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“ผลของแลคโตบาซิลลัสและบิฟิโดแบคทีเรียม ต่อการยับยั้งคลอสตริเดียม ดิฟฟิซิล ในโมเดลการติดเชื้อของหนูทดลอง”

“Effect of *Lactobacillus* and *Bifidobacterium* on the inhibition of Clostridium difficile in mouse infection model”

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Abstract in Thai

คำสำคัญ : คลอสตริเดียม ดิฟฟิซิล/ แลคโตบาซิลลัส / บิฟิโดแบคทีเรียม /โพรไบโอติกส์/ไส้รั่ว/ไซโตไคน์ที่เกี่ยวข้องกับการอักเสบ

คลอสตริเดียม ดิฟฟิซิล เป็นสาเหตุหลักที่ทำให้เกิดการท้องเสียและลำไส้อักเสบที่เกี่ยวข้องกับการใช้ยาปฏิชีวนะในผู้ป่วยที่ได้รับยาแบบออกฤทธิ์กว้าง การทำลายจุลินทรีย์ประจำถิ่นโดยยาปฏิชีวนะมีผลให้คลอสตริเดียม ดิฟฟิซิล สามารถตั้งถิ่นฐานและหลั่งสารพิษที่มีผลให้เกิดไส้รั่วและการสร้างไซโตไคน์ที่เกี่ยวข้องกับการอักเสบ คณะผู้วิจัยได้ตรวจพิสูจน์แล็คโตบาซิลลัสและบิฟิโดแบคทีเรียมจำนวน 6 สายพันธุ์ที่สามารถลดการเกิดไส้รั่วและการอักเสบที่เกิดจากคลอสตริเดียม ดิฟฟิซิล ในหลอดทดลอง ในการศึกษานี้คณะผู้วิจัยมีวัตถุประสงค์เพื่อหาผลของการใช้เชื้อสายพันธุ์เหล่านี้ทั้งแบบเดี่ยวหรือใช้ร่วมกัน ต่อการยับยั้งการติดเชื้อคลอสตริเดียม ดิฟฟิซิล ในหนูทดลอง C57BL/6 การให้เชื้อแล็คโตบาซิลลัส แรมโนซัส L34 (1x106 เซลล์) เป็นเวลาวัน จากวันที่ให้ยาคลินดามัยซินจนถึงวันก่อนฆ่าหนู มีผลให้ลดการตาย การเปลี่ยนแปลงน้ำหนักตัว การท้องเสีย ไส้รั่ว และพยาธิสภาพของหนู และยับยั้งการสร้างไซโตไคน์ที่เกี่ยวข้องกับการอักเสบได้แก่ MIP-2, KC, IL-1β และ TNF-α ทั้งในเนื้อเยื่อลำไส้และซีรั่ม การให้เชื้อแล็คโตบาซิลลัส เคซิไอ L39, B13,B106 บิฟิโดแบคทีเรียม บิฟิดุม NB42 หรือ บิฟิโดแบคทีเรียม ซูโดแคเทนูลาตัม NB48 แบบเดี่ยวสามารถลดการตาย การเปลี่ยนแปลงน้ำหนักตัว และการท้องเสียในหนูทดลอง การให้เชื้อแล็คโตบาซิลลัส เคซิไอ L39, B13 และ B106 สามารถยับยั้งการสร้างไซโตไคน์ที่เกี่ยวข้องกับการอักเสบบางชนิดในเนื้อเยื่อลำไส้และซีรั่ม ยังไม่ได้ทดสอบผลของการให้บิฟิโดแบคทีเรียมต่อการยับยั้งการสร้างไซโตไคน์ เฉพาะแล็คโตค็อกเทล (แล็คโตบาซิลลัส แรมโนซัส L34 และ แล็คโตบาซิลลัส เคซิไอ L39) สามารถลดความรุนแรงของโรค ส่วนบิฟิโดค็อกเทล (บิฟิโดแบคทีเรียม บิฟิดุม NB42 และ บิฟิโดแบคทีเรียม ซุโดแคเทนูลาตัม NB48) หรือ แล็คโต-บิฟิโดค็อกเทล (แล็คโตบาซิลลัส แรมโนซัส L34, แล็คโตบาซิลลัส เคซิไอ L39, บิฟิโดแบคทีเรียม บิฟิดุม NB42 และ บิฟิโดแบคทีเรียม ซุโดแคเทนูลาตัม NB48) ไม่ลดความรุนแรงของโรค เชื้อสายพันธุ์เหล่านี้เป็นโพรไบโอติกส์ที่น่าจะใช้ได้ผลกับการติดเชื้อคลอสตริเดียม ดิฟฟิซิล

Abstract in English

Keywords: *Clostridium difficile/Lactobacillus / Bifidobacterium / probiotics /leaky gut/ inflammatory cytokines*

