CHAPTER I

INTRODUCTION

Background and Rationale

The members of genus *Lactobacillus* are gram-positive, non-motile, non-sporulating, non-catalase producing, rod-shaped bacteria. Genus *Lactobacillus* includes 175 species (www.dsmz.de) that are aerobic or facultatively anaerobic or strictly anaerobic. *Lactobacillus* species are members of food and dairy industrial lactic acid bacteria. Lactobacilli are acid tolerant and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product [1]. Lactobacilli are microbiota of the gastrointestinal tract of humans and animals [1, 2, 3, 4]. Several species have been isolated and identified from humans [2, 3], animals [5, 6], dairy and fermented food products [7-9]. Lactobacilli can resist gastric and bile salts, adhere to intestinal tissues, colonize intestinal tract and are relatively harmless [10]. In addition, they are easy to cultivate in bulk and have a long history of safe use in food and fermented products [11]. Host specificity is a feature of colonization by individual species. For example, *L. acidophilus*, *L. fermentum* and *L. plantarum* are commonly found in the feces of humans [12].

Probiotics are defined as "non-pathogenic live microbial feed or food supplements that beneficially affects the host by improving its gastrointestinal microbial balance" [13]. Lactobacilli have been used as probiotics against gastrointestinal infection [12, 13] and inflammatory bowel disease [14, 15]. The most commonly used and the best studied probiotics against gastrointestinal disorders are

currently the *Bifidobacterium* spp. and lactic acid-producing bacteria, particularly *Lactobacillus* spp. [16]. *Lactobacillus* strains of human origin are most suitable used as probiotics [10] because some health-promoting benefits may be species-specific and microorganisms may perform optimally in the species from which they were isolated [17]. Several lactobacilli of human origin are being exploited commercially, i.e., *L. rhamnosus* GG [18], *L. casei* Shirota [19] and *L. acidophilus* LA-1 [20].

Infections with gastrointestinal pathogens continue to be major health problem worldwide, especially in children, undernourished, hospitalized and immunocompromised individuals [21]. The most common agents that are responsible for diarrhea of infectious origin include *Escherichia* and *Salmonella* [22, 23]. The same bacterial genera, together with *Shigella*, *Campylobacter* and *Vibrio* spp., are often recognized as the causative agents of diarrhea in children in developing countries [22]. In addition, antibiotic-associated diarrhea, the most common gastrointestinal side effect of antibiotic therapy, is often associated with *Clostridium difficile* infection in adults and children [24]. Moreover, gastrointestinal infection with *Helicobacter pylori* is associated with gastritis, gastric and duodenal ulcers and a risk factor for cancer [21]. This organism can be found in 70-90 % of the population in developing countries and in 25-50 % in developed countries [25]. A lack of mucosal vaccine and the widespread emergence of antibiotic-resistant microorganisms have stimulated the quest for alternative methods of infection control [21, 24]. The use of probiotics have been suggested as a safer alternative to chemotherapy in the management of gastrointestinal disorders caused by infectious agents and one with the potential for preventing such disorders [22].

There are many proposed mechanisms by which *Lactobacillus* may protect the host from gastrointestinal disorders. Much work remains to classify the mechanisms of action of particular *Lactobacillus* against particular pathogens. In addition, the same probiotic lactobacilli may inhibit different pathogens by different mechanisms [16]. The following mechanism by which *Lactobacillus* may protect the host against gastrointestinal infection, include production of antimicrobial substances [26, 27], blocking of adhesion sites, degradation of toxin receptor and modulation of immunity [16].

