CHAPTER V

DISCUSSION

It has been documented that *Lactobacillus* isolates derived from humans are probably the optimal candidates and most suitable for beneficial effects in human beings because they are well adapted to conditions in the human gut environment [108]. In addition, some health promoting benefits may be species-specific [108]. The fecal microbiota includes many members of the intestinal microbiota [4, 62] and fecal isolates are a subset of all intestinal bacteria and may not be recoverable in feces (maybe bacteria are adherent to intestinal wall). In many studies, human *Lactobacillus* strains have been isolated from fecal samples [4, 22, 30]. Therefore, we proposed to search for probiotic *Lactobacillus* isolates recovered from human feces and screen for probiotic properties such as inhibition of gastrointestinal pathogens or modulation of tumor necrosis factor-α (TNF-α) production.

Inhibition of pathogen growth is regarded as an important property for probiotics [110, 174]. In this study, *Lactobacillus* species were isolated from human volunteers who were not on antibiotic treatment and were not consuming yogurts for at least two months prior to fecal sampling. This strategy would avoid obtaining *Lactobacillus* from commercial products and killing of sensitive *Lactobacillus* strains by antibiotics in gastrointestinal tract. *Lactobacillus* isolates of healthy human volunteers were investigated for antagonistic activities against gastrointestinal pathogens including gram-negative bacteria such as *S.* Typhimurium, *Sh. flexneri, V. cholerae, C. jejuni,* EHEC, EPEC, ETEC, EIEC and gram-positive bacteria such as

C. difficile. The general inhibitory actions of most *Lactobacillus* isolates were due to acid production rather than the production of bacteriocin-like metabolites [173]. Consequenctly, low glucose MRS medium containing 0.2% glucose (modified MRS: MMRS) was used to restrict the extent of acid production in this experiment. The pH value of MMRS after cultivation with *Lactobacillus* isolates was pH 5.5-6.0 and neutralized to pH 6.8. This pH is difference from non-neutralized MRS after cultivation with *Lactobacillus* isolates (pH 4.0). The pH 4.0 showed strong inhibitory activity to almost pathogens. However, after acidic factor (pH 4.0) was excluded by neutralized with NaOH, no such inhibitory reactions were observed for any of the cultures (data not shown). Our results suggested that the inhibitory effects versus several pathogens were due to acid production which was similar to the result obtained by Lin et al [175]. They demonstrated that several strains of *L. plantarum* inhibited food-borne pathogens due to acid production (pH 3.7-4.4) by using an agar well diffusion assay. However, the inhibitory effects were slight when antimicrobial products were neutralized to pH 7.0 [175].

As shown in Table 4 and 5, four *Lactobacillus* strains including SB42-6, BJ48-5, RT49-5 and RT49-7 demonstrated weak (12-14 mm) antagonistic activities against *Vibrio cholerae* non-O1 by neutralized supernatants of MMRS which was lower than the one obtained from non-neutralized supernatants of MMRS (15-16 mm). However, 4 strains showed no antagonistic effects against other pathogens. The inhibitory effects of *Lactobacillus* strains were variable even within the same species [159, 176]. Our result suggested that the inhibitory effects of those 4 strains were possibly due to combination of organic acids and probably antimicrobial compounds.

Several *Lactobacillus* strains displayed antibacterial activities via the production of organic acids such as lactic acid, acetic acid and propionic acid and other metabolites such as hydrogen peroxide and short chain fatty acids [4]. Also, specific antibacterial compounds such as reuterin produced from *L. reuteri* which showed strong inhibition to several pathogens [88] have been identified [84]. In addition, the other important antimicrobial substances known as bacteriocins were produced by several species of *Lactobacillus*. *L. plantarum* has been reported to produce various types of plantaricins such as plantaricins S and T [177]. Plantaricins S showed inhibitory activity against gram-positive bacteria including *C. tyrobutyricum*, *Enterococcus faecalis* and *Propionibacterium* spp. No action was observed against gram-negative bacteria [177]. *L. plantarum* 423 produced a plantaricin 423, which showed inhibitory activity to several food spoilage bacteria and food-borne pathogens, including *Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria spp.* and *Staphylococcus* spp. [178]. *Lactobacillus acidophilus* TK9201 produced acidocin A which was active against different species of *Enterococcus, Pediococcus, Streptococcus,* and *Listeria monocytogenes* [179], while Lactococcin MMFII produced from *Lactococcus lactis* MMFII was active against different species of *Enterococcus* and *Lactococcus* [180].