Clostridium difficile is a major cause antibiotic-associated diarrhea and colitis in patients with broad-spectrum antibiotic therapy. A disruption of the gut microbiota by using antibiotics results in colonization with *C. difficile* and release of toxins that cause leaky gut and the production inflammatory cytokines. We identified six specific strains of *Lactobacillus* or *Bifidobacterium* are able to reduce leaky gut and inflammation caused by *C. difficile in vitro*. In this study, we aim to investigate the effect of these strains either alone or in combination on the inhibition of *C. difficile* infection in a C57BL/6 mouse model. The administration of *L. rhamnosus* L34 ($1x10^6$ cells) for 4 days at the day of clindamycin injection to the day before sacrifice (D-1 to D2) reduced mortality, body weight change, diarrhea, gut leakage and pathology of mice and also suppressed the production of tissue and systemic inflammatory cytokines including MIP-2, KC, IL-1β and TNF-α. The administration of $1x10^8$ cells of *L. casei* L39, *L. casei* B13, *L. casei* B106, *B. bifidum* NB42 or *B. pseudocatenulatum* NB48 alone reduced mortality, body weight change and diarrhea in mice. *L. casei* L39, *L. casei* B13 and *L. casei* 106 administration also reduced the production of some tissue and systemic inflammatory cytokines. The effect of *Bifidobacterium* spp. on cytokine production was not determined yet. Only Lacto cocktail *(L. rhamnosus* L34 and *L. casei* L39) attenuated the disease severity whereas Bifido cocktail (*B. bifidum* NB42 or *B. pseudocatenulatum* NB48) or Lacto-Bifido cocktail (*L. rhamnosus* L34, *L. casei* L39, *B. bifidum* NB42, *B. pseudocatenulatum* NB48) did not. These strains are promising probiotics for *C. difficile* infection.

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List of Abbreviations

IL: Interleukin
CDI: *Clostridium difficile* infection
CDAD: *Clostridium difficile* -associated diarrhea
AAD: Antibiotic-associated diarrhea
MRS: deMan Rogosa Sharp
CO_2: Carbon dioxide
H_2: Hydrogen
N_2: Nitrogen
CFU: Colony forming unit
°C: Degree Celsius
ATCC: American type culture collection
Ml: Milliliter
µm: Micrometers
SD: Standard deviation
et al.: et alii
g: Gram
h: Hour
mM: Millimolar
mg: Milligram
rpm: Round per minute
µl: Microliter

Introduction

Clostridium difficile is an anaerobic, gram-positive spore forming bacillus which causes diarrhea and colitis [1]. The clinical symptom varies from asymptomatic to mild self-limited diarrhea and severe pseudomembranous colitis [2]. These symptoms result from the release of two protein exotoxins: toxin A (Tcd A), a 308-kD enterotoxin and toxin B (Tcd B), an approximately 270-kD cytotoxin [3, 4]. The pathogenesis of *C. difficile*-associated disease (CDAD) involves the disruption of gut microbiota by antibiotics which permits *C. difficile* colonization if the individual is exposed to the organism or its spores. Toxigenic strains of *C. difficile* produces two protein exotoxins: toxin A (TcdA), a 308-kD enterotoxin and toxin B (TcdB), an approximately 270-kD cytotoxin [3, 4]. TcdA and TcdB bind to specific receptors on the surface of the intestinal epithelium cells, internalized and stimulate the production of interleukin (IL)-8 [5]. Both toxins modify and inactivate Rho proteins via glucosylation of Rho GTPases resulting in the rearrangement of actin cytoskeleton, disruption of tight junctions, rounding up of cell, cell death and loss of intestinal epithelium barrier function [6, 7]. Previous studies showed that *Clostridium difficile* toxins cause tight junction disruption which was observed from the decrease in transepithelial electrical resistance and the increased paracellular permeability in epithelial cell lines incubated with toxins [8-10]. Disruptions of tight junctions facilitates TcdA and TcdB to cross the epithelium, reaching and activating monocytes, macrophages and mast cells to secrete several inflammatory cytokines, such as interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α), IL-1 and IL-6.These inflammatory cytokines, especially IL-8 cause neutrophil and lymphocyte influx resulting in pseudomembrane formation and diarrhea [11, 12].

We previously reported the ability of *Lactobacillus rhamnosus* L34 (LR-L34) and *Lactobacillus casei* L39 (LC-L39) to suppress *C. difficile*-induced IL-8 production in HT-29 colonic epithelial cells [13]. *L. rhamnosus* L34 is also able to increase tight junction (TJ) integrity and prevent *C. difficile* -induced TJ disruption of Caco-2 colonic epithelial cells [14]. Moreover, we identified three strains of *Bifidobacterium* including *Bifidobacterium bifidum*-NB42 (BB-NB42) and *Bifidobacterium psedocatenulatum*-NB48 (BP-NB48) with the ability to increase TJ integrity and demonstrated that BB-NB-42 can prevent *C. difficile* -induced TJ disruption Caco-2 colonic epithelial cells [15]. We thus aim to investigate the effect of these lactobacilli and bifidobacteria either alone or in combination on the inhibition of *C. difficile* infection in a mouse infection model.

