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ชื่อโครงการ (ไทย) การตรวจพิสูจน์โพรไบโอติกแลคโตบาซิลลัสที่สามารถกดการตอบสนองทางภูมิคุ้มกันที่ทำให้เกิดการอักเสบในทางเดินอาหาร

(อังกฤษ) **Identification of Probiotic *Lactobacillus* with Anti-Inflammatory Activity in the Gastrointestinal Tract**

หน่วยงาน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

คณะผู้วิจัย รองศาสตราจารย์ ดร. สมหญิง ธัมวาสร
Professor Dr. James Versalovic

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บทคัดย่อ

การตรวจพิสูจน์โพรไบโอติกแลคโตบาซิลลัส ที่สามารถกดการตอบสนองทางภูมิคุ้มกันที่ทำให้เกิดการอักเสบในทางเดินอาหาร

เพื่อตรวจพิสูจน์โพรไบโอติกแลคโตบาซิลลัสสำหรับใช้เป็นอิมมูโนโพรไบโอติกส์เพื่อบรรเทาหรือรักษาโรคทางเดินอาหารที่เกิดจากการอักเสบ เช่น โรคกระเพาะอาหารและลำไส้ส่วนต้นที่เกิดจากเฮลิโคแบคเตอร์ ไพโลไร และ โรคที่เกิดจากคลอสตริเดียม ดิฟฟีไซล์ ผู้วิจัยเลือกใช้วิธีทดสอบความสามารถของแลคโตบาซิลลัสในการปรับเปลี่ยนการสร้างอินเตอร์ลิวคิน-8 ในเซลล์เยื่อบุทางเดินอาหาร โดยนำแลคโตบาซิลลัสที่แยกได้จากกระเพาะอาหารจำนวน 87 isolates มาทดสอบความสามารถในการปรับเปลี่ยนการสร้างอินเตอร์ลิวคิน-8 ใน AGS cells ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร และ แลคโตบาซิลลัสที่แยกได้จากอุจจาระจำนวน 37 isolates มาทดสอบความสามารถในการปรับเปลี่ยนการสร้างอินเตอร์ลิวคิน-8 ใน HT-29 cells ที่ถูกกระตุ้นด้วยคลอสตริเดียม ดิฟฟีไซล์ ซึ่งสร้าง toxin A พบว่าน้ำเลี้ยงเชื้อที่ปราศจากเซลล์ของแลคโตบาซิลลัสที่แยกได้จากกระเพาะอาหารจำนวน 12 isolates สามารถกดการสร้างอินเตอร์ลิวคิน-8 ใน AGS cells ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไรในการทดลอง 3 ครั้ง เชื้อส่วนใหญ่ (7/12) คือ *Lactobacillus salivarius* ซึ่งเป็นจุลชีพประจำของมนุษย์ นอกนั้นคือ *L. plantarum* และ *L. casei* group ซึ่งเป็นจุลชีพที่พบเป็นครั้งคราว น้ำเลี้ยงเชื้อที่ปราศจากเซลล์ของแลคโตบาซิลลัสที่แยกได้จากอุจจาระจำนวน 11 isolates สามารถกดการสร้างอินเตอร์ลิวคิน-8 ใน HT-29 cells ที่ถูกกระตุ้นด้วยคลอสตริเดียม ดิฟฟีไซล์ในการทดลอง 1 ครั้ง เมื่อนำเชื้อ 4 isolates มาทดสอบซ้ำได้ผลยืนยันแบบเดิม แลคโตบาซิลลัสกลุ่มนี้คือ *L. rhamnosus* 3 isolates และ *L. casei* 1 isolate

จะนำแลคโตบาซิลลัสสายพันธุ์ที่สามารถกดการสร้างอินเตอร์ลิวคิน-8 มาแยกแยะทัยป์เพื่อบ่งบอกสายพันธุ์ด้วยวิธีทางอณูชีววิทยา คัดเลือกสายพันธุ์ที่จำเพาะมาทดสอบกลไกการทำงานในการปรับเปลี่ยนวิถีสื่อสัญญาณของโฮสท์เซลล์ตามที่เสนอในแผนวิจัย และทดสอบ cytokine profile ที่สร้างในเซลล์เยื่อบุเมื่อถูกปรับเปลี่ยนด้วยโพรไบโอติกแลคโตบาซิลลัส

คำสำคัญ : แลคโตบาซิลลัส/ อิมมูโนโพรไบโอติกส์/ เฮลิโคแบคเตอร์ ไพโลไร / คลอสตริเดียม ดิฟฟีไซล์ / อินเตอร์ลิวคิน-8 / การปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกัน

Abstract

Identification of Probiotic *Lactobacillus* with Anti-Inflammatory Activity in the Gastrointestinal Tract

In order to identify probiotic *Lactobacillus* for potential use as immunoprobiotics for the amelioration or treatment of inflammatory diseases in the gastrointestinal tract such as *Helicobacter pylori*-induced gastro-duodenal diseases and *Clostridium difficile*-associated disease (CDAD), we chose to test for the ability of human-derived *Lactobacillus* to modulate interleukin-8 (IL-8) production in gastrointestinal epithelial cells. Eighty-seven gastric biopsy-derived *Lactobacillus* isolates were tested for their ability to modulate IL-8 production in *H. pylori*-stimulated AGS cells and 37 feces-derived isolates were tested in HT-29 cells stimulated with toxin A-positive *C. difficile*. Cell-free conditioned media of 12 *Lactobacillus* spp. obtained from gastric biopsy were found to suppress IL-8 production in *H. pylori*-stimulated AGS cells in three separate experiments. The majority of these isolates (7/12) belongs to *L. salivarius* which is an indigenous flora of humans, whereas the rest belongs to transient flora such as *L. plantarum* and *L. casei* group. Cell-free conditioned media from 11 *Lactobacillus* spp. obtained from infant feces significantly inhibited IL-8 production in *C. difficile*-stimulated HT-29 cells in one experiment. Four of these 11 *Lactobacillus* spp. were re-tested and the result confirmed the inhibitory effect of these 4 *Lactobacillus* spp. These lactobacilli were 3 isolates of *L. rhamnosus* and one isolate of *L. casei*.

These IL-8 suppressing *Lactobacillus* will be subjected to molecular typing for strain identity. Selected strains will be tested for their mechanism of action in the modulation of host signaling pathway as proposed in the research proposal. The probiotic candidate strains will also be tested for cytokine profile they elicit in the specific epithelial cells.

Keywords: *Lactobacillus / immunoprobiotics/ Helicobacter pylori /Clostridium difficile /* IL-8/ immunomodulation

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	bovine serum albumin
CFU	colony forming unit
CO_2	carbon dioxide
°C	degree Celsius
EDTA	ethylenediamine tetra-acetic acid
et al.	et alii
h	hour
H_2	hydrogen gas
H_2SO_4	sulfuric acid
IL-8	interleukin-8
LCM	*Lactobacillus* conditioned media
µg	microgram
µl	microliter
µm	micrometer
min	minute
ml	milliliter
mM	millimolar
N	normality
N_2	nitrogen gas
nm	nanometer
PBS	phosphate buffer saline
PPI	proton pump inhibitor
SD	standard deviations
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
v/v	volume per volume
w/v	weight per volume

INTRODUCTION

Probiotics are defined by the Food and Agricultural Organization as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (1, 2). Members of the genus *Lactobacillus* are mainly used as probiotic microorganisms and have long been available to the consumers in food products. At present, probiotics are gaining widespread inclusion as new prevention strategies or therapies for various gastrointestinal diseases according to the results of multiple meta-analyses and systematic reviews (3). Probiotic properties are strain specific, and each probiotic strain may contribute beneficial effect to host through different mechanisms. Mechanisms of probiosis include remodeling of microbial communities and suppression of pathogens, immunomodulation by up-regulation of anti-inflammatory factors, immunomodulation by suppression of proinflammatory factors, enhancement of immunity, effect on epithelial cell differentiation, and proliferation and promotion of intestinal barrier function (3). Our previous study demonstrated the immunomodulatory activities of a number of *Lactobacillus* spp. isolated from feces of healthy adults (4). These human-derived *Lactobacillus* either suppressed or stimulated tumor necrosis factor (TNF) production in lipopolysaccharide-activated THP-1 monocytes. In addition to these isolates, we obtained more *Lactobacillus* spp. from gastric biopsy of dyspeptic patients and infant feces. We are, therefore, interested in the characterization of immunomodulatory properties of these *Lactobacillus* for potential use as immunoprobiotics for inflammatory diseases in the gastrointestinal tract. The diseases of our interest are *Helicobacter pylori*-induced gastro-duodenal diseases and *Clostridium difficile*-associated disease (CDAD).