The effective results have been performed on the ability of *Lactobacillus* strains to prevent or treat gastrointestinal infections. In randomized, placebo-controlled study, the incidence of nosocomial diarrhea in *Lactobacillus* GG supplement children was found to be significantly lower than that observed in children given placebo [28]. *Lactobacillus* GG has also been reported as effective in the treatment of *C. difficile* relapsing diarrhea, without side effects, in adults and children [29]. Intestinal lactobacilli have been shown to confer the inhibitory effect against different pathogenic *C. difficile* strains *in vitro* [30]. Effective treatment with the other lactobacilli strain has been demonstrated in *L. reuteri* which can reduce the duration and severity of diarrhea in infants with diarrhea [31]. Human studies have also provided evidences that the oral consumption of specific strain of lactobacilli may be effective in mediating protection against *H. pylori* infections [32]. Similar results have been shown that there was a significant reduction in the density of *H. pylori* and the severity of gastric inflammation in volunteers infected with *H. pylori* following the intake of fermented milk containing *L. johnsonii* LAI [33], *L. salivarius* was found to be potentially effective against *H. pylori* and reduce the inflammatory response in

H. pylori-infected gnotobiotic murine model after oral administration [34]. The inhibitory effects of *L. paracasei* and *L. acidophilus* have been demonstrated *in vitro* on the growth of both *Escherichia coli* and *Salmonella enteritidis* [22]. Similar result has been reported that enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), *Klebsiella pneumoniae*, *Shigella flexneri*, *S* Typhimurium, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *C. difficile* were inhibited by *L. rhamnosus* [35]. In mice which had been fed a diet containing *L. rhamnosus,* it was found that there was a lower cumulative morbidity and bacterial translocation rates following infection with *E. coli* O157:H7 [23]. It has been shown that lactobacilli isolated from commercial probiotic consortium had inhibitory effect against *E. coli* O157:H7 and *S.* Typhimurium [36]. The antagonistic activity of *Lactobacillus* against gastrointestinal pathogens is possibly due to the antimicrobial substances produced by the probiotic strains. *L. reuteri* have been found to produce reuterin [27], while several *Lactobacillus* strains produces various types of bacteriocins for example: *L. acidophilus* produces lactacin [26]. Lactobacilli also produce hydrogen peroxide and organic acids such as lactic and acetic acids, which inhibit growth of many pathogenic gram-negative bacteria [37].

Increasing of pro-inflammatory cytokines and chemokines is one of the leading causes of the pathogenesis of inflammatory diseases in the gut such as peptic ulcer from *H. pylori* [38], pseudomembranous colitis from *C. difficile* [39] and inflammatory bowel disease [40]. Tumor necrosis factor alpha (TNF-α) represents a pro-inflammatory cytokine and plays an important role in inflammatory bowel disease [41]. Stimulation of Toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR) by pro-inflammatory mediators such as lipopolysaccharide (LPS) of gram negative

bacteria [42] leads to the activation of nuclear factor kappa B (NF-κB) signaling pathway [42] that results in the induction of TNF-α production [42, 43]. Consequently, the inflammatory reaction is amplified [44]. A healthy homeostasis in the gut milieu may thus be achieved by optimizing the balance of pro-inflammatory and anti-inflammatory cytokines [44].

Lactobacillus species have been demonstrated to effectively suppress TNF-α production [5, 45, 46] and inflammation during chronic colitis in several studies [47, 48]. *L. rhamnosus* GG-conditioned media specifically inhibit TNF-α production in LPS-activated macrophages [45]. Interestingly, *L. rhamnosus* GG-conditioned media also decreases TNF-α production of *H. pylori* and *H. hepaticus*-activated macrophages [45]. *L. paracasei* and *L. reuteri* recovered from mice without colitis displayed TNF-α inhibitory properties on LPS-stimulated macrophage [5]. Combination of these two strains reduced intestinal inflammation in *H. hepaticus*-challenged IL-10 deficient mice and the levels of pro-inflammatory colonic cytokine, TNF- α, were lowered in *Lactobacillus*-treated mice [47]. In addition, administration of *L. reuteri* to IL-10 deficient mice resulted in decrease of colitis in treated animals [48].

Lactobacillus species are considered to have beneficial effects on the human health by interference with infection of gastrointestinal pathogens and ability to suppress inflammatory responses in the gastrointestinal tract. Therefore, several species of lactobacilli are used as probiotics for treatment and prevention of gastrointestinal disorders [49]. However, each probiotic *Lactobacillus* species may have an individual mechanism of action. The inhibitory activity of *Lactobacillus* is variable even within the same or different species and the underlying mechanism remains unclear. Consequently, selection and characterization of optimal

Lactobacillus strains against gastrointestinal pathogens and anti-inflammatory cytokines responses required further investigation.