Materials & Methods

Bacterial strains and culture conditions

Lactobacillus and *Bifidobacterium* in this study (Table1) were obtained from stock culture kept at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. *Lactobacillus* and *Bifidobacterium* strains were stored in deMan Rogosa Sharpe (MRS) broth (Oxoid, Hampshire, UK) containing 20% (v/v) glycerol and brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) containing 20% (v/v) glycerol, respectively, at -80℃. Lactobacilli and bifidobacterial were cultured on MRS agar and BHI agar, respectively, in an anaerobic chamber (Concept Plus, Ruskinn Technology, UK) (10% CO_2, 10% H_2, and 80%N_2) at 37℃ for 24 h. *Clostridium difficile* ATCC BAA1870 (tcdA positive, tcdB-positive, cdtB positive, Toxinotype IIIb, Ribotype 027, PFGE Type NAP1, REA Type B1 8; ATCC, Manassas, VA, USA) was cultured on Brucella agar (Becton, Dickinson, France) at 37℃ for 48 h under anaerobic conditions as mentioned above.

Table 1. *Lactobacillus* and *Bifidobacterium* in this study

Strains	Source	IL-8 suppression [a]	Intestinal integrity enhancement [b]	Prevention of tight junction disruption [c]	References
L. rhamonosus L34	Infant feces	(+)	(+)	(+)	[13, 14]
L. rhamonosus L31	Infant feces	(-)	(-)	(-)	[13, 14]
L. casei L39	Infant feces	(+)	(+)	(+)	[13, 14]
L. casei B13	Gastric biopsy	(+) This study [d]	ND	ND	[16]
L. casei B106	Gastric biopsy	(+) This study [d]	ND	ND	[16]
B. bifidum NB42	Breast milk	(+) This study [d]	(+)	(+)	[15]
B. pseudocatenulatum NB48	Breast milk	(+) This study [d]	(+)	(+)	[15]

[a] IL-8 suppression in *Clostridium difficile* –stimulated HT-29 colonic epithelial cells

[b] Tested by the measurement of transepithelial electrical resistance (TEER)

[c] Prevention of tight junction destruction by *C. difficile*

[d] See supplementary data

ND, not determined

Animals and *C. difficile* animal model

The US National Institutes of Health (NIH) protocols (NIH publication protocols #85-23, revised 1985) were followed. Male, 6 weeks old C57BL/6 mice were used. The animal protocol (#006/2560) was approved by the Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All animals were housed in an air-conditioned room designed for infectious animals under a 12:12 h light-dark cycle. Mice were provided with a standard diet and ad libitum access to tap water.

Mice were randomly separated into different groups (eight mice per group) and subjected to the experiments as follows.

A. Mouse model of *C. difficile* infection. A mouse model of CDI was performed as previously described with minor modification [17, 18]. Briefly, five hundred microliters of a cocktail of antibiotics (Sigma-Aldrich, St. Louis, MO, USA) containing gentamicin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg) and vancomycin (4.5 mg/kg) were given to mice twice a day for 3 days by oral gavages (Figure 1). A single dose of clindamycin (10 mg/kg) was then injected intraperitoneally (D-1). Subsequently, mice were gavaged with either 0.5 ml of normal saline (NSS) in antibiotics control group, or $1x10^{10}$ CFU/ml *C. difficile* in NSS in groups of C. difficile-infected mice at day 0 and day 1 (D0 and D1).

B. Treatment of *C. difficile*-infected mice with *Lactobacillus* and/or *Bifidobacterium* strains

C. difficile-infected mice were treated with *Lactobacillus* and *Bifidobacterium* strains in Table 1 either alone or in combination at various doses and time points as will be shown in the results. Groups of mice were divided as shown in Table 2.

Table 2. Groups of mice in this study

Group of mice	**Number of mice/Group**
Non-infected mice (treated with only antibiotics)	8
C. difficile-infected mice (treated with *C. difficile* and PBS)	8
C. difficile-infected mice treated with *Lactobacillus* and/or *Bifidobacterium*	8

At the end of the experiment, blood collection was performed via tail vein nicking at D1 (3h after FITC-dextran administration) for gut permeability determination. Mice were sacrificed through cardiac puncture under isoflurane anesthesia when moribund or at D3 with blood collection. The

cecum and colon sections were collected at the time of sacrifice, by cutting a haft of cecum and 1 cm of colon for histopathology examination. One part of cecum and colon were collected to test tissue inflammation by cytokine analysis. The rest of tissue were recorded by weight and stored at -80ºC for *C. difficile* quantification by real-time PCR.

Evaluation of *C. difficile* infection (CDI) in mice

Mice symptoms observation

Mice were monitored to observe the symptoms of CDI (i.e., diarrhea, hunching and wet tail) [19] and survival rate and record their body weight every day.