Mante et al. [181] demonstrated the mechanism of action of antimicrobial substances of *L. plantarum* as follows. Cell free supernatants were digested with proteinase K and pronase E. For hydrogen peroxide, supernatants were treated with catalase enzyme and for acid production as antimicrobial substances, supernatants were neutralized with sodium hydroxide to raise their pH to 6.8. The result revealed that the addition of catalase and proteolytic enzyme to the supernatants did not have

an effect on their inhibitory activity. They concluded that the antimicrobial effects of *L. plantarum* were attributed to acid productions [181]. The same result revealed that supernatants of *Lactobacillus* isolates from infant feces were reduced in antibacterial activity when catalase was added and supernatants were neutralized to pH 6.5 [4].

Many studies have been reported that *Lactobacillus* strains from human feces displayed antagonistic activity against several pathogens [4, 121]. *Lactobacillus* strains isolated from feces inhibited *E. coli*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Bacillus cereus* [4], *Clostridium difficile* and *E. coli* O157:H7 [121]. Some *Lactobacillus* species inhibited *Clostridium difficile* due to hydrogen peroxide and lactic acid. *Lactobacillus* P93 showed antibacterial activity due to organic acids and antimicrobial peptide productions [30].

However, our results of weak inhibitory activity may be due to the antimicrobial substances were too diluted in culture supernatants or the bactericidal effect of bacteriocin-like substances was active only in acidic condition [182]. Anyhow, the culture supernatants were concentrated 10 times, but the inhibitory activity was not different from un-concentrated one.

The antimicrobial activity testing by using agar well diffusion assay was not practical to allow some *Lactobacillus* species to produce antimicrobial substances especially, *L. reuteri* which resides in the gastrointestinal tract of healthy humans and animals [88, 183] and needs glycerol in metabolic pathway to produce reuterin [27]. Reuterin is formed during the anaerobic growth on a mixture of glucose and glycerol by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin [27]. Reuterin has a very broad spectrum of antimicrobial activity. It was found to have antibacterial, antifungal, antiprotozoal and antiviral activity [85-88].

Consequently, we investigated reuteirn production by using overlay method according to the instruction by Biogaia AB, Stockholm, Sweden [158]. *Lactobacillus* spotted plate was overlaid with soft agar containing glycerol and incubated anaerobically at 37°C for 1 hr for reuterin production and detected by the DNPH solution and KOH solution. As shown in Figure 2, reuterin was not detected in all of 437 testing *Lactobacillus* isolates whereas *L. reuteri* SD2112 positive control of reuterin producing strain capable to produce reuterin. This result suggested that in all 437 *Lactobacillus* isolates, there was no *L. reuteri* or there was *L. reuteri* which produced reuterin in very low amounts and could not detected by this method.

An agar spot method overlaid with soft agar containing glycerol for reuterin production, and each target strain of pathogen was used to investigate antimicrobial activities of all those 437 isolates which could not be detected by the method described above. In this study, the results showed that 144 *Lactobacillus* isolates varied in their ability to inhibit *V. cholerae*, and 32 of these 144 strains also showed different activities against *S. enterica*. Several strains showed more inhibitory activity than that obtained by agar well diffusion assay. This method may allow for the direct determination of antimicrobial substances which are secreted directly to the surrounding environment by *Lactobacillus* spots [88]. However, antibacterial activity of these strains demonstrated differential activities and showed no reproducibility on both target strains. The antimicrobial activity of four strains including SB42-6, BJ48-5, RT49-5 and RT49-7, as described in agar well diffusion assay, also showed antibacterial effect toward both *V. cholerae* and *S. enterica*.

The results obtained by Spinler et al. [88] demonstrated that four human-derived *L. reuteri* showed differential inhibitory activities against enterohemorrhagic

E. coli, enterotoxigenic *E. coli, S. enterica, Sh. sonnei* and *V. cholerae* by the agar spot assay, and these *L. reuteri* strains showed varied amounts of reuterin production [88]. The ability to produce large quantities of reuterin was not correlated with a strain's inhibitory properties on enteric pathogens [88].