Helicobacter pylori is the important pathogen that infects the stomach of humans around the world. This pathogen is the main cause of gastritis, peptic ulceration, gastric adenocarcinoma, and gastric lymphoma. The second highest cause of cancer deaths worldwide is gastric adenocarcinoma due to a combination of high incidence rate, aggressive disease course, and lack of effective treatment (5). *H. pylori* persistence is the most important step of pathogenesis. After many years of infection and inflammation, ulcers can take place mainly in mid- or late-adulthood. Eventually, after an even longer period of chronic inflammation and epithelial damage, gastric adenocarcinoma occurs in late adulthood (6). *H. pylori* colonization is known to

induce several cytokines regulating accumulation and cellular activation of neutrophils and mononuclear cells (7-10). Among the cytokines caused by *H. pylori* in gastric epithelium, interleukin-8 (IL-8) plays a major role in the mucosal inflammation. The presence of chronic inflammation is associated with gastric cancer that was well described in many studies (11-13). IL-8 is known as a potent neutrophil chemotactic and activating agent (14-18). A variety of cell types such as monocytes, fibroblasts, endothelial and epithelial cells can produce IL-8 (16, 18). There is the evidence showing that *H. pylori*-infected and gastric cancer patients have increasing levels of IL-8 in gastric epithelium of both corpus and antrum (19). Some studies demonstrated the stimulation of IL-8 production by *H. pylori* in gastric epithelial cell lines *in vitro* (20, 21). These evidences suggest that IL-8 is an important cytokine induced by *H. pylori.*

Treatment of choice to eradicate *H. pylori* infection usually consists of 2 antibiotics and a proton pump inhibitor (PPI) and the most successful regimens have achieved eradication rate of 75-90% (22). However, the cure rates following standard therapies are reducing because of antibiotics resistance and poor compliance due to side effects of the medications (23). For this reason, considerable interest has focused in developing low-cost, large-scale alternative approaches to prevent or decrease *H. pylori* infection (24). Probiotics have been proposed as a useful adjunctive therapy to improve either side effect or the rates of eradication (3).

Probiotics could modify the immunologic response of the host by interacting with epithelial cells and modulating the secretion of inflammatory cytokines. This interaction would lead to reduction of gastric activity and inflammation (25). In the previous study of probiotic lactobacilli, *L. salivarius* showed the inhibition of the *H. pylori*-stimulated production of IL-8 by gastric epithelial cell *in vitro*. In addition, *H. pylori* could not colonize the stomach of *L. salivarius*-implanted mice *in vivo*(26). Another *in vivo* study, *L. johnsonii* La1 has been demonstrated to attenuate *H. pylori*-associated gastritis in mice (27). This *L. johnsonii* La1 could also reduce levels of proinflammatory chemokines such as MIP-2, chemokine in inflammatory states and a potent murine neutrophil chemoattractants (28). Viable *L. gasseri* OLL2716 (LG21) showed suppression of *H. pylori*-induced IL-8 production *in vitro*, and also in human gastric mucosa (29). Recently, *L. bulgaricus* has been demonstrated for its ability to

inhibit *H. pylori* LPS -induced IL-8 production by inhibition of the Toll-like receptor (TLR) 4 pathway *in vitro* (30). These studies support the possibility of using lactobacilli to modulate *H. pylori*-induced IL-8 production.

Clostridium difficile is the predominant cause of post-antibiotic diarrhea and life-threatening pseudomembranous colitis (31). The incidence of CDAD has been increasing rapidly in the last 10 years especially in the elderly (32, 33). The situation is more complicated with the increase in disease severity, mortality (32, 34), and treatment failure (35, 36) due to the emergence of a hypervirulent *C.difficile* strain (37, 38). The main virulence factors are two large exotoxins, toxin A (TcdA) and toxin B (TcdB), which are cytotoxic and cause apoptotic and necrosis cell death. Both toxins can disrupt tight junctions and trigger the production of various inflammatory mediators from the intestinal epithelial cells, mast cells and macrophages. These activities result in fluid secretion into the intestine, inflammation and the accumulation of neurophils (39, 40). TcdA has been shown to stimulate the production of interleukin 8 (IL-8), which is a potent chemoattractant and activates neutrophils (41), in human intestinal epithelial cells (42, 43) and monocytes (44).

Metronidazole or vancomycin is currently used for the treatment of CDAD. Despite antimicrobial therapy, up to 20% of patients will have a recurrent CDAD which is very difficult to treat (45). Probiotic administration as an adjunct to antibiotic therapy has been under study. Use of probiotics such as *Saccharomyces boulardii* (46, 47), *L. rhamnosus* GG (48,49,50), *L. plantarum* 299v (51,52) were found benefit for recurrent CDAD but the mechanism of action of these probiotics has never been demonstrated.

Since both *Helicobacter pylori*-induced gastro-duodenal diseases and CDAD have pathologic conditions that include increased mucosal IL-8 level, we are interested to investigate whether human-derived *Lactobacillus* could regulate IL-8 production by epithelial cells. We selected AGS cells as a gastric epithelial cell model and HT29 cells as an intestinal cell model and explored the immunomodulatory effect of *Lactobacillus* on IL-8 production. Gastric biopsy-derived *Lactobacillus* isolates were tested for the ability to modulate IL-8 production in *H. pylori*-stimulated AGS cells and feces-derived isolates were tested in HT29 cells stimulated with toxin A-positive *C. difficile.*

MATERIALS AND METHODS

Lactobacillus culture and conditioned media preparation

Eighty-seven *Lactobacillus* spp. were isolated from gastric biopsy of dyspeptic patients (53), 37 *Lactobacillus* spp. were isolates from infant feces (54), and *L. saerimneri* was isolated from adult feces (4). *Lactobacillus* conditioned media were prepared as previously described (4). Bacterial cultures were grown anaerobically (10% CO_2, 10% H_2, and 80% N_2) in de Man, Rogosa, Sharpe (MRS) broth in anaerobic chamber (Concept Plus, Ruskinn technology, UK) at 37°C for 24 h. The OD_{600} of culture was determined by using spectrophotometer (Bio-Rad Smart Spec™ Plus), adjusted to 0.1 in MRS broth and incubated under anaerobic condition for 48 h. Culture broth was centrifuged at 4,000 g for 10 min at 4°C and the collected supernatant was filtered through 0.22 μm filter (Minisart, Germany) and concentrated by speed-vacuum drying (Speed vacuum, Savant instruments, USA). The pellet was resuspended in an equal volume of tissue culture medium RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA, USA) and McCoy's medium (Gibco-Invitrogen, Carlsbad, CA, USA) for testing with AGS and HT-29 cells, respectively. These conditioned media were stored at -20°C until analysis.

Cell line and *H. pylori* culture condition

AGS human gastric adenocarcinoma epithelial cells (ATCC CRL-1739) were obtained from the American Type Culture Collection (Manassas, VA, USA). AGS cells were grown in 25 ml of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen, USA) at 37°C in a 5% CO_2 atmosphere in a 75-cm^2 tissue culture flask. After culture for 2 days, a monolayer of more than 80% confluence was obtained as visualized with an inverted microscope. After removal of tissue culture medium, these adherent cells were detached from the flask with 3 ml of 0.25% (v/v) Trypsin in 1 mM EDTA (Gibco-Invitrogen, USA) at 37°C for 5-8 min. The detached cells in Trypsin-EDTA solution was then suspended in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen, USA) and the cell suspension was used for co-culture assay.

H. pylori ATCC 43503 (*vacA* s1a-m, *cagA* $^{+}$, *ureC* $^{+}$) was grown on Columbia Blood agar (Oxoid, England) supplemented with 7% (v/v) horse serum (Gibco, New Zealand) and 7% (v/v) sheep blood at 37° C for 48 h at 37°C in sealed culture boxes(Mitsubishi Gas Chemical [MGC], New York, NY) containing a microaerobic atmosphere (6-12% O_2, 5-8% CO_2) generated by Anaero Pack-Micro Aero (MGC). Bacterial cells were then transferred to and resuspended in antibiotic-free RPMI medium. The bacterial suspension was adjusted to the density of No. 6 McFarland standard and used for co-culture assay.

***Lactobacillus*:AGS IL-8 Bioassay**

AGS suspension was seeded at a density of $1.0x10^5$ cell/ml (20,000 cells per well) in a volume 200 µl in each well of a 96-well flat-bottom tissue culture plate (NUNCLON D, Denmark) and pre-incubated at 37°C in a humidified 5% CO_2 incubator. After 24 h incubation, the culture supernatant was removed and 200 µl of fresh tissue culture medium was added. The assay was optimized for peak production of IL-8 after stimulating AGS cells with *H. pylori*. Cell-free *Lactobacillus* conditioned media (LCM) was added in AGS cell culture in appropriate well at the concentration of 5% v/v prior to the addition of $3x10^7$ CFU/ml viable *H. pylori* ATCC 43503 ($6.0x10^6$ CFU per well). This experiment allows a multiplicity of infection of 300 of *H. pylori* to AGS cells. The plate was incubated under 5% CO_2 at 37°C for 24 h. and cell culture supernatants were collected by centrifugation at 125xg for 7 min at 4°C for IL-8 measurement. **Add control.**

Cell line and *Clostridium difficile* culture condition

HT-29 human colon adenocarcinoma cell line (ATCC HTB-38) were obtained from the American Type Culture Collection (Manassas, VA, USA). HT-29 cells were grown in McCoy's 5a Modified Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen, USA) at 37°C under 5% CO2 atmosphere in a 75-cm2 tissue culture flask. After culture for 2 days, a monolayer of more than 80% confluence was obtained as visualized with an inverted microscope. After removal of tissue culture medium, these adherent cells were detached from the flask with 3 ml of 0.25% (v/v) Trypsin in 1 mM EDTA (Gibco-Invitrogen, USA) at

37°C for 5-8 min. The detached cells in Trypsin-EDTA solution was then suspended in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen, USA) and the cell suspension was used for co-culture assay.