Gut leakage measurement

Intestinal epithelial permeability defect (gut leakage) in mice was determined by fluorescein isothiocyanate-dextran (FITC-dextran) assay. Briefly, 12.5 mg/0.5ml of FITC-dextran (molecular weight 4.4 k Da, Sigma, St. Louis, MO, USA) was orally gavaged at day3 (D3) and blood was collected through cardiac puncture under isoflurane anesthesia at 3h later. Blood was centrifuged and serum was kept in -80°C until analysis. Serum FITC-dextran was measured by the fluorospectrometry (NanoDrop 3300; Thermo Scientific, Wilmington, DE, USA) with the excitation and emission wavelength at 485 and 523 nm, respectively, against a standard curve of serially diluted FITC-dextran in phosphate buffer solution (PBS).

Cytokines measurements in sera and tissues

To test the effect of *Lactobacillus* and *Bifidobacterium* to suppress inflammation in mice, cytokines in sera and cecum and colon tissues were measured. Chemokine including macrophage inflammatory protein 2 (MIP-2), keratinocyte chemoattractant (KC), TNF-α and IL-1β in sera were measured with the ELISA assays (PeproTech, NJ, USA). MIP-2 and KC are homologs of human IL-8. All assays were performed according to the manufacturer's protocol. Cecum and colon tissues were collected at sacrifice, weighed, homogenized (Ultra-Turrax homergenizer, IKA, Staufen, Germany) in 500 µl of PBS containing protease inhibitor, and then centrifuged at 12,000 xg for 10 min at 4ºC. The supernatant was collected and stored at -80°C until analysis. Tissue cytokines (MIP-2, KC, TNF-α and IL-1β) as the representatives of local inflammatory responses were measured with the ELISA assays.

Quantification of *Clostridium difficile* in mouse cecum and colon

The presence of *C. difficile* in mice was enumerated by quantitative real-time PCR. DNA was extracted from in cecum and colon content by using High Pure PCR Template Preparation Kit (Roche, USA) according to the manufacturer's instruction and quantitated by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc, USA). The previously described tcdB-specific primers

tcdB-F [5'-GGAAAAGAGAATGGTTTTATTAA-3'] and tcdB-R [5'-ATCTTTAGTTATAA CTTTGACATCTTT-3'] from the conserved 5' region of tcdB were used to generate a 160-bp fragment [19]. The standard curve was created by the Light Cycler software using 10-fold serial dilutions of *C. difficile* DNA equivalents to copy number. The profiling standard curve was indicated as a graph of crossing point (Cp) vs. bacterial copy number. PCR reaction was performed in 20 μl mixture containing 4 μl of Faststart DNA LightCycler®FastStart DNA Master^{PLUS}SYBR Green I (Roche, Germany), 9 μl of nuclease-free water, 1 μl of each 10μM primer, and 5 μl of plasmid template. PCR reaction was performed as following by 45 cycles of 95°C for 10s, 55°C for 10s, 72°C for 25s with LightCycler® 2.0 instrument (Roche, Germany). The amplified product was measured by a SYBR green fluorescent signal using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche, Germany). Melting curve was analyzed after amplification step as the following: 0 s at 95°C; 1 min at 55°C; 0 s at 95°C until all product was denatured. *C. difficile* quantification was calculated by using the standard curve and shown in bacterial copy numbers.

Histopathology of mouse cecum and colon

Mice were sacrificed and cecum and the proximal 2/3 of colon were fixed in 10% buffered formalin and stored at room temperature until placed in 10% ethanol. Tissue from each mouse was placed in separate tissue cassette, then processed and paraffin embedded. Slides with two levels of sections from each paraffin block were routine-stained with haematoxylin and eosin (H&E). The histological severity of enteritis was graded using a scoring system reported previously by Reeves et al. It was used to determine epithelial damage (score 0–4), edema (score 0–4) and cellular infiltration (score 0–4) and assigned a total score between 0 and 12. [20, 21].

Fecal microbiome

The microbiota analysis protocol was performed as previously reported [22]. Caecal content samples from individual mice were used for metagenomic DNA extractions, with 3 independent extractions of 0.25 g performed per sample. The DNA Isolation Kit (MoBio, Carlsbad, CA, USA) was used to extract total nucleic acid. Metagenomic DNA quality was assessed by agarose gel electrophoresis and nanodrop spectrophotometry. Universal prokaryotic 515F (forward; (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (reverse; 5'-GGACTACHVGGGTWTCTAAT-3'), with appended 5' Illumina adapter and 3' Golay barcode sequences, were used for 16S rRNA gene V4 library construction [22]. Independent triplicate PCR experiments were performed and pooled to prevent stochastic PCR bias. The 16S rDNA amplicons of 381 base pairs (bp) were purified by the GenepHlow^{TM} Gel Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), and quantified with Picogreen (Invitrogen, Eugene, Oregon, USA). Each samples at 240 ng were pooled for sequencing by Miseq300

platform (Illumina, San Diego, CA, USA) [23]. Raw sequences were quality screened by Mothur's MiSeq platform procedures [24]. Quality screening steps including the removal of reads that have (i) ambiguous bases, (ii) >1 mismatch in the reverse primer sequence, (iii) >10 homopolymers, (iv) a minimum quality score of <35 over a 50-bp window, (v) a read length of <350 bases, and (vi) chimeric sequence. Quality sequences were aligned and assigned taxon (operational taxonomic unit, OTU) based on a default parameter as previously published [24]. Samples were normalized to an equal sampling depth (N=118121 reads per sample) [24]. Good's Coverage was used to estimate the OTU coverage by sequencing (all samples showed 99.7-100% coverage). Non-metric multidimensional scaling (NMDS) was used to analyze OTU structural patterns.