However, *Lactobacillus* strains in our study showed weak inhibitory effects, had narrow spectrum activity against gastrointestinal pathogens and lacked reproducible results. These *Lactobacillus* strains therefore may not be suitable for further investigations characterizing antimicrobial substances.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine and mediated inflammation and regulate immune function. It has been implicated in the pathogenesis of a wide range of human diseases including sepsis, diabetes, cancer, osteoporosis, autoimmune diseases, and inflammatory bowel diseases [184]. TNF-α is a homotrimer of 157 amino acid subunits produced by macrophages and many different cell types including monocytes, macrophages, T lymphocytes, B lymphocytes, NK cells and other types of cells [184].

In this study, we also proposed to look for probiotic *Lactobacillus* strains of human origin which have probiotic properties in modulation of pro-inflammatory cytokine (TNF-α) production *in vitro*. THP-1 human monocytic leukemia cell line was used in this *in vitro* study, and lipopolysaccharide (LPS) was employed for activation of the cells. As shown in Tables 8-12 and Figures 5-9, all 46 isolates displayed abilities to modulate TNF-α production. Without LPS, some *Lactobacillus* slightly induced TNF-α production but most of them did not activate TNF-α production. With LPS-activated THP-1 cells, the inhibitory activities of forty-six isolates varied in each isolate from 8-65% inhibition (Table 13 and Figure 10).

Twelve isolates displayed weak TNF-α inhibitory activities (8-20% inhibition), twenty-four isolates displayed moderate TNF-α inhibitory activities (21-40% inhibition). Ten isolates displayed high TNF-α inhibitory activities (more than 40% inhibition). Interestingly, in this highly inhibitory group, one isolate (TH58 strain) exhibited the most potent TNF-α inhibitory activity (65% inhibition).

The TNF-α inhibitory activity were different in each isolate, therefore strains which showed TNF-α inhibitory activity ≥ 25% inhibition were chosen to evaluate the ability to inhibit TNF-α production in comparison with the TNF-α stimulatory strain of TH14. As expected, twelve isolates significantly inhibited TNF-α protein production and varied between 32-68% among isolates. The TH58 strain still showed the strongest TNF-α inhibitory activity in LPS-activated THP-1 monocytic cells. The magnitude of TNF-α suppression varied among the isolates, indicating that these potential probiotic isolates were functionally different [5]. This study performed at least three times in triplicate and displayed reproducible results. Therefore, these 12 *Lactobacillus* strains were chosen for further studies.

Several *Lactobacillus* species can produce immunoregulatory factors, known as immunomodulins [45, 185], which down-regulated TNF-α production [5, 45]. This suggested that anti-inflammatory strains possibly produced some immunoregulatory factors which were able to suppress TNF- α production *in vitro* [45, 185]. Similar results have been shown in *L. rhamnosus* GG [45] and different *Lactobacillus* species which were able to inhibit TNF-α secretion in macrophages [46]. Murine lactobacilli from mice without colitis significantly inhibited TNF-α production in LPS-activated murine macrophages [5]. Some lactic acid bacteria secreted anti-inflammatory metabolites

that inhibited TNF-α production by peripheral blood mononuclear cells and suppressed NF-κB activation in LPS-activated THP-1 cells [186].

Probiotic *Lactobacillus* administration can help restore microbial homeostasis in the gut, down-regulated intestinal inflammation and ameliorated diseases [41, 187]. They have been tested in the prevention and treatment of IBD including Crohn's disease and ulcerative colitis [188, 189]. Several beneficial effects of probitics *Lactobacillus* in the treatment of IBD have been reported. *L. acidophilus* combined with *B. animalis* subsp. *lactis* can reduce clinical symptom in IBD patients [189] and *L. rhamnosus* GG showed significantly improvements of clinical outcomes in children active Crohn's disease [190]. *L. casei* DN-1140 has been shown to inhibit TNF-α production in cultured mucosal explants from Crohn's disease [148, 149]. VSL#3, probiotics includes four different *Lactobacillus* strain, two *Bifidobacterium* and *Streptococcus thermophilus* have been effective for induction of remission with no adverse effects in treatment of patients with mild to moderate ulcerative colitis [191].