Toxin-producing *Clostridium difficile* clinical isolate was grown on Brucella agar (Oxoid, England) at 37°C for 48 h in an anaerobic atmosphere. Bacterial cells were harvested and resuspended in antibiotic-free McCoy's medium. The bacterial suspension was adjusted to the density of No. 6 McFarland standard and used for

***Lactobacillus* : HT-29 IL-8 Bioassay**

HT-29 cell suspension was seeded at a density of $1.0x10^5$ cell/ml (20,000 cells per well) in a volume 200 µl in each well of a 96-well flat-bottom tissue culture plate (NUNCLON D, Denmark) and pre-incubated at 37° C in a humidified 5% CO_2 incubator. After 24 h incubation the culture supernatant was removed and 200 µl of fresh tissue culture medium was added. The assay was optimized for peak production of IL-8 after stimulating HT-29 cells with *C. difficile.* Cell-free *Lactobacillus* conditioned media (LCM) was added in HT-29 cell culture in appropriate well at the concentration of 5% v/v prior to the addition of $3x10^7$ CFU/ml viable *Clostridium difficile* ($6.0x10^6$ CFU per well). This experiment allows a multiplicity of infection of 300 of *C. difficile* to HT-29 cells. The plate was incubated under 5% CO_2 at 37°C for 24 h. and cell culture supernatants were collected by centrifugation at 125xg for 7 min at 4°C for IL-8 measurement.

IL-8 analyses by Quantitative ELISA

IL-8 level in the culture supernatant was determined by using the sandwich quantitative enzyme-linked immunnosobent assay (DuoSet, R&D Systems, USA) according to the manufacturer's instructions. Briefly, 96-well tissue microtiter plates (MAXISORP, Nunc, Denmark) were coated overnight at 4°C with 100 µl per well of mouse anti-human IL-8 antibodies (R&D System, USA) as capturing antibodies. After incubation, plates were washed three times with 400 µl per well of wash buffer (PBS pH 7.2-7.4 containing 0.05% [v/v] Tween 20). To reduce non-specific binding, wells were blocked with 300 µl per well of reagent diluent (0.1% BSA [Sigma, USA], 0.05% Tween 20 in Tris-buffered Saline, pH 7.2 - 7.4) for 1 h and washed three times.

The plates were added 100 µl of sample or standard recombinant human IL-8 in an appropriate well. The recombinant human IL-8 was diluted to prepare seven point standards by 2-fold serial dilutions. Plates were incubated 2 h at room temperature. After washing three times, 100 µl of biotinylated goat anti-human IL-8 (R&D System, USA) were added in each well and the plate was incubated for 2 h at room temperature. The plates were then washed three times and 100 µl of streptavidin-conjugated horse radish-peroxidase was added to each well and incubated for 20 min by avoiding the exposure to direct light. After washing three times, 100 µl of substrate solution composed of mixture of equal volume of reagent A (H_2O_2) and color reagent B (tetramethylbenzidine, TMB) (R&D Systems, USA) was added to each well. The plate was incubated for 20 min at room temperature avoiding exposure to direct light and 100 µl of stop solution (2 N H_2SO_4, MERCK, Germany) was added to each well. Absorbance was determined with microplate reader (MULTISKAN EX PRIMARY EIA V. 2.1-0) at 450 nm with background subtraction at 570 nm.

Cell viability assay

Cell viability was evaluated by the Trypan-Blue (Gibco-Invitrogen) exclusion assay. Briefly, cell suspension was mixed with 0.4 w/v trypan blue solution (1:1) and then visually examined to determine whether cells take up or exclude dye. The number of stained cells and total of cells were counted on a hemocytometer under a phase-contrast microscope within the $1mm^2$ area. The procedure was stained dead cells but not unstained viable cells. The percentage of viable cells was calculated from the ratio of viable cells over total cells.

Statistical analysis

All experiments were performed at least in triplicate and the results were reported as mean and standard deviations (SD). The data were analysed using the Student's *t*-test with one-tailed distribution and considered statistically significant at p-value ≤ 0.05.

RESULTS

IL-8 production by *H. pylori*-stimulated AGS cells and immunomodulatory effect of *L. saerimneri*

AGS cells secreted increased amount (>10-fold) of IL-8 after 24 h-coincubation with *H. pylori* compared to cells exposed to bacterial media control (MRS) alone and MRS sometimes significantly suppressed the IL-8 production. (Tables 1-7). *L. saerimneri*, a novel human-derived *Lactobacillus* isolated from adult feces which was previously demonstrated for the ability to suppress TNF production in *E. coli* LPS-stimulated THP-1 cells (4), was tested for its ability to modulate IL-8 production in AGS cells. Cell-free conditioned medium from *L. saerimneri* did not inhibit IL-8 production and was used as a negative control in the experiment as shown in Tables 1-7.

Immunomodulatory effects of gastric biopsy-derived *Lactobacillus* spp.

Eighty-seven *Lactobacillus* spp. were tested for their ability to modulate IL-8 production. These lactobacilli were previously identified by sequence analysis of 16S rRNA gene and tested for their ability to modulate TNF production in *E. coli* LPS-stimulated THP-1 cells (53) and the results of their TNF- immunomodulatory effects were also shown in Tables 1-7. Cell-free conditioned media from 21 *Lactobacillus* spp. significantly inhibited IL-8 production in various magnitudes as shown in Tables 1-7. The inhibition of IL-8 production by these *Lactobacillus* spp. did not have cytotoxic effects on AGS cells as determined by Trypan Blue dye exclusion assay. The percentage of viable cells in each sample was > 80%. These 21 *Lactobacillus* spp. were re-tested for their immunomodulatory effects in at least 2 additional experiments each in triplicate. The result confirmed the inhibitory effect of 12 *Lactobacillus* spp. and also indicated that in the absence of *H. pylori*, the condition media did not significantly stimulate IL-8 production. Most of the IL-8-suppressing isolates (11 out of 12) inhibited TNF production in LPS-stimulated THP-1 cells (Table 8, Figures 1-3). These lactobacilli were 2 isolates (B90 and XB7) of *L. plantarum*, 7 isolates (B91, B109, B110, B55, B60, B102 and B37) of *L. salivarius*, and 3 isolates (B106, B107

and B103) of *L. casei* group. The result of colony morphology and TNF immunomodulating properties of these 12 isolates which was previously done (53) was shown in Table 9.

IL-8 production by *C.difficile*-stimulated HT-29 cells and immunomodulatory effect of infant feces -derived *Lactobacillus* spp.

HT-29 cells by themselves produced IL-8 in McCoy's 5a Modified Medium and bacterial media control (MRS) itself significantly suppressed the IL-8 production. Co-incubation of HT-29 cells with *C. difficile* for 24 h demonstrated increased production (2-to 8-fold) of IL-8 compared to cells exposed to MRS alone (Tables 10-12). Thirty-seven *Lactobacillus* spp. isolated from infant feces were tested for their ability to modulate IL-8 production. These lactobacilli were previously identified by sequence analysis of 16S rRNA gene and tested for their ability to modulate TNF production in *E. coli* LPS-stimulated THP-1 cells (54) and the results of their TNF-immunomodulatory effects were also shown in Tables 10-12. Cell-free conditioned media from 11 *Lactobacillus* spp. significantly inhibited IL-8 production in various magnitudes as shown in Tables 10-12. The inhibition of IL-8 production by these *Lactobacillus* spp. did not have cytotoxic effects on HT-29 cells as determined by Trypan Blue dye exclusion assay. The percentage of viable cells in each sample was > 80%.Four of these 11 *Lactobacillus* spp. were re-tested for their immunomodulatory effects in at least 2 additional experiments each in triplicate. The result confirmed the inhibitory effect of these 4 *Lactobacillus* spp. (Table 13, Figure 5). These lactobacilli were 3 isolates of *L. rhamnosus* (L33, L34 and L35) and one isolate of *L. casei* (L39).

Table 1. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no.1-16 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code (no.)	Sample		48h LCM		48h LCM with *H. pylori*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
RPMI	media control	-	44.60	10.44	2095.67	11.55		
MRS	media control	-	80.69	22.53	2086.00	46.94	0	0.3733
TH58	*L. saerimneri*	55.56*	98.68	8.06	2161.33	43.52	0	0.0556
B60 (1)	*L. salivarius*	48.03*	123.52	18.17	936.08	145.92	55.13***	0.0001
B61 (2)	*L. fermentum*[a]	0	95.98	6.50	2173.00	32.91	0	0.0291
B62 (3)	*L. salivarius*[a]	0	117.21	10.20	1886.18	330.43	9.58	0.1792
B64 (4)	*L. plantarum*	74.90**	144.27	13.14	290.37	356.35	86.08[y]	0.0013
B66 (5)	*L. fermentum*	0	146.20	5.09	1952.54	304.10	6.40	0.2471
B67 (6)	*L. plantarum*	39.86*	119.74	37.73	84.48	(-)	95.9[y]	-
XB68 (7)	*L. gasseri*	63.64***	113.68	38.42	217.62	168.48	89.57[y]	0.0000
RPMI	media control	-	79.54	2.70	2089.04	89.77		
MRS	media control	-	97.31	8.88	1806.17	85.30	13.54**	0.0084
TH58	*L. saerimneri*	55.56*	129.09	15.76	1879.81	137.81	0	0.2376
XB41 (8)	*L. gasseri*	30.16*	134.07	10.73	1880.56	72.75	0	0.1572
B99 (9)	*L. fermentum*[a]	0	117.48	15.07	1910.74	157.06	0	0.1841
B101 (10)	*L. salivarius*	38.69*	111.12	5.02	1468.56	199.38	18.69*	0.0271
B102 (11)	*L. salivarius*[b]	38.91***	103.17	9.01	1568.34	13.34	13.17**	0.0044
B105 (12)	*L. fermentum*	0	100.68	14.11	1784.34	84.92	1.21	0.3846
B106 (13)	*L. casei*	35.09*	178.88	115.94	567.93	423.91	68.56**	0.0039
B108 (14)	*L. fermentum*[a]	0	103.26	4.18	1685.07	87.47	6.70	0.0806
B109 (15)	*L. salivarius*[b]	16.35*	103.70	8.41	1503.87	86.33	16.74**	0.0063
B110 (16)	*L. salivarius*[b]	45.91*	104.93	10.83	1403.68	135.17	22.28**	0.0060