Statistical analysis

All experiments were performed three times each in triplicate and the results were reported as mean or standard error of mean (SEM) ± standard deviation (SD). The data were analyzed in GraphPad Prism 5 using the unpaired t test with one-tailed distribution and considered statistically significant at a *P*-value ≤ 0.05, unless otherwise stated.

Results

1. Development of mouse model of *C. difficile* infection using *C. difficile* ATCC BAA 1870

A C57BL/6 mouse model of *C. difficile* infection as shown in Figure 1 resulted in the development of clinical signs characterized by diarrhea (wet tail and traces of liquid stool), weight loss, hunched posture and sometimes death (Figure 2). Mice in antibiotics control group did not show signs of disease and also showed 100 percent survival. Percent survival of *C. difficile*- infected group was significantly different from antibiotics control group ($p<0.05$, Figure 2A). *C. difficile*-infected mice had weight loss (Figure 2B) and approximately 80% had diarrhea for 2 days (Figure 2C).

Analysis of serum cytokines and intestinal leakage in dead mice and alive mice showed that the levels of serum IL-1β, TNF-α, MIP-2 and KC in dead mice were significantly higher than those in alive mice (Figure 3A-D). In addition, the levels of serum FITC-dextran of dead mice were significantly higher than those of alive mice (Figure 3E). The data suggested that the severity or death of mice depend on the increase of serum cytokines and intestinal leakage.

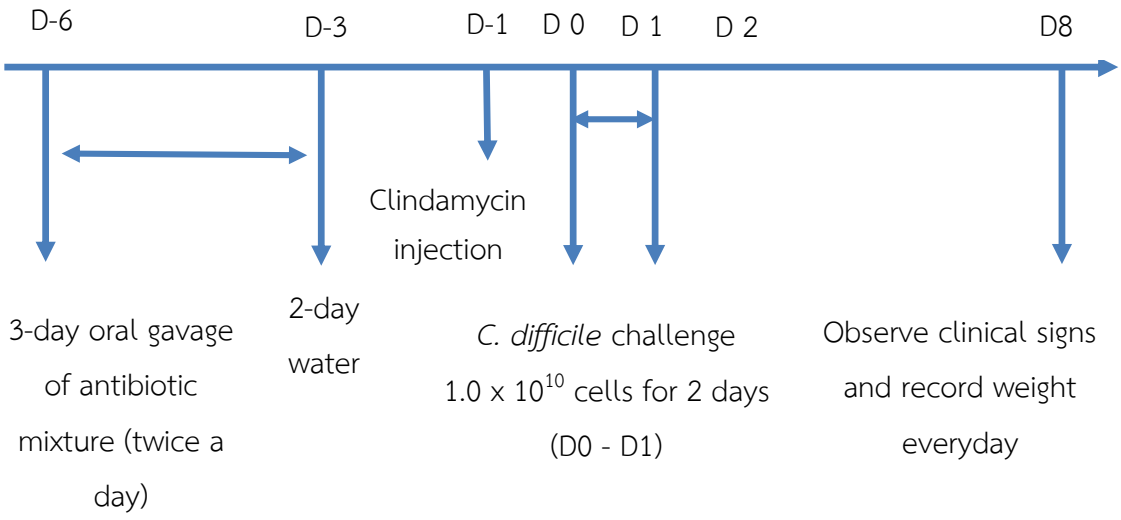


Figure 1. Experimental scheme for primary *C. difficile* infection (CDI) mouse model