We also found that the immunomodulatory activity of TH58 was optimal in LCM collected at 48 hr cultivation (Table 16 and Figure 13). Based on growth characteristics of TH58 strain, more than 24-28 hr time point represented late logarithmic phase, >28-40 hr represented stationary phase and more than 40 hrs of culture indicated early decline phase for TH58. The microbes synthesized amounts of various metabolic intermediates or metabolites in two kinds [192]. The primary metabolites were produced via the major metabolic pathways and were essential for microbial functions such as production of amino acids and organic acids. These pathways occurred during mid-logarithmic through mid-stationary phase of growth.

The secondary metabolites are by-products of metabolism such as vitamins, antibiotics and steroids which occurred during mid-stationary phase through the decline phase of growth [192]. After 24 hr until 48 hr, TH58 were grown into late-logarithmic through stationary and decline phase which covered primary and secondary metabolic pathways. Therefore, it was possible that these time points were optimal for TH58 strain to produce more immunoregulatory factors which optimally suppressed TNF-α production *in vitro*.

Stimulation of a pattern recognition receptors (PRRs) such as Toll-like receptor 4 (TLR4) by pro-inflammatory mediators such as lipopolysaccharide (LPS), one of a pathogen-associated molecular pattern (PAMP) of gram-negative bacteria [42] led to the activation of transcription factors such as nuclear factor-κB (NF-κB) and activating protein-1(AP-1) [42, 184, 193], resulting in the induction of cytokines such as TNF-α, IL-12, IL-1, IL-6 and IL-8.

The NF-κB proteins are composed of homodimers and heterodimers. There are five subunits of the NFκB family in mammals: p50 (NFκB1), p65 (RelA), c-Rel, p52 and RelB3. The p50/p65 (NFκB1/RelA) heterodimers and the p50 homodimers are the most common dimers found in NF-κB signaling pathway [194]. The p50/p65 heterodimer is referred as NF-κB [194]. NF-κB regulates expression of many immune and inflammatory genes associated with rheumatoid arthritis and inflammatory bowel disease [193]. In the majority of cells, NF-κB exists in an inactive form in the cytoplasm bound to the inhibitory IκB proteins. Stimulation of the cells with various stimuli results in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins lead to release of NF-κB (p50/p65 heterodimer) which subsequently translocation to the nucleus for the DNA binding [193]. NF-κB is expressed by almost

all cell types can be activated by a wide variety of cellular stimuli, including inflammatory cytokines, such as interleukin-1 or TNF-α, infection with invasive microbes such as *Salmonella* [195] or viruses, components of bacterial cell walls, such as lipopolysaccharide, peptidoglycan, and by other forms of cellular stress including oxidative stress and DNA double-strand breaks [196].

In this study, to examine a possible mechanism of TH58 mediated suppression of TNF-α production, the potential transcriptional regulation, NF-κB transcription factor was initially investigated by NF-κB p65 ELISA. As displayed in Table 14 and Figure 16, TH58, the most potent TNF-α inhibitory strain had no effect on the level of active NF-κB in nuclei of LPS-activated THP-1 cells. In addition, TH58, in absence of LPS, did not activate the NF-κB transcription factor complex. In contrast, TH14, TNF-α stimulatory strain induced NF-κB activation by itself without stimulation by LPS. None of other tested control strains significantly suppressed NF-κB activation in LPS-activated THP-1 human monocytic cells.