[a] indicates TNF stimulation, [b] indicates TNF inhibition and stimulation, [y] indicates cell viability of less than 80%

Table 2. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no. 17-32 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code (no.)	Sample		48h LCM		48h LCM With *H. pylori*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
RPMI	media control	-	70.21	5.50	2093.39	176.57		
MRS	media control	-	104.76	13.77	1653.33	74.63	21.02*	0.0157
TH58	*L. saerimneri*	55.56*	130.67	9.96	1938.05	179.28	0	0.0534
B84 (17)	*L. fermentum*[a]	0	157.34	14.98	2120.86	189.66	0	0.0158
B87 (18)	*L. plantarum*	41.91**	96.32	21.20	302.95	337.48	81.68[y]	0.0026
B90 (19)	*L. plantarum*	17.45*	121.73	10.95	423.68	296.97	74.37**	0.0024
B92 (20)	*L. fermentum*[a]	0	124.61	4.55	2186.00	109.82	0	0.0024
B93 (21)	*L. oris*[a]	0	163.02	3.30	2056.20	111.81	0	0.0066
XB95 (22)	*L. gasseri*	24.76**	157.65	1.19	1849.93	196.23	0	0.1280
XB96 (23)	*L. gasseri*	34.23***	161.22	14.22	2026.34	88.43	0	0.0052
RPMI	media control	-	83.16	5.25	1550.00	392.68		
MRS	media control	-	115.02	7.52	1285.79	358.19	17.05	0.2042
TH58	*L. saerimneri*	55.56*	122.68	11.97	1819.18	560.66	0	0.1104
XB7 (24)	*L. plantarum*	57.23***	224.14	5.75	609.71	61.95	52.58**	0.0096
B21 (25)	*L. salivarius*	47.47***	109.22	13.80	2067.94	196.98	0	0.0100
B25 (26)	*L. fermentum*	0	98.80	10.90	1909.36	270.01	0	0.0281
B26 (27)	*L. mucosae*[a]	0	141.46	6.13	1670.25	752.86	0	0.2290
B29 (28)	*L. fermentum*[a]	0	130.87	15.46	2134.75	99.07	0	0.0050
XB30 (29)	*L. gasseri*[a]	0	143.58	15.06	2073.89	173.64	0	0.0087
B31 (30)	*L. fermentum*[a]	0	109.08	8.96	1552.76	221.86	0	0.1428
B33 (31)	*L. fermentum*[a]	0	114.12	17.66	2082.68	90.11	0	0.0060
B35 (32)	*L. fermentum*[a]	0	91.25	5.32	2129.00	12.49	0	0.0043

[a] indicates TNF stimulation, [b] indicates TNF suppression and stimulation, [y] indicates cell viability of less than 80%

Table 3. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no. 33-41 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code (no.)	Sample		48h LCM		48h LCM With *H. pylori*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
RPMI	media control	-	56.35	2.81	2130.37	71.13		
MRS	media control	-	94.41	23.41	1908.86	104.82	10.40*	0.0194
TH58	*L. saerimneri*	55.56*	87.47	2.49	1935.34	6.88	0	0.3425
B107 (33)	*L. casei*	34.26*	86.01	8.46	952.25	773.35	50.11*	0.0500
B6 (34)	*L. plantarum*	35.60**	139.25	12.63	1353.64	410.26	29.09*	0.0428
B7 (35)	*L. plantarum*	74.52**	169.56	24.33	1331.25	451.93	30.26*	0.0486
XB45 (36)	*L. gasseri*	25.31*	65.59	8.89	1838.71	90.44	3.67	0.2149
B70 (37)	*L. plantarum*	81.91**	69.99	8.39	1626.61	62.75	14.79**	0.0081
RPMI	media control	-	68.41	8.37	2037.76	31.94		
MRS	media control	-	84.80	4.44	2129.54	35.16	0	0.0143
TH58	*L. saerimneri*	55.56*	100.12	7.42	2060.56	188.92	3.24	0.2839
B74 (38)	*L. salivarius*	44.18*	101.34	9.72	1027.01	826.09	51.77*	0.0410
B103 (39)	*L. casei*	21.48***	112.55	55.58	890.00	651.15	58.21*	0.0151
B43 (40)	*L. salivarius*[a]	0	89.21	12.44	1483.00	680.12	30.36	0.0877
B85 (41)	*L. salivarius*[a]	18.33	90.92	12.10	1606.86	396.65	24.54*	0.0427

[a] indicates TNF stimulation

Table 4. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no. 42-52 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code (no.)	Sample		48h LCM		48h LCM with *H. pylori*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
RPMI	media control	-	54.70	3.45	1862.77	16.74		
MRS	media control	-	77.93	6.91	1948.79	75.51	0	0.0632
TH58	*L. saerimneri*	55.56*	97.00	3.27	1999.41	43.98	0	0.1863
XB77 (42)	*L. gasseri*	16.30	115.24	10.19	1891.76	163.64	2.93	0.3064
B78 (43)	*L. salivarius*	0	85.61	3.72	2017.10	24.88	0	0.1055
B16 (44)	*L. salivarius*[a]	0	77.36	5.48	1829.01	103.13	6.15	0.0899
B32 (45)	*L. salivarius*[a]	0	79.46	4.55	1841.41	68.35	5.51	0.0709
B36 (46)	*L. agilis*	62.76*	114.80	3.56	1603.67	350.28	17.71	0.0853
XB40 (47)	*L. gasseri*	82.41**	113.57	17.52	1701.88	242.27	12.67	0.0836
RPMI	media control	-	53.64	1.82	1912.52	120.77		
MRS	media control	-	79.45	6.38	2054.88	29.00	0	0.0590
TH58	*L. saerimneri*	55.56*	106.22	20.04	1964.09	3.27	4.42	0.0029
XB94 (48)	*L. gasseri*	28.05*	105.42	10.87	2005.37	64.95	2.41	0.1472
XB19 (49)	*L. gasseri*	0	104.37	6.00	2084.80	34.43	0	0.1569
XB48 (50)	*L. gasseri*	18.05*	110.88	6.75	2105.38	77.52	0	0.1751
XB49 (51)	*L. gasseri*[a]	0	112.28	3.76	2021.69	141.55	1.62	0.3555
B53 (52)	*L. salivarius*	0	111.46	7.72	2182.95	19.06	0	0.0015

[a] indicates TNF stimulation

Table 5. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no.53-62 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code	Sample		48h LCM		48h LCM plus *H. pylori*		% IL-8 suppression	*p*-value
	Lactobacillus species	% TNF suppression	Conc. (pg/ml)	SD	Conc. (pg/ml)	SD		
RPMI	media control	-	67.87	1.36	785.99	89.02		
MRS	media control	-	81.54	1.40	1028.94	226.61	0	0.0795
TH58	*L. saerimneri*	55.56*	91.55	2.00	1346.93	64.48	0	0.0398
B5 (53)	*L. fermentum*[a]	0	75.58	2.98	1342.59	149.81	0	0.0581
B8 (54)	*L. salivarius*	76.31***	39.40	3.08	61.61	1.15	94.01[y]	0.0009
B9 (55)	*L. fermentum*	0	93.81	7.00	1040.55	193.81	0	0.4747
B13 (56)	*L. casei*	16.45*	34.99	2.43	61.92	9.75	93.98[y]	0.0009
B14 (57)	*L. fermentum*[a]	0	78.03	3.41	979.41	300.52	4.81	0.4154
B15 (58)	*L. mucosae*[a]	0	84.18	4.46	957.75	281.24	6.92	0.3750
RPMI	media control	-	74.34	6.16	1546.27	232.57		
MRS	media control	-	71.04	1.63	1212.43	191.07	21.59	0.0636
TH58	*L. saerimneri*	55.56*	75.27	4.92	1523.10	142.08	0	0.0433
B18 (59)	*L. oris*[a]	0	72.13	0.97	1857.58	313.30	0	0.0191
B20 (60)	*L.fermentum*	0	70.27	4.80	1745.61	131.40	0	0.0082
B22 (61)	*L. oris*[a]	0	77.61	1.48	1352.82	217.15	0	0.2239
B24 (62)	*L. fermentum*[a]	0	67.16	1.74	1115.73	174.03	7.98	0.2761
RPMI	media control	-	74.34	6.16	1546.27	232.57		
MRS	media control	-	71.04	1.63	1212.43	191.07	21.59	0.0636
TH58	*L. saerimneri*	55.56*	75.27	4.92	1523.10	142.08	0	0.0433
B18 (59)	*L. oris*[a]	0	72.13	0.97	1857.58	313.30	0	0.0191
B20 (60)	*L.fermentum*	0	70.27	4.80	1745.61	131.40	0	0.0082
B22 (61)	*L. oris*[a]	0	77.61	1.48	1352.82	217.15	0	0.2239
B24 (62)	*L. fermentum*[a]	0	67.16	1.74	1115.73	174.03	7.98	0.2761