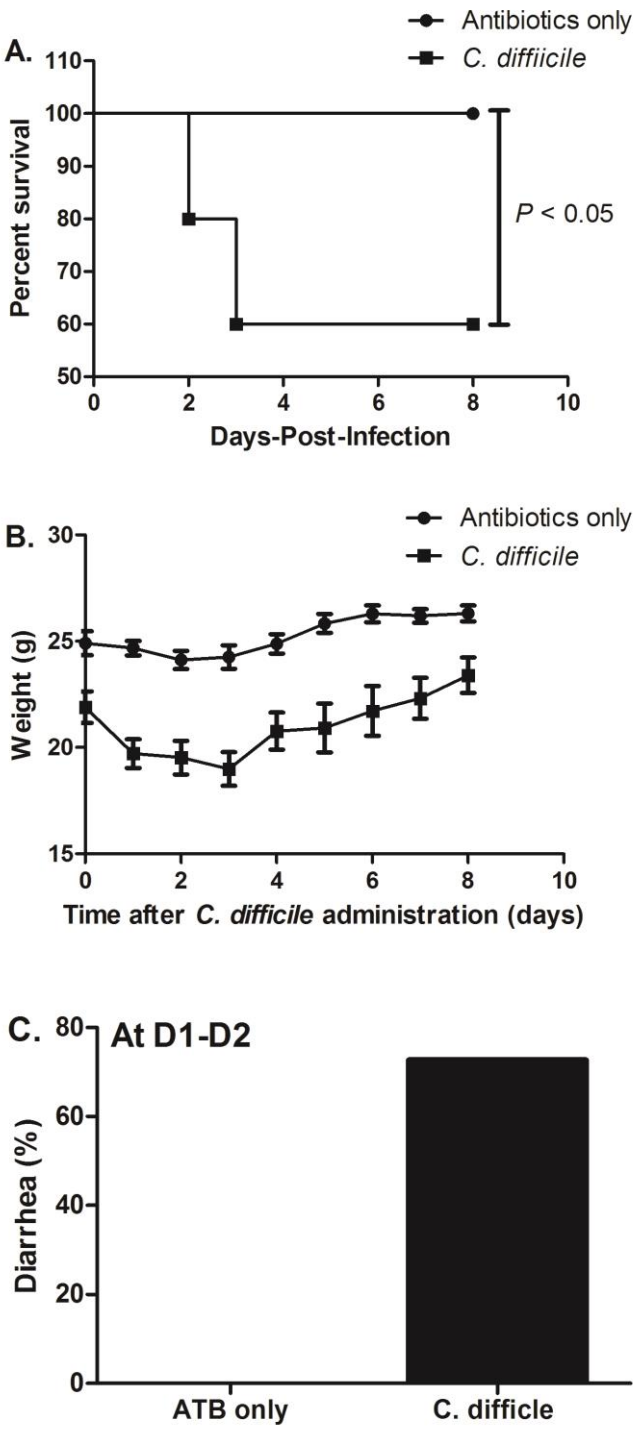
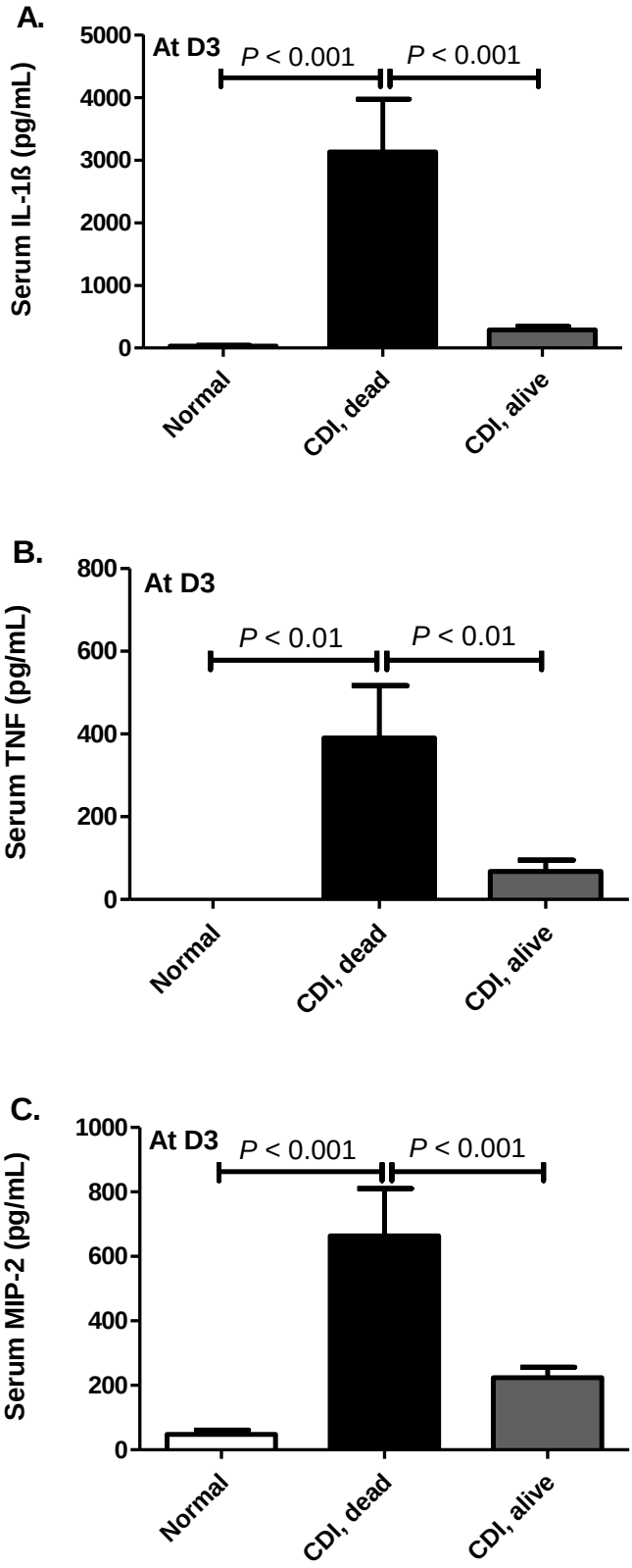


Figure 2. Development of *C. difficile* infection after challenge with *C. difficile* BAA1870. A; the percentage of survivors is presented at day 0 to day 8. B; weight of mice, C; the percentage of diarrhea mice at day1-2.