Similar results have been shown that *Lactobacillus rhamnosus* GG, which inhibited TNF-α production, did not affect NF-κB activation because other NF-κB regulated genes such as IL-12 were not decreased [197]. Kim et al. [198] have demonstrated that granulocyte-colony stimulating factor (G-CSF) secreted by *L. rhamnosus* GG- and *L. rhamnosus* GR-1-exposed THP-1 cells suppressed TNF-α production induced by lipopolysaccharide through a paracrine route. The suppression of TNF production by G-CSF was mediated through activation of signal transducer and activator of transcription (STAT)-3, subsequently inhibiting activation of c-Jun-N-terminal kinases (JNKs) in THP-1 cells. In addition, Lin et al. [199] demonstrated that *L. reuteri* MM4-1A suppressed TNF- production in TLR4-activated

Mono-Mac-6 cells and TLR2-activated THP-1 cells. Regulation by *L. reuteri* MM4-1A did not affect NF-κB activation but down-regulated activator protein-1 (AP-1) activation (active AP-1 is a heterodimer, composed of phospho c-Jun and c-Fos) by preventing the phosphorylation of c-Jun by c-Jun-N-terminal kinases (JNKs).

However, mechanisms of *Lactobacillus*-mediated suppression of TNF-α production via NF-κB have been reported. Some *Lactobacillus* species and some lactic acid bacteria suppressed pro-inflammatory cytokine production by inhibiting NF-κB activation [(185, 186, 197, 200)]. Iyer et al. [(201)] demonstrated that *L. reuteri* suppressed TNF-induced NF-κB activation in myeloid-derived cells (KBM5) by stabilization of IκBα and inhibition of nuclear translocation of NF-κB p65 [(201)]. *L. bulgaricus* decreased expression of nuclear NF-κB p65 in gut epithelial cells [(45)]. In addition, lactic acid bacteria inhibited NF-κB activation in THP-1 cells [(35)] and *L. reuteri* inhibited translocation of NF-κB to the nuclei of Hela cells [(46)].

The regulation of TNF-α production has been reported at the level of transcription, translation and secretion [(202, 203)]. In THP-1 monocytic cells, transcriptional activation of TNF-α by LPS requires the activation of a distinct set of transcription factors binding to at least two regions of the TNF-α promoter which include NF-κB, early growth response1 (Egr-1) protein and activator protein-1 (AP-1) binding sites [(204, 205)]. While the exact array of transcription factors interacting with the TNF-α promoter is to some extent cell and species specific [(206)], recruitment of NF-κB, Egr-1, as well as increased c-Jun binding to a CRE/AP-1 binding site appear to be required for full activation of TNF-α expression in most types of macrophages

[204, 205]. Activation of each of these nuclear transcription factors is mediated by specific LPS-mediated signaling cascades [207].

The results of these investigators suggested that the possible mechanisms of TH58-mediated suppression of TNF-α production did not include regulation of NF-κB transcription factor activation but TH58 may affect other mechanisms such as inhibition of c-Jun-N-terminal kinase (JNK) activation [198] or suppression of AP-1 transcription factor [199], transcription, translation or secretion [42, 202, 203]. In addition, LPS or lipoteichoic acid (LTA) may stimulate NF-κB or AP-1 and TH58 may block AP-1 [199], but if cells are stimulated with TNF, then TH58 may block NF-κB instead of AP-1 [201]. However, the possible mechanisms also require further investigation.

Bacterial characteristics such as Gram stain, colony appearance, and growth in media were useful for characterization of *Lactobacillus* species. *Lactobacillus* TH58, the most potential probiotics strain was characterized for general characteristics, probiotic properties such as acid and bile tolerance, and their abilities to grow in aerobic conditions. Cells and colonies of TH58 looked similar to general lactobacilli which have regular, white round colony and can grow well in MRS selective media [1].

Acid and bile tolerance are properties that indicate the abilities of probiotic microorganisms to survive the passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and bile acids at the beginning of the small intestine [208, 209]. The survival of bacteria in the gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in the stomach is 0.9, but the presence of food raises the pH value to 3.0 [210]. In this study, TH58 strain was tested for acid and bile tolerance, and the results showed that TH58 strain could survive after incubation for

3 hr at pH 2.5 (decreased by 3 log values), but this strain survived well at pH 3.5, 4.5 which showed no log difference when compared to MRS control. No growth occurred after incubating at pH 1.5. Prasad et al. [211] demonstrated that screening of 200 lactic acid bacterial strains, yielded four resistant strains that were able to survive at pH 3.0 for 3 hr. Xanthopoulos [212] studied 20 *Lactobacillus* strains isolated from infant feces and found that three *L. paracasei* subsp. *paracasei* strains and one *L. rhamnosus* strain were unaffected by the low pH. Pennacchia et al [209] reported that 28 of 150 *Lactobacillus* strains from fermented sausages showed a survival at pH 2.5 for 3 hr.