[a] indicates TNF stimulation, [y] indicates cell viability of less than 80%

Table 6. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no.63-75 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code	Sample		48h LCM		48h LCM plus *H. pylori*		% IL-8 suppression	*p*-value
	Lactobacillus species	% TNF suppression	Conc. (pg/ml)	SD	Conc. (pg/ml)	SD		
RPMI	media control	-	92.42	1.54	826.05	50.29		
MRS	media control	-	145.41	12.36	486.18	45.45	41.14***	0.0005
TH58	*L. saerimneri*	55.56*	164.14	30.03	710.01	242.00	0	0.0952
B2 (63)	*L. fermentum*[a]	0	256.33	55.72	880.02	243.76	0	0.0257
B4/2 (64)	*L. salivarius*[a]	0	244.93	57.14	706.10	187.46	0	0.0598
RPMI	media control	-	65.81	8.99	1749.31	204.49		
MRS	media control	-	77.35	3.87	1720.20	135.61	1.66	0.4236
TH58	*L. saerimneri*	55.56*	111.17	1.96	1933.67	59.75	0	0.0336
B38 (65)	*L. fermentum*[a]	0	90.60	3.45	1898.00	104.24	0	0.0731
B39 (66)	*L. fermentum*[a]	0	84.20	7.24	1997.20	62.53	0	0.0163
B42 (67)	*L. fermentum*	0	72.16	9.31	1615.38	258.16	6.09	0.2836
B44 (68)	*L. fermentum*[a]	0	69.15	7.90	1973.49	92.62	0	0.0279
RPMI	media control	-	69.87	6.62	2173.89	180.40		
MRS	media control	-	111.16	8.36	1206.24	283.48	44.51**	0.0038
TH58	*L. saerimneri*	55.56*	165.40	20.11	2019.03	110.68	0	0.0049
B72 (69)	*L. fermentum*[a]	0	175.57	16.80	2209.33	170.94	0	0.0032
B73 (70)	*L. salivarius*	53.30**	113.22	33.60	134.10	(-)	88.88[y]	-
XB75 (71)	*L. fermentum*	0	136.93	15.49	2329.33	100.86	0	0.0015
B76 (72)	*L. fermentum*[a]	0	159.71	16.79	2377.00	92.46	0	0.0012
B79 (73)	*L. mucosae*[a]	0	196.77	9.45	2306.33	140.02	0	0.0019
B82 (74)	*L. fermentum*[a]	0	162.95	7.48	2185.67	135.29	0	0.0028
B83 (75)	*L. fermentum*	0	131.99	5.33	2036.40	226.10	0	0.0083

[a] indicates TNF stimulation, [y] indicates cell viability of less than 80%

Table 7. Immunomodulatory effects of gastric-derived *Lactobacillus* spp. no.76-87 on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; TH58, negative control of non-IL-8 suppressing strain; SD, standard deviation; significantly different from MRS media control: ***p<0.001, **p<0.01,*<0.05.

Code	Sample		48h LCM		48h LCM plus *H. pylori*		% IL-8 suppression	*p*-value
	Lactobacillus species	% TNF suppression	Conc. (pg/ml)	SD	Conc. (pg/ml)	SD		
RPMI	media control	-	36.58	2.40	1564.62	90.10		
MRS	media control	-	55.71	3.32	1643.74	84.62	0	0.1649
TH58	*L. saerimneri*	55.56*	69.41	2.25	1596.04	59.91	2.90	0.2351
B121 (76)	*L. mucosae*[a]	0	87.44	4.69	1812.51	70.80	0	0.0285
B91 (77)	*L. salivarius*[b]	58.86*	60.13	16.78	600.36	109.17	63.48***	0.0001
B98 (78)	*L. fermentum*[a]	0	80.86	10.92	2062.33	35.36	0	0.0007
RPMI	media control	-	72.88	3.62	1596.36	406.41		
MRS	media control	-	98.24	9.28	982.80	133.74	38.43*	0.0340
TH58	*L. saerimneri*	55.56*	102.56	11.69	1563.89	295.20	0	0.0180
B46 (79)	*L. fermentum*	0	90.53	3.53	1890.40	304.76	0	0.0046
B47 (80)	*L. salivarius*	55.34*	99.74	5.86	1175.40	317.82	0	0.1941
B52 (81)	*L. salivarius*[b]	40.12***	107.19	2.25	331.02	58.09	66.32[y]	0.0008
B54 (82)	*L. mucosae*	0	105.13	7.92	1440.44	308.19	0	0.0389
B57 (83)	*L. murinus*	57.35*	86.61	2.59	674.29	156.27	31.39*	0.0301
XB58 (84)	*L. gasseri*	36.85*	95.76	6.93	744.03	127.17	24.30*	0.0443
RPMI	media control	-	69.32	9.89	2394.59	45.12		
MRS	media control	-	123.49	28.74	2159.63	73.05	9.81**	0.0045
TH58	*L. saerimneri*	55.56*	147.95	21.03	2079.22	211.97	3.72	0.2841
B23 (85)	*L. salivarius*	39.62**	104.42	5.22	1790.08	246.57	17.11*	0.0338
B37 (86)	*L. salivarius*	25.86	138.03	25.04	1366.82	465.83	36.71*	0.0218
B55 (87)	*L. salivarius*[b]	35.99*	137.69	7.59	1624.80	112.25	24.77**	0.0011

[a] indicates TNF stimulation, [b] indicates TNF suppression and stimulation, [y] indicates cell viability of less than 80%

Table 8. Inhibitory effects of 12 gastric-derived *Lactobacillus* spp. on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; RPMI, AGS media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, ***p<0.001, **p<0.01,*<0.05. The experiments were performed in at least 3 times, each in triplicate.

Code	Sample		48h LCM		48h LCM plus *H. pylori*		% IL-8 suppression	*p*-value
	Lactobacillus species	% TNF suppression	IL-8 conc. (pg/m)	SD	IL-8 conc. (pg/ml)	SD		
MRS	media control	-	81.01	24.56	1734.99	149.82		
B90	*L. plantarum*	17.45*	101.92	20.17	749.44	400.03	56.80**	0.0081
MRS	media control	-	81.01	24.56	1734.99	149.82		
B91	*L. salivarius*	58.86*	89.71	34.37	1018.06	406.2	41.32*	0.0228
MRS	media control	-	86.44	9.54	1922.98	125.04		
B106	*L.casei* group	35.09*	130.65	43.25	1018.41	452.26	47.04*	0.0144
MRS	media control	-	77.56	19.83	1820.16	152.79		
B107	*L. casei* group	34.26*	87.97	5.41	1257.57	265.51	30.91*	0.0168
MRS	media control	-	91.43	7.82	1874.31	59.01		
B109	*L. salivarius*	18.14	103.7	8.41	1187.79	526.05	36.63*	0.0440
MRS	media control	-	92.91	13.54	1785.93	145.97		
B110	*L. salivarius*	45.91*	95.79	11.91	1419.32	103.15	20.53**	0.0051
MRS	media control	-	100.15	21.06	1992.13	145.06		
B55	*L. salivarius*	35.99*	99.19	38.84	1500.85	302.24	24.66*	0.0321
MRS	media control	-	95.58	24.19	2051.18	129.43		
B60	*L. salivarius*	48.03*	107.09	18.87	1196.59	429.93	41.66*	0.0150
MRS	media control	-	86.68	9.83	1961.50	162.06		
B102	*L. salivarius*	38.91***	112.58	8.44	1692.22	124.00	13.73*	0.0421
MRS	media control	-	72.81	15.21	1907.36	245.54		
B103	*L. casei*	21.48***	103.20	8.66	1098.62	509.27	42.40*	0.0342
MRS	media control	-	96.61	23.33	1481.43	575.79		
XB7	*L. plantarum*	57.23***	142.41	39.02	684.56	138.88	53.79*	0.0401
MRS	media control	-	95.21	24.77	2003.12	245.48		
B37	*L. salivarius*	25.86	107.9	26.19	1234.96	132.94	38.35**	0.0044

Table 9. Colony morphology and TNF immunomodulatory effects of IL-8 inhibitory *gastric-derived Lactobacillus* spp. ***p<0.001,**<0.01,*<0.05;TNF stimulation indicates TNF concentration >400 pg/ml, high indicates TNF concentration >700 pg/ml.

No.	*Lactobacillus* species	Code	Colony morphology	%TNF inhibition of		TNF Stimulation of	
				24-h LCM	48-h LCM	24 -h LCM	48- h LCM
1	*L. salivarius*	B91	Large, round, entire, convex, white, pearl-like	35.98	58.86*	yes	yes
2	*L. salivarius*	B109	Medium, round, entire, convex, white, pearl-like	16.35**	18.14	no	yes
3	*L. salivarius*	B110	Medium, undulated, umbonate, white, butter-like	0	45.91*	yes (high)	yes (high)
4	*L. salivarius*	B55	Medium, round, entire, convex, white, pearl-like	0	35.99*	no	yes (high)
5	*L. salivarius*	B60	Medium, oval, entire, convex, white, pearl-like	0	48.03*	no	no
6	*L. salivarius*	B102	Medium, round, entire, convex, white, pearl-like	0	38.91***	yes	yes
7	*L. salivarius*	B37	Medium, round, entire, convex, white, pearl-like	17.79	25.86	no	no
8	*L. plantarum*	B90	Medium, round, entire, convex, yellow, smooth	52.2*	63.64***	no	no
9	*L. plantarum*	XB7	Medium, round, entire, convex, white, pearl-like	77.26***	57.23***	no	no
10	*L. casei* group	B103	Medium, round, entire, raised, white, pearl- like	0	21.48***	no	no
11	*L. casei* group	B106	Medium, round, entire, convex, white, pearl-like	51.93**	35.09*	no	no
12	*L. casei* group	B107	Medium , round, entire, convex, white, pearl-like	11.49*	34.26*	no	no

Figure 1. Inhibitory effects of gastric-derived *Lactobacillus* spp. (B90, B91 and B106) on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, **p<0.01,*<0.05. The experiments were performed in at least 3 times, each in triplicate.