A.
At D3
P < 0.001
P < 0.001
Serum IL-1ß (pg/mL)
5000
4000
3000
2000
1000
0
Normal
CDI, dead
CDI, alive
B.
At D3
P < 0.01
P < 0.01
Serum TNF (pg/mL)
800
600
400
200
0
Normal
CDI, dead
CDI, alive
C.
At D3
P < 0.001
P < 0.001
Serum MIP-2 (pg/mL)
1000
800
600
400
200
0
Normal
CDI, dead
CDI, alive

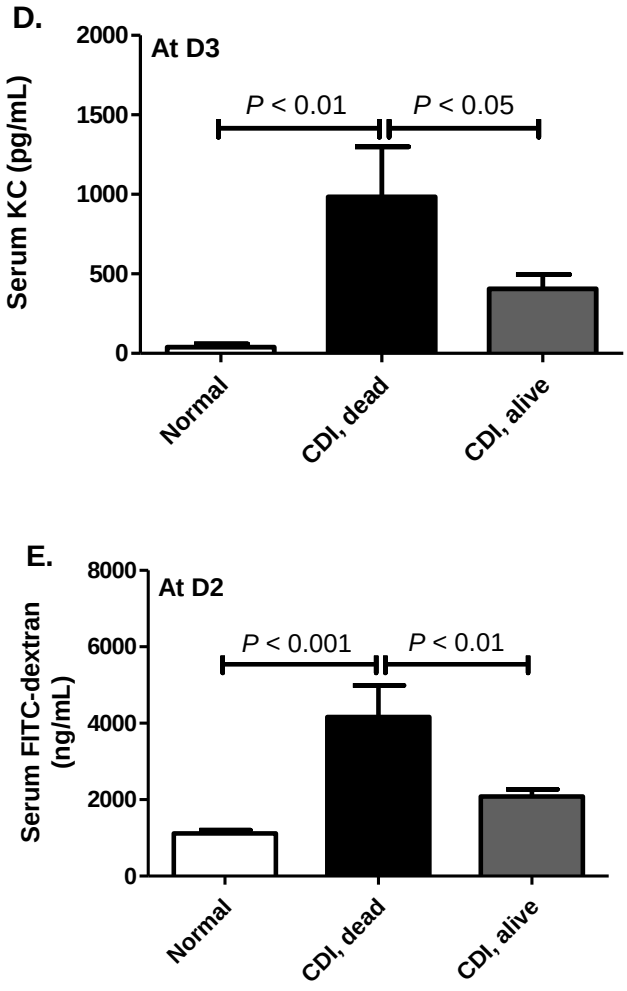


Figure 3. The correlation between death and serum cytokines or FITC-dextran. A-D; the level of serum cytokines IL-1β, TNF-α, MIP-2 and KC, respectively.

2. **Effects of *L. rhamnosus* L34 (LR-L34) treatment on *C. difficile* infection in mice**

We have tried using high dose (10^{10} cells/mouse) of LR-L34 and found that it led to more or worse symptoms in mice compared with *C. difficile*-infected mice (as shown in the progress report).

We then used a lower dose of LR-L34 to study its effects on *C. difficile*-infected mice. LR-L34 $1.0x10^8$ cells were given simultaneously with *C. difficile* at day0 and day1 (D0 and D1) as shown in Figure 4. LR-L31, a matching strain without the ability to suppress IL-8 *in vitro* was used as a negative control. It was found that the application of LR-L34 increased percent survival in mice as compared with *C. difficile*-infected group while LR-L31 did not (Figure 5). None of the mice in antibiotics control group was dead but approximately 20% had diarrhea. Percent survival of LR-L34 -treated group (92.85 %) was significantly different (p= 0.01) as compared with *C. difficile*-infected group (50%) and LR-L31-treated group (50%) (Figure 5). Treatment with LR-L34 slightly increased body weights of mice at day1-

3 (D1-D3) (Figure 6A), reduced diarrhea as compared with LR-L31 and *C. difficile*-infected group (Percent diarrhea was 28.57, 66.67 and 85.71, respectively) (Figure 6B), significantly (p= 0.01) reduced the level of serum FITC-dextran as compared with *C. difficile*-infected group (Figure 6C), reduced the levels of serum cytokines IL-1β, TNF-α, MIP-2 and KC (Figure 7A-D). Treatment of LR-L31 also reduced the levesl of serum FITC-dextran (Figure 6C) and suppressed serum cytokine MIP-2, and KC ($p< 0.05$) (Figure 7A-D).