The bile in human intestine is also an important which affects the viability of *Lactobacillus*. In the human gastrointestinal tract, the mean bile concentration is 0.3% w/v [213]. Viability of TH58 strain was decreased by 3.5 log differences in 1% and 2% bile bovine whereas in 3%, 4%, 5% bovine bile, the viable counts were decreased by about a 4.5 log difference when compared to MRS bacterial media control. Pennacchia et al. [209] reported that most of the 150 *Lactobacillus* strains from fermented sausages were able to grow in the presence of 0.3% bile salts.

In this study a total of 4 anti-pathogenic and 14 immunomodulatory *Lactobacillus* strains were subjected to characterize by API 50 CHL, 16S rRNA gene sequencing, pyrosequencing and rep-PCR genotyping. The use of phenotypic identification alone is less reliable than the use of genotypic analysis [214]. The highest identity closely related to 100% was used for species identification.

The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of the 16S rRNA gene, to provide distinguishing and statistically valid measurements [215]. Universal primers are usually chosen as

complementary to the conserved regions at the beginning of the gene and at either of the 540-bp region or at the end of the whole sequence [215]. For the species identification, the 16S rRNA gene sequence is much easier to determine and thus has become the new gold standard [216, 217]. In practice a range of about a 0.5 to 1% difference or 99 to 99.5% similarities is often used for species identification [215, 218]. DNA sequencing of rRNA genes appear to be sufficient for the identification of most lactobacilli [5].

Bacterial 16S rRNA consists of eight highly conserved regions with nine variable regions. The V1-V3 regions of the gene are documented as species-specific sequences [219]. The pyrosequencing is focused on the V1 and V3 regions of the 16S rRNA gene. PCR primers are designed in the conserved regions flanking the V1 and V3 regions [166]. The advantage of pyrosequencing is accuracy, flexibility, parallel processing and easily automated [220].

In our study, 4 anti-pathogenic strains SB42-6, BJ48-5, RT49-5 and RT49-7 were identified *as L. plantarum* or *L. plantarum* group [221] including *L. plantarum, L. pentosus* and *L. paraplantarum* based on phenotypic and genotypic characteristics, respectively.

In immunomodulatory strains, phenotypic identification by API did not yield definitive species identification for each strain, whereas 16S rRNA gene sequencing yielded species identifications and provided the same results. Almost all strains displayed with similarity closed to or equal to 100%. Based on phenotypic characteristics, 8 strains were identified as *L. plantarum* or *L. plantarum* group. TH33 was identified as *L. salivarius* based on phenotypic and genotypic characteristics. Based on phenotypic characteristics, TH43 was identified as *L. paracasei* spp

paracasei, while genotypic characteristics yielded identification as *L. plantarum* or *L. plantarum* group. TH58, the strongest TNF-α inhibitory strain showed less identity to several species of *Lactobacillus, P. damnosus and W. viridescens* by phenotypic characteristics, while it was identified as *L. saerimneri* by genotypic characteristics. TH62 was identified as *L. brevis* by phenotypic studies, while by genotypic characteristics it was identified as *L. plantarum* or *L. plantarum* group. The immunostimulatory strain, TH14 was identified as *Leu. lactis* by phenotypic characteristics, while by genotypic characteristics, it was identified as *L. ruminis*. TH64, non-anti-inflammatory and non-immunostimulatory strain was identified as *L. brevis* by phenotypic characteristics, while by genotypic characteristics, it was identified as *W. cibaria*. These findings indicated that *Lactobacillus* species identification by biochemical tests is inadequate for *L. ruminis* and *L. saerimneri*, in contrast to *L. plantarum*.