In Thailand, probiotics investigations have been documented for applications in animal feed but there has been no report of human-derived probiotic *Lactobacillus* for potential use in gastrointestinal disorders.

In this study, *Lactobacillus* strains were isolated from fecal samples of healthy human volunteers and investigated for their two main probiotic properties to inhibit gastrointestinal pathogens and to suppress TNF-α production in THP-1 monocytic cells stimulated with purified *Escherichia coli*-derived lipopolysaccharide (LPS). The most potent TNF-α inhibitory strain was chosen to study the effects to NF-κB signaling pathway. Strains which have probiotics properties were chosen to be characterized by API 50 CHL, 16S rRNA gene sequencing, pyrosequencing and genomic fingerprinting with repetitive element-based PCR (rep-PCR) as well as phylogenetic relationships based on 16S rRNA gene sequencing and rep-PCR genotyping. The *Lactobacillus* strains which have been genotyped and characterized for their probitic mechanisms of action may be useful for the potential use as probiotics for the treatment and prevention of gastrointestinal disorders.

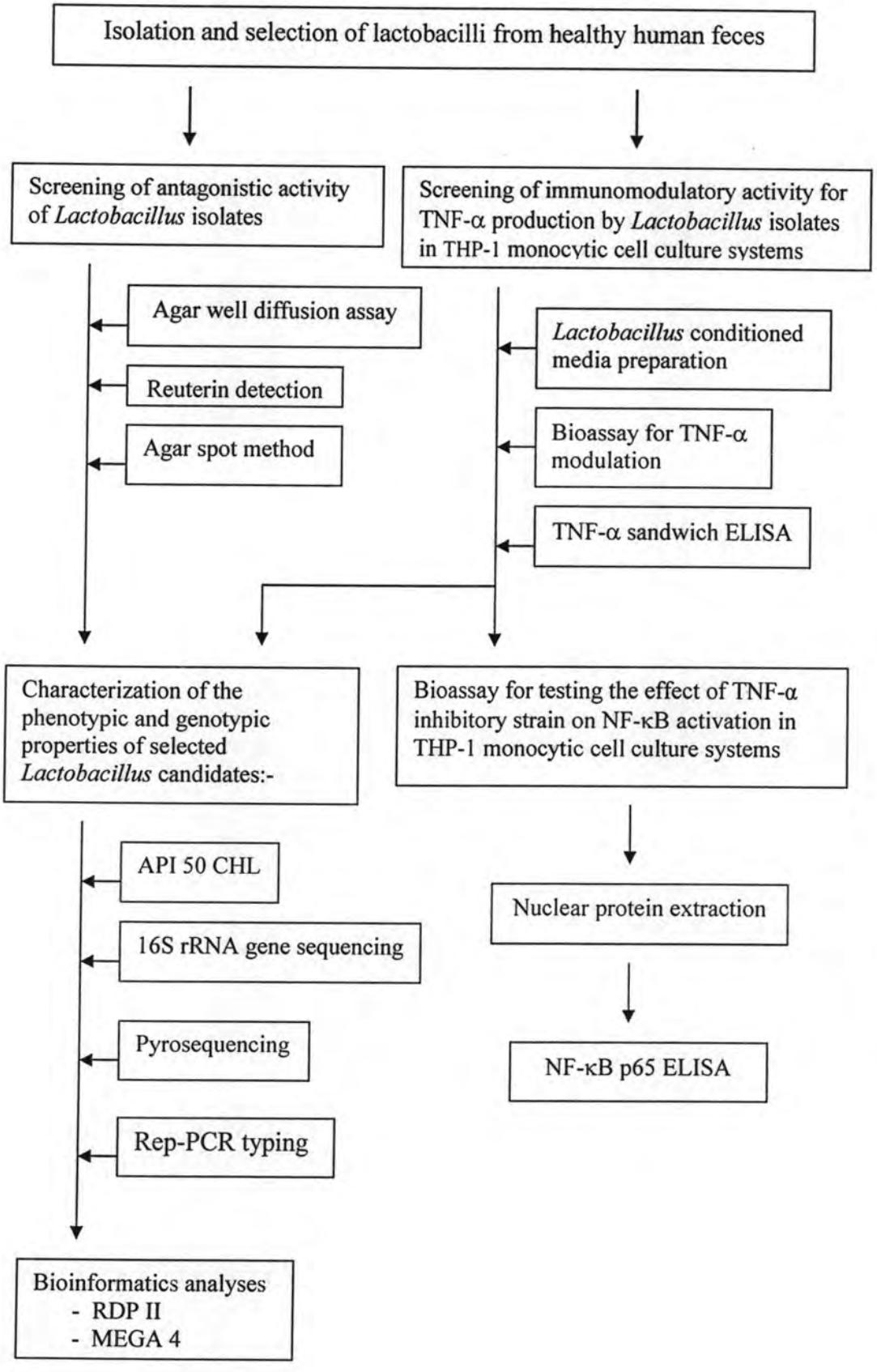
Hypothesis

Specific probiotic *Lactobacillus* strains in nature are capable of inhibiting gastrointestinal pathogens and/or modulating pro-inflammatory cytokine responses. Screening of *Lactobacillus* for these probiotic properties with appropriate methods will enable us to find novel strain(s) of probiotic *Lactobacillus* for further application.

Objectives

1. Search for *Lactobacillus* isolates which possess antagonistic activities against gastrointestinal pathogens
2. Search for *Lactobacillus* isolates that are able to modulate pro- inflammatory cytokine responses *in vitro*
3. Study the phenotypic and genotypic characteristics of selected *Lactobacillus* candidates

Flow Chart of Conceptual Framework



Expected Outcome

1. Human-derived *Lactobacillus* isolates with antagonistic activities against gastrointestinal pathogens for the potential use as a source of Thai probiotics
2. Human-derived *Lactobacillus* isolates which reduce pro-inflammatory cytokine responses for the potential application as probiotics for chronic gastrointestinal inflammatory diseases