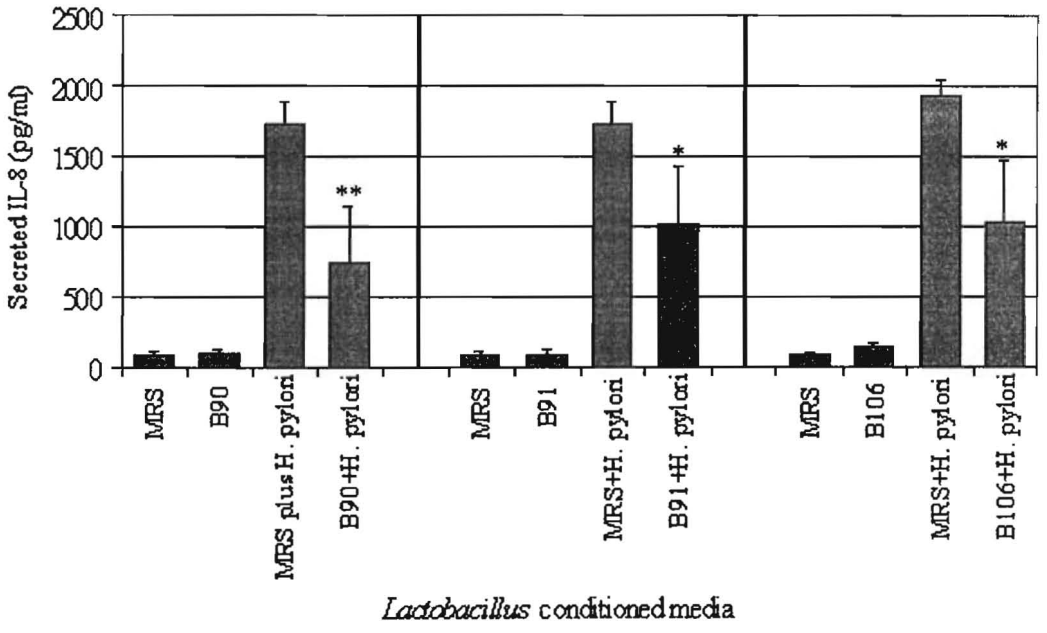


Figure 2. Inhibitory effects of gastric-derived *Lactobacillus* spp. (B107, B109 and B110) on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, **p<0.01,*<0.05. The experiments were performed in at least 3 times, each in triplicate.

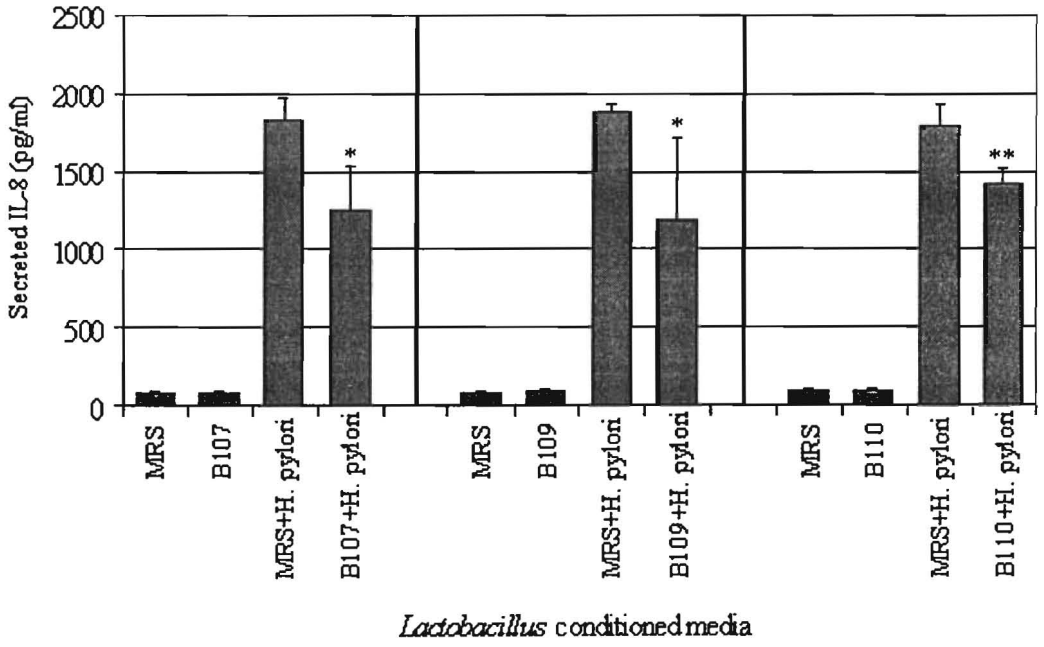


Figure 3. Inhibitory effects of gastric-derived *Lactobacillus* spp. (B55, B60 and B102) on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, *<0.05. The experiments were performed in at least 3 times, each in triplicate.

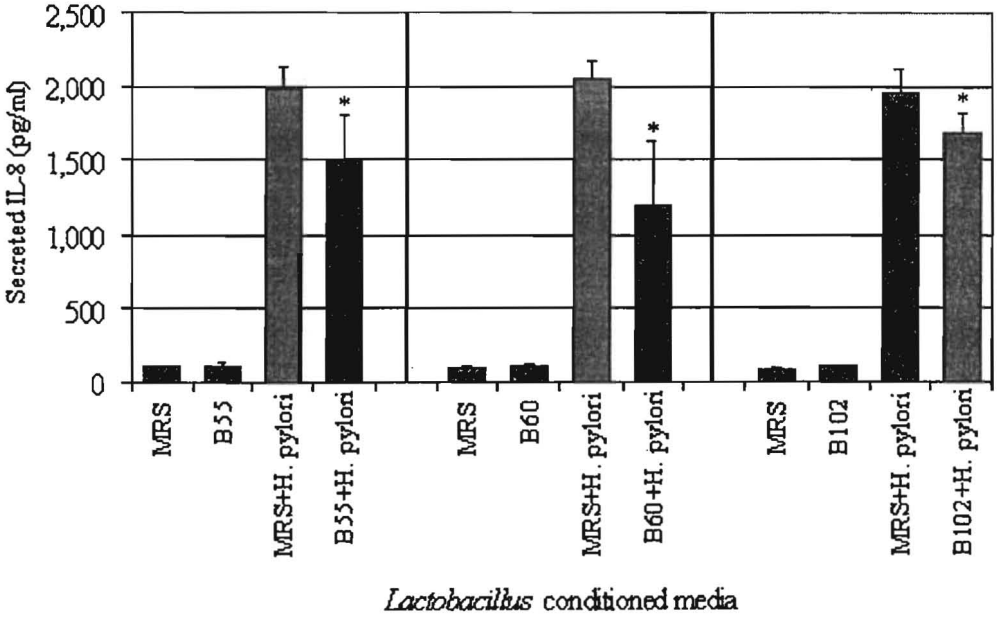


Figure 4. Inhibitory effects of gastric-derived *Lactobacillus* spp. (B103, XB7 and B37) on IL-8 production in *H. pylori*-stimulated AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, **p<0.01,*<0.05. The experiments were performed in at least 3 times, each in triplicate.

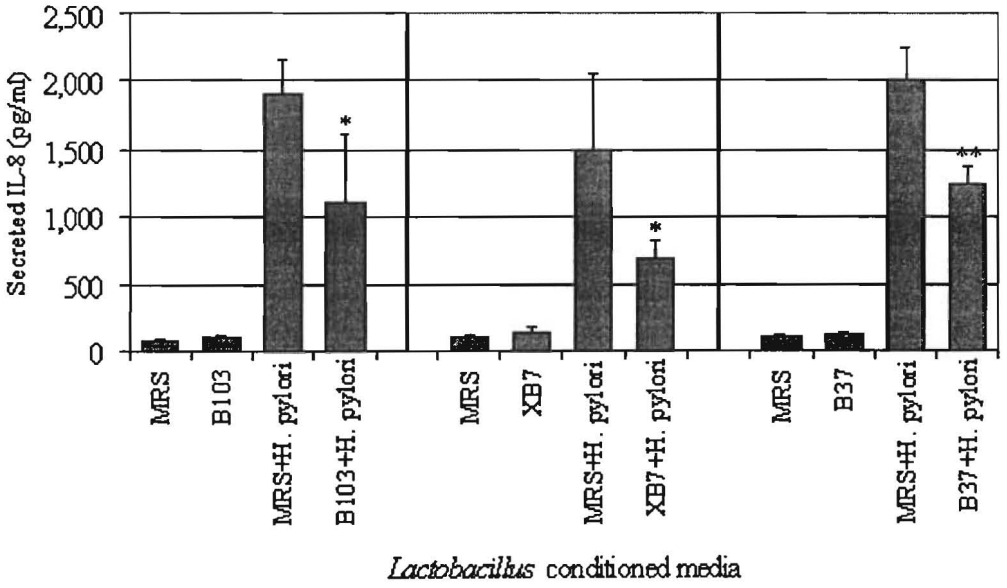


Table 10. Immunomodulatory effects of infant feces-derived *Lactobacillus* spp. No 1-16 on IL-8 production in *C. difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; McCoy's, HT-29 media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control: **p<0.01,*<0.05.