Based on genotypic characteristics, anti-pathogenic isolates were identified as *L. plantarum*, whereas anti-inflammatory strains were identified as 3 species including *L. plantarum, L. salivarius and L. saerimneri*. The result obtained by Abrno et al. [(70)] revealed that *L. plantarum* was the major species of human gastrointestinal mucosa, and it was capable to adhere to human colonic cell lines [(70)]. *L. plantarum* groups (*L. plantarum*, *L. pentosus* and *L. paraplantarum*) had high similarities in 16S rRNA gene and phenotypic similarities [(221)]. TH58 strain, the most potent TNF-α inhibitory activity was identified as *L. saerimneri*, a species first isolated from pigs by Pederson and Ross in 2004 [(222)]. The result obtained by Pena et al.[(5)] revealed that several strains of intestinal *L. reuteri* from mice without colitis were able to suppress TNF-α production by macrophages, whereas *L. johnsonii*

isolated from mice susceptible to colitis were not able to inhibit TNF-α production [5]. These finding indicated that immunomodulatory effects may be species specific [223].

In this study, we also investigated genetic relationships between 12 anti-inflammatory strains with one immunostimulatory strain and one non anti-inflammatory and non-immunostimulatory strain based on 16S rRNA gene sequences. These findings indicated that 3 distinct clusters among the *Lactobacillus* species including 10 strains of *L. plantarum* cluster, *L. saerimneri* and cluster of *L. salivarius* which closely related to immunostimulatory strain of *L. ruminis*, whereas non anti-inflammatory and non-immunostimulatory strain of *Weissella cibaria*, was out of *Lactobacillus* groups. These results suggested that functional differences with respect to modulation of TNF-α production possibly correlated with genetic variation among lactobacilli [5].

Genetic fingerprinting is very useful for differentiating bacterial isolates [224]. Versalovic et al. [167] described a method for fingerprinting bacterial genomes by examining strain-specific pattern obtained from PCR amplification of repetitive elements present within bacterial genomes. The DNA fragments are ranging in size from 200 bp to over 2,000 bp [172]. The technique is easy to perform and can be applied to large or small numbers of isolates. Rep-PCR has considerably better discriminatory power than restriction analyses of the 16S rRNA gene or the 16S-23S spacer region [225]. Furthermore, studies which compared rep-PCR to other typing methods such as multilocus enzyme electrophoresis [226], biochemical characterization [227], or ribotyping [225] showed that rep-PCR was superior to these methods [224]. Several studies have shown that rep-PCR correlates well with the gold standard of molecular typing, pulsed-field gel electrophoresis (PFGE) results but, with slightly

less discriminatory power [228, 229]. Rep-PCR is fast becoming the most widely used method of DNA typing [224]. Rep-PCR has been useful and reliable for the identification and typing of various *Lactobacillus* species [230-232].

We performed whole genomic fingerprinting by rep-PCR [5] which has been recognized as an effective method for bacterial strain typing [5, 167]. In 4 anti-pathogenic strains of *L. plantarum*, 3 strains displayed 100% similarity whereas, 1 strain was showed 90% similarity to those 3 strains. All 4 stains were heterogeneous with *L. reuteri* strains which showed approximately 55% similarity.

Based on 16S rRNA gene sequencing, immunomodulatory strains, 10 *L. plantarum* isolates displayed within the same cluster, whereas, rep-PCR fingerprinting divided isolates into 3 clusters including TH43, TH45, TH61, TH62 which displayed 97% similarity. Cluster of TH39 were closely related to TH47 and displayed 90% similarity and cluster of TH24, TH48 and TH49 were closely related together and displayed 94-97% similarity. The most potent TNF-α inhibitory strain, TH58, displayed 78%, 70% and 60% similarity to TH39 and TH47, SD2112, MM4-1A and TH14, respectively.

These results indicated that some *L. plantarum* isolates from different persons which displayed similar functions and appeared to be closely related subspecies. Although, among those 3 clusters indicated subspecies variations with 60% similarity. TH58, *L. saerimneri* was genetically heterogeneous from those 10 *L. plantarum*, anti-inflammatory strains; *L. reuteri* MM41-A, anti-inflammatory strain; TH14, TH64 and SD2112, non-anti-inflammatory strains. Some researchers demonstrated that *L. reuteri* from the different part of the same healthy mice which displayed the same function to inhibit TNF-α production have DNA fingerprinting

which variations in subspecies [5]. Whereas, *L. johnsonii* from colitis and healthy mice displayed DNA fingerprinting appeared to be different strains [5].