3. Strains for further studies to understand host-microbe interactions
4. Novel strains of probiotic *Lactobacillus* with applications to be patented

Research Methodology

1. Isolation of lactobacilli from 64 human fecal samples
2. Selection of *Lactobacillus* isolates by presumptive test including gram stain, catalase test and vancomycin susceptibility test
3. Antagonistic activities of 510 *Lactobacillus* isolates against 10 gastrointestinal pathogens by agar well diffusion method
4. Reuterin detection of 437 *Lactobacillus* isolates
5. Antagonistic activities of 437 *Lactobacillus* isolates against *Vibrio cholerae* and *Salmonella enterica* by agar spot method
6. Preparation of *Lactobacillus* conditioned media (LCM) of 46 *Lactobacillus* isolates in MRS bacterial culture media
7. Bioassay for modulation of TNF-α production by 46 LCM of *Lactobacillus* isolates in THP-1 monocytic cell culture systems

8. Assessment of TNF-α levels by TNF-α sandwich ELISA
9. Bioassay for the effect of TNF inhibitory strain on NF-κB activation in THP-1 monocytic cell culture systems
10. NF-κB nuclear protein extraction
11. Assessment of NF-κB using NF-κB p65 ELISA
12. Phenotypic characterization of the most potent TNF-α inhibitory strain including growth curve, acid tolerance, bile tolerance and aerotolerance
13. Phenotypic characterization of selected *Lactobacillus* strains by API 50 CHL
14. Genotypic characterization of selected *Lactobacillus* strains using 16S rRNA gene sequencing
15. Genotypic characterization of selected *Lactobacillus* strains by pyrosequencing
16. Species identification of selected *Lactobacillus* strains based on 16S rRNA gene sequences using sequences match program at Ribosomal Database Project II
17. Species identification of selected *Lactobacillus* strains based on pyrosequencing using sequences match program at Ribosomal Database Project II
18. Phylogenetic tree of selected *Lactobacillus* strains based on 16S rRNA gene sequencing using MEGA 4.0 software package
19. Genotyping of selected *Lactobacillus* strains by rep-PCR DNA typing and analyses by DiversiLab software version 3.3