol and gently inverting the tube several times to wash the DNA pellet followed by centrifugation at 13,000 x g

for 2 min. The ethanol was aspirated and the tube was drained on clean absorbent paper. The pellet was air-dried for 15 min or placed in 37°C incubator. Rehydration solution (50 µl) was added to each DNA pellet tube and the DNA was rehydrated by incubation at 65°C for 1 hr. Genomic DNA was stored at 2-8°C. DNA concentrations were determined by absorbance spectrophotometry using the Nanodrop-1000 (NanoDrop Technologies, Wilmington, DE, USA). Genomic DNA was determined by 1% agarose gel (ISC Bioexpress, USA) electrophoresis in TAE buffer (Biorad, USA) with 135 mA and subsequently visualized by ultraviolet illumination using ChemiImager 500: ACQUIR software, after staining with ethidium bromide. All DNA solutions were stored at -20°C for further studies.

11.2 16S rRNA gene sequencing

Genotypic characteristic by 16S rRNA gene sequencing was performed according to the method as previously described [5]. Briefly, the template DNA for sequencing of the 16S rRNA gene was amplified using forward primers 16S-8F (5′-AGA GTT TGA TCY TGG YTY AG-3′) and reverse primers 16S-1541R (5′-AAG GAG GTG WTC CAR CC-3′). Polymerase chain reactions (PCR) were performed in a total volume of 50 µl containing 2 µl of genomic DNA, 1.5 µL of 10 µM of each primer, 0.5 µl of 5 U/µl of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1.5 µl of 10 µM each deoxynucleoside triphosphate, 5 µl of 10x reaction buffer and 3 µl of $MgCl_2$. Amplification of 16S rRNA gene was performed under the following PCR conditions: 95°C for 5 min; 35 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 5 min with a GeneAmp PCR system 2700 (Applied Biosystems, Foster

City, CA, USA). PCR products (approx. 1,500 bp in size) of 16S rRNA genes were amplified and purified by QAIGEN MinElute PCR Purification Kit (QIAGEN, USA). Nucleotide sequencing of 16S rRNA genes were performed with two sets of primers as described above and determined by the dideoxynucleotide chain termination method according to the instructions of the SeqWright DNA Technology Services, Houston, Texas, USA. Sequence analysis was performed using the sequence match program at the Ribosomal Database Project (RDP-II; http://rdp.cme.msu.edu/html). The closest relatives of the 16S rRNA gene sequences were evaluated, and a similarity of ≥ 98% to 16S rRNA gene sequences of type strains was used as the criterion for identification. Phylogenetic analyses were conducted by using MEGA version 4.0 [161] and multiple sequence alignments were performed using the Clustal W program [162] and neighbour-joining (NJ) clustering were applied. Robustness of the trees was obtained by bootstrap re-sampling with 1,000 replicates.

11.3 Pyrosequencing

Genotypic characteristic by pyrosequencing as previously described [163-165] was performed following protocols of the Department of Pathology at Texas Children's Hospital [166]. Variable V1 and V3 regions of the 16S rRNA gene were chosen as targets. V1 was amplified with V1 primers; Bio-pBR: 5′ -biotin- GAA GAG TTT GAT CAT GGC TCA G –3′, and 5′-TTA CTC ACC CGT CCG CCA CT-3′. Variable V3 was amplified with V3 primers; Bio-B-V3b: 5′-Biotin-ACG ACA GCC ATG CAG CAC CT-3′ and pJBS.V3: 5′-GCA ACG CGA AGA ACC TTA CC-3′. These primers were designed in the conserved regions flanking the V1 and V3 regions. Polymerase chain reactions were performed in a total volume of 50 μl

containing 1 µl of genomic DNA 10 ng/µL, 1 µl of 10 µM of each primer, 0.25 µL of Amplitaq DNA polymerase 5 U/µl (Applied Biosystems, Foster City, CA, USA), 4 µl of deoxynucleoside triphosphate, 5 µl of 10x reaction buffer, 5 µl of $MgCl_2$ and 1 µl of DNA sample (concentration of 10 ng/µl). Amplification reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the following PCR conditions: 95°C for 10 min; 35 cycles of 95°C for 40 s, 55°C for 40 s, 72°C for 60 s with a final extension of 72°C for 60 s. Biotinylated PCR product was mixed with binding buffer and Streptavidin Sepharose HP beads (Amersham Bioscience, USA). The single stranded PCR product was then hybridized to pyrosequencing primer; V1 (5′-TTA CTC ACC CGT CCG CCA CT-3′) and V3 (5′-GCA ACG CGA AGA ACC TTA CC-3′) and dissolved in annealing buffer, substrate, enzyme and dNTPs for the sequencing reactions and the sequencing was performed with Pyrosequencer. The light signal was detected during DNA synthesis and seen as a peak in the resulting program. Approximately 20-45 bases of the sequences were analyzed by using the sequence match program at the Ribosomal Database Project II (http://rdp.cme.msu.edu/html).

11.4 rep-PCR genotyping

Repetitive element based-PCR (rep-PCR) as previously described [167-172] was performed according to the protocol of the Department of Pathology at Texas Children's Hospital. Briefly, genomic DNA (25 ng/µl) was amplified using the DiversiLab *Lactobacillus* kit (Spectral Genomics, Inc., Houston, TX, USA). The amplification reactions were performed in a total volume of 25 µl containing 2 µl of genomic DNA, 0.5 µl of 2.5 U of *Taq* DNA polymerase (AmpliTaq, Applied

Biosystems, USA), 2.5 µl of 10x PCR buffer (Applied Biosystems) 2 µl of *Lactobacillus* specific primer (DiversiLab *Lactobacillus* Kit, USA) and 18 µl of rep-PCR master mix (DiversiLab DNA Fingerprinting kit: *Lactobacillus* Kit, USA). The thermal cycling conditions were performed as follows: initial denaturation of 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; extension at 70°C for 90 s; and a final extension at 70°C for 3 min. The amplified products were separated in a microfluidics DNA LabChip® device (LabChip device; Caliper Technologies, Inc., USA) and detected in the model B 2100 bioanalyzer (Agilent Technologies, Inc., CA, USA.) by added 9 µl of gel-dye mix containing 400 µl of DNA gel matrix (DNA DiversiLab Chip Reagents; Spectral Genomics, USA) and 20 µL of DNA Dye concentrate (DNA DiversiLab Chip Reagents; Spectral Genomics, USA) into the DNA chip, and 1 µL of PCR product was added into each of the sample wells. DNA standard markers (DNA DiversiLab Chip Reagents) were used for normalization of each sample run, and 1 µl of the Chip Kit molecular weight ladder were used. Dendrograms and scatterplots were generated by using the DiversiLab software version 3.3 (http://tch.diversiLab.com) with the Pearson correlation coefficient to determine distance matrices and the unweighted-pair group method with arithmetic mean (UPGMA) to create dendrograms.