Code (no.)	Sample		48h LCM		48h LCM plus *C. difficile*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
McCoy's	Media control	-	109.07	4.20	1158.55	129.31		
MRS	Media control	-	81.19	2.05	598.07	178.34	48.38**	0.0058
L1 (1)	*L. gasseri*	0	76.60	3.23	475.31	124.98	20.53	0.1921
L2 (2)	*L. gasseri*	22.28	114.53	28.03	817.50	47.30	0	0.0542
L5 (3)	*L. salivarius*	6.51	68.32	2.57	523.89	84.58	12.4	0.2753
L6 (4)	*L. fermentum* [a]	0	76.93	7.05	454.92	114.61	23.94	0.1535
L9 (5)	*L. gasseri* [a]	0	76.66	11.49	517.47	106.70	13.48	0.2693
McCoy's	Media control	-	227.24	43.53	1793.57	22.49		
MRS	Media control	-	189.74	14.83	1078.15	371.52	39.89*	0.0408
L13 (6)	*L. ruminis* [a]	7.26	638.00[x]	201.36	1329.88	189.48	0	0.1774
L15 (7)	*L. fermentum* or *L. mucosae*	-	166.03	4.06	685.76	90.71	36.40	0.0751
L18 (8)	*L. fermentum* [a]	8.15	151.01	7.87	688.58	137.67	36.13	0.0819
L21 (9)	*L. fermentum*	-	170.62	7.00	594.32	75.30	44.88*	0.0458
L24 (10)	*L. mucosae* or *L. fermentum* [a]	0	219.48	38.14	1094.13	188.41	0	0.4751
L25 (11)	*L. gasseri*	2.39	170.82	36.07	789.98	234.58	26.73	0.1597
L27 (12)	*L. gasseri* [a]	0	178.75	27.75	742.75	124.64	31.11	0.1062
L28 (13)	*L. gasseri* [b]	7.44*	571.14[x]	116.11	926.52	152.63	14.06	0.2744
L34 (14)	*L. rhamnosus*	38.79*	156.92	23.44	516.43	181.41	52.10*	0.0391
L35 (15)	*L. rhamnosus*	53.19*	162.00	28.09	538.07	142.38	50.09*	0.0392
L3 (16)	*L. gasseri*	33.54	162.96	6.81	496.40	62.41	53.96*	0.0278

[a] indicates TNF stimulation (> 400 pg/ml TNF), [b] indicates TNF inhibition and stimulation, [x] indicates IL-8 stimulation

Table 11. Immunomodulatory effects of infant feces-*derived Lactobacillus* spp. no. 17-29 on IL-8 productions in *C. difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; McCoy's, HT-29 media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control: ***p< 0.001,**p<0.01,*<0.05

Code (no.)	Sample		48h LCM		48h LCM plus *C. difficile*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
McCoy's	Media control	-	81.32	14.71	517.47	100.87		
MRS	Media control	-	79.23	0.77	318.15	27.20	38.52*	0.0149
L41 (17)	*L. gasseri*	0	81.15	13.51	413.56	54.55	0	0.0267
L8 (18)	*L. salivarius*	26.77*	94.15	2.79	428.90	50.81	0	0.0146
L10 (19)	*L. gasseri*	65.27*	76.14	3.81	361.41	46.43	0	0.1181
L11 (20)	*L. salivarius*	48.92*	79.22	13.82	413.36	95.83	0	0.0866
L20 (21)	*L. gasseri*	51.32*	77.50	7.23	403.13	42.58	0	0.0218
McCoy's	Media control	-	137.57	25.23	1080.75	102.20		
MRS	Media control	-	91.22	6.45	837.30	188.78	22.52	0.0605
L23 (22)	*L. salivarius*	41.14*	83.46	1.65	722.45	153.23	13.72	0.2296
L26 (23)	*L. gasseri*[b]	23.56*	82.09	7.30	720.43	200.76	13.96	0.2517
McCoy's	Media control	-	284.37	36.96	2062.48	84.125		
MRS	Media control	-	216.24	34.21	1716.83	147.11	16.76*	0.0121
L17 (24)	*L. salivarius*[b]	24.55*	204.05	16.72	1631.84	231.50	4.95	0.3010
L19 (25)	*L. vaginalis*[b]	61.13*	189.11	2.03	1236.36	99.99	27.99**	0.0047
L22 (26)	*L. salivarius*[b]	28.01*	273.48	80.17	1993.60	79.73	0	0.0229
L30 (27)	*L. gasseri*	23.42*	206.63	17.98	1213.66	43.10	29.31**	0.0024
L33 (28)	*L. rhamnosus*	25.66*	172.89	5.26	1235.02	89.24	28.06**	0.0042
L38 (29)	*L. gasseri*	0	208.92	10.61	1360.20	56.16	20.77**	0.0086

[b] indicates TNF inhibition and stimulation

Table 12. Immunomodulatory effects of infant feces-*derived Lactobacillus* spp. no. 30-37 on IL-8 productions in *C. difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; McCoy's, HT-29 media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control: ***p< 0.001,**p<0.01,*<0.05.

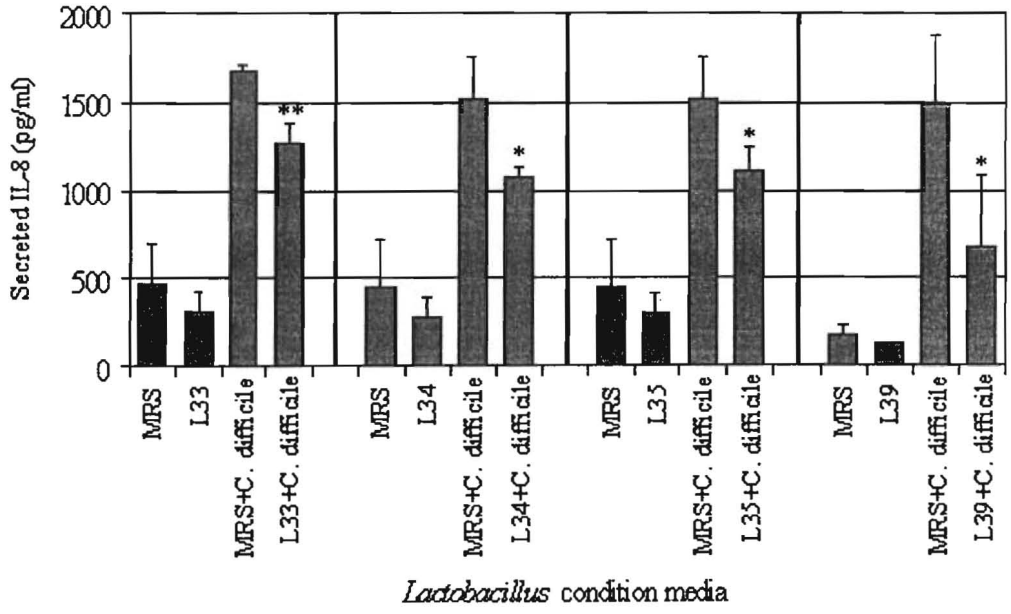
Code (no.)	Sample		48h LCM		48h LCM plus *C. difficile*		IL-8 suppression (%)	*p*-value
	Identity	TNF suppression (%)	IL-8 conc. (pg/ml)	SD	IL-8 conc.	SD		
McCoy's	Media control	-	128.29	11.15	1512.98	136.94		
MRS	Media control	-	143.34	7.29	1246.89	26.63	17.59*	0.0149
L40 (30)	*L. salivarius*[a]	0	171.10	16.76	1343.59	65.88	0	0.0390
L29 (31)	*L. gasseri*	28.45*	179.85	4.23	1317.61	128.33	0	0.2015
L31 (32)	*L. rhamnosus*	40.29*	158.50	4.99	1267.01	241.55	0	0.4464
L32 (33)	*L. gasseri*	36.53*	162.50	1.74	1445.57	131.07	0	0.0309
L7 (34)	*L. fermentum*[a]	0	154.09	11.48	1076.25	58.28	13.69**	0.0050
L12 (35)	*L. fermentum*[a]	0	151.31	16.04	1103.81	184.39	11.48	0.1271
L14 (36)	*L. mucosae*	0	162.67	3.69	1033.86	169.16	17.09*	0.0487
L39 (37)	*L. casei*	66.70*	135.25	16.15	707.27	463.30	43.28	0.0571

[a] indicates TNF stimulation (> 400pg/ml TNF)

Table 13. Immunomodulatory effects of 4 infant feces-derived *Lactobacillus* spp. on IL-8 production in *C. difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; McCoy's, HT-29 media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, ***p< 0.001,**p<0.01,*<0.05.

Code	Sample		48h LCM		48h LCM with *C. difficile*		% IL-8 suppression	*p*-value
	Lactobacillus species	% TNF suppression	Conc. (pg/m)	SD	Conc. (pg/ml)	SD		
MRS	Media control	-	471.75	221.28	1673.48	37.53		
L33	*L. rhamnosus*	25.66*	309.83	118.64	1267.11	112.74	24.28**	0.0020
MRS	Media control	-	447.45	263.37	1516.84	233.78		
L34	*L. rhamnosus*	38.79*	283.13	108.23	1073.38	60.69	29.24*	0.0168
MRS	Media control	-	447.45	263.37	1516.84	233.78		
L35	*L. rhamnosus*	53.19*	300.59	113.72	1112.17	124.58	26.68*	0.0286
MRS	Media control	-	174.54	54.04	1478.00	400.29		
L39	*L. casei*	66.70*	127.98	9.25	674.21	410.70	54.38*	0.0361

Figure 5. Inhibitory effects of 4 infant feces-derived *Lactobacillus* spp. on IL-8 production in *C. difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; McCoy's, HT-29 media control; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, *<0.05. The experiments were performed in at least 3 times, each in triplicate.



DISCUSSION

The gastrointestinal tract of humans are colonized by a large number of bacteria at birth and throughout life, with remarkable age-specific changes. These resident flora do not normally have any adverse effects and some of them have been shown to be necessary for maintaining the well-being of their host (56). The gut flora especially *Lactobacillus* and *Bifidobacterium* are, therefore, the important source of probiotics for human use. It has been documented that several *Lactobacillus* species produce immunoregulatory factors, known as immunomodulins, which may modulate cytokine production (57). Among the inflammatory cytokines, IL-8 is important in mediating inflammation in *Helicobacter pylori*-induced gastro-duodenal diseases and *Clostridium difficile*-associated disease (CDAD).

In this study, none of gastric-derived *Lactobacillus* spp. stimulated IL-8 production in *H. pylori*-activated AGS human gastric epithelial cells. Some isolates (12/87) suppressed IL-8 production in a contact-independent manner, suggesting the possibility that these isolates may secrete immunomodulins. Similar result was previously reported that viable *Lactobacillus bulgaricus* or its MRS culture supernatant inhibited IL-8 production in SGC-7901 cells treated with *H. pylori* lipopolysaccharide via inhibition of the TLR4 pathway (30). In addition, live *Lactobacillus gasseri* OLL2716 (LG21) was reported to suppress *H. pylori*-induced IL-8 production in MKN45 gastric epithelial cells and within gastric mucosa of *H. pylori*-infected patients (29).

Most of the IL-8 inhibitory isolates (11/12) in this study also suppressed TNF production. The majority of these isolates (7/12) belongs to *L. salivarius* which is an indigenous (autochtonous) flora of humans, whereas the rest belongs to transient (allochthonous) flora such as *L. plantarum* and *L. casei* group (58). The IL-8 inhibitory property of *Lactobacillus* spp. is strain-specific according to the results shown in Tables 1-7 demonstrating that the isolates of the same species had different immunomodulating activities. From a total of 23, 8 and 4 isolates of gastric-derived *L. salivarius, L. plantarum* and *L. casei* group; 7, 2 and 3 isolates suppressed IL-8, respectively. In addition, these isolates had different characteristics of colony morphology and TNF immunomodulation (Table 9). Most of *L. salivarius* which harbor IL-8-inhibitory activity (isolates B91, B109, B110, B55 and B102) have been

demonstrated to inhibit and stimulate TNF production in different magnitudes, whereas one isolate (B60) only inhibited TNF production and one isolate (B37) had no TNF- immunomodulatory activity (53). Two *L. plantarum* isolates (B90 and XB7) which inhibited IL-8 production seem to be different strain as the colony of B90 was yellow and that of XB7 was white. These two strains also inhibited TNF production and interestingly, all 8 gastric-derived *L. plantarum* had TNF-inhibitory activities (Tables 1-7). Among 3 isolates (B106, B107 and B103) of IL-8 - suppressing *L. casei* group, B106 and B107 seem to be the same strain as they were both isolated from the same human subject and their colonies were similar (Table 9). The other isolate (B13, Table 5) produced conditioned media toxic to AGS cells, resulting in the cell viability of only 20.08%. In addition to this toxic LCM-producing *L. casei* group strain B13, some isolates of *L. plantarum* (B64 and B67, Table 1 and B78, Table 2), *L. salivarius* (B8, Table 5; B73, Table 6 and B52, Table 7) and one isolate of *L. gasseri* (XB68, Table 1) also produced LCM which were toxic resulting in the AGS cell viability of 50-70%.

On the contrary to gastric-derived *Lactobacillus* isolates of which none stimulated IL-8 production, *L. ruminis* which is an infant-feces isolate demonstrated IL-8 stimulation (Table 10). This bacterial species also stimulated TNF production in THP-1 monocytic cells. *L. ruminis* was known to be an indigenous human flora and an isolate from adult-feces has been recently shown to have immunostimulatory effect by our investigation (4).The isolates of which conditioned media were confirmed to suppress *C. difficile*-induced IL-8 production belongs to *L. rhamnosus* and *L. casei,* the species of transient flora (Table 13). The ability of the other 7 isolates for IL-8 suppression was yet to be confirmed. Some of these isolates such as *L. gasseri* were among indigenous *Lactobacillus* species in humans. In the evaluation of probiotics to treat first or recurrent episodes of CDAD, most case series and case reports have shown favorable results with *L. rhamnosus* GG or *Saccharomyces boulardii* (59). The mechanism of action of both probiotics was partly understood in term of their anti-inflammatory effects and the modulation of host signaling pathway. *L. rhamnosus* GG and its conditioned media inhibited IL-1β-induced IL-8 production in Caco-2 human colon adenocarcinoma cells, at least in part, by inhibition of the NF-κB signaling pathway (60). *S. boulardii* culture supernatant inhibited IL-8 production stimulated by *C. difficile* toxin A or IL-1β by blocking the activation of Erk1/2 MAP kinases (61).

Indigenous human flora are considered to be appropriate for the source of probiotics strains. It has been noted that indigenous microbial strains that naturally persist in humans are tested by nature for their functionality in the gastrointestinal tract, possibly resulting in the possession of of adaptive traits to benefit the human host (62). Our findings of indigenous *Lactobacillus* species with immunomodulating property should provide candidate strains for further characterization of their mechanism of action in the amelioration or prevention of specific diseases.

CONCLUSION AND SUGGESTION

Our data indicate that specific strains of human-derived *Lactobacillus* spp. were able to modulate the production of cytokine involved in gastrointestinal inflammatory responses. *L. salivarius, L. plantarum* and *L. casei* group found in human stomach suppressed IL-8 production stimulated by *H. pylori* in AGS gastric epithelial cells and *L. rhamnosus* and *L. casei* isolated from infant feces did in *C. difficile*-stimulated HT29 colon epithelial cells. On the contrary, an infant feces-derived *L. ruminis* strain by itself stimulated IL-8 production in HT 29 cells. These IL-8 suppressing strains will be subjected to molecular typing for strain identity. Selected strains will be tested for their mechanism of action in the modulation of host signaling pathway as proposed in the research proposal. The probiotic candidate strains will also be tested for cytokine profile they elicit in the specific epithelial cells. Those results will provide scientific evidences for the selection of probiotics strain for health and specific disease. In addition to *in vitro* tests, *in vivo* study using appropriate animal model should be performed to prove the benefits of probiotics. Those strains which fulfill probiotics criteria will then be candidates for further studies in humans. These potential probiotic Thai strains are promising ones as they are well-adapted and naturally selected to persist in the gastrointestinal tract of Thai population.

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CURRICULUM VITAE

NAME : Somying Tumwasorn

ADDRESS : Department of Microbiology
Faculty of Medicine, Chulalongkorn University
Bangkok 10330, Thailand
Telephone : 662-256-4132
FAX : 662-252-5952
E-mail address : fmedsts@md.chula.ac.th
somying.T@chula.ac.th

POSITION : Associate Professor
Head, Bacteriology Division and Diagnostic Molecular Bacteriology Laboratory

EDUCATION:

Ph.D.	Immunology & Medical Microbiology, University of Florida, College of Medicine, Gainesville, Florida, USA, 1988
M.S.	Microbiology, Kasetsart University, Bangkok, Thailand, 1977
B.S. (Hons)	Food Science & Technology, Kasetsert University, Bangkok, Thailand, 1975

TRAINING:

Computer Applications in Molecular Biology. International Center For Genetic Engineering and Biotechnology, Trieste, Italy. 22 July – 2 August 1991

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PATENTS:

"Cloned *Porphyromonas gingivalis* genes and probes for the detection of periodontal disease" Patent Issued by United States Patent and Trademark Office: November 3, 1998. Patent No.: 5,830,710

Inventors: Ann Prokulske-Fox, Somying Tumwasorn, Guylaine Lepine, Naiming Han, Marilyn Lantz, Joseph M. Patti

CURRICULUM VITAE

NAME : James Versalovic

ADDRESS : Department of Pathology and Immunology
Baylor College of Medicine and Texas Children's Hospital,
Feigin Center, Suite 830
1102 Bates Avenue
Houston, Texas 77030
Phone : 832-824-2213
Fax : 832-824-1165
E -mail: jamesv@bcm.edu

POSITION : Professor of Pediatrics, Molecular Virology and Microbiology and Molecular and Human Genetics; Milton J. Finegold Professor, Department of Pathology and Immunology
Head, Department of Pathology
Chief, Pathology Service
Director, Texas Children's Microbiome Center

EDUCATION:
M.D. Baylor College of Medicine, Houston, TX, 1995
Ph.D. Baylor College of Medicine, Houston, TX (Cellular and Molecular Biology), 1994
B.A. High Honors (Plan II Honors Program) The University of Texas at Austin, Austin, TX, 1985

POSTDOCTORAL TRAINING:

Digestive Diseases Research Fellowship, Baylor College of Medicine, Houston, TX
Clinical Pathology Residency, Massachusetts General Hospital, Boston, MA
Pathology Clinical Fellowship, Harvard Medical School, Boston, MA
Comparative Medicine Fellowship, Massachusetts Institute of Technology, Cambridge, MA

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Peña JA, Rogers AB, Ge Z, Ng V, Li SY, Fox JG and **Versalovic J.** Probiotic *Lactobacillus* diminishes *Helicobacter hepaticus*-induced inflammatory bowel disease in IL-10-deficient mice. Infect Immun 2005;73:912-920.

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" Fingerprinting bacterial strains using repetitive DNA sequence amplification"
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