In order to test ability of LR-L34 to suppress local cytokine production, tissue cytokines were determined. Treatment with LR-L34 significantly diminished the levels of IL-1β and TNF-α in colon tissue while non-significantly diminished KC level (Figure 8A,B,D). LR-L31 also suppressed IL-1β and TNF-α in colon tissue of survivor mice.

Mice infected with *C. difficile* showed the swelling of ileum, cecum and colon with small amount of intestinal content. Treatment with *L. rhamnosus* LR-L34 reduced swelling of the intestine and also increased cecum and colon content. Survivor mice after treatment with LR-L31 reduced swelling of intestine about 40%. These results were shown in Figure 9.

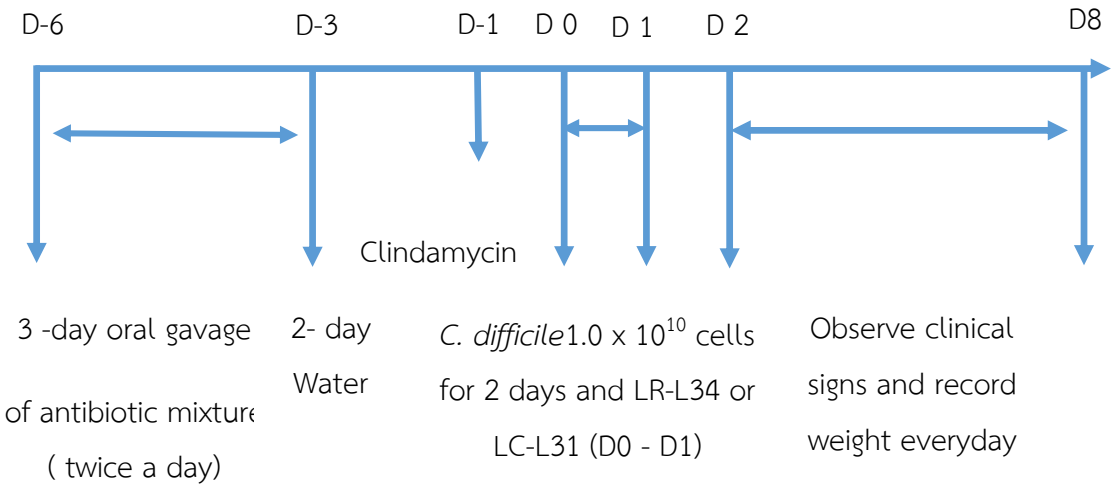


Figure 4. Experimental scheme for simultaneous treatment of LR-L34 or LR-L31 (1.0×10^8 cells/mouse) in *C. difficile* – infected mice

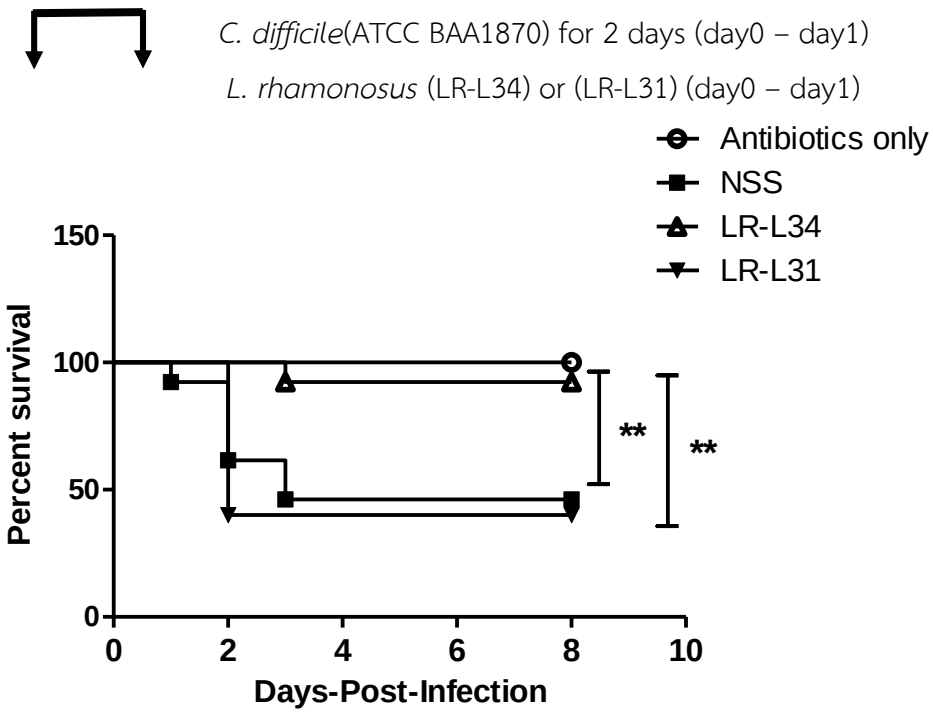


Figure 5. The percentage of survivors in each group at day 0 to day 8. (*C. difficile*- infected mice were treated simultaneously with LR-L34 or LR-L31 (1.0×10^8 cells/mouse) (p=0.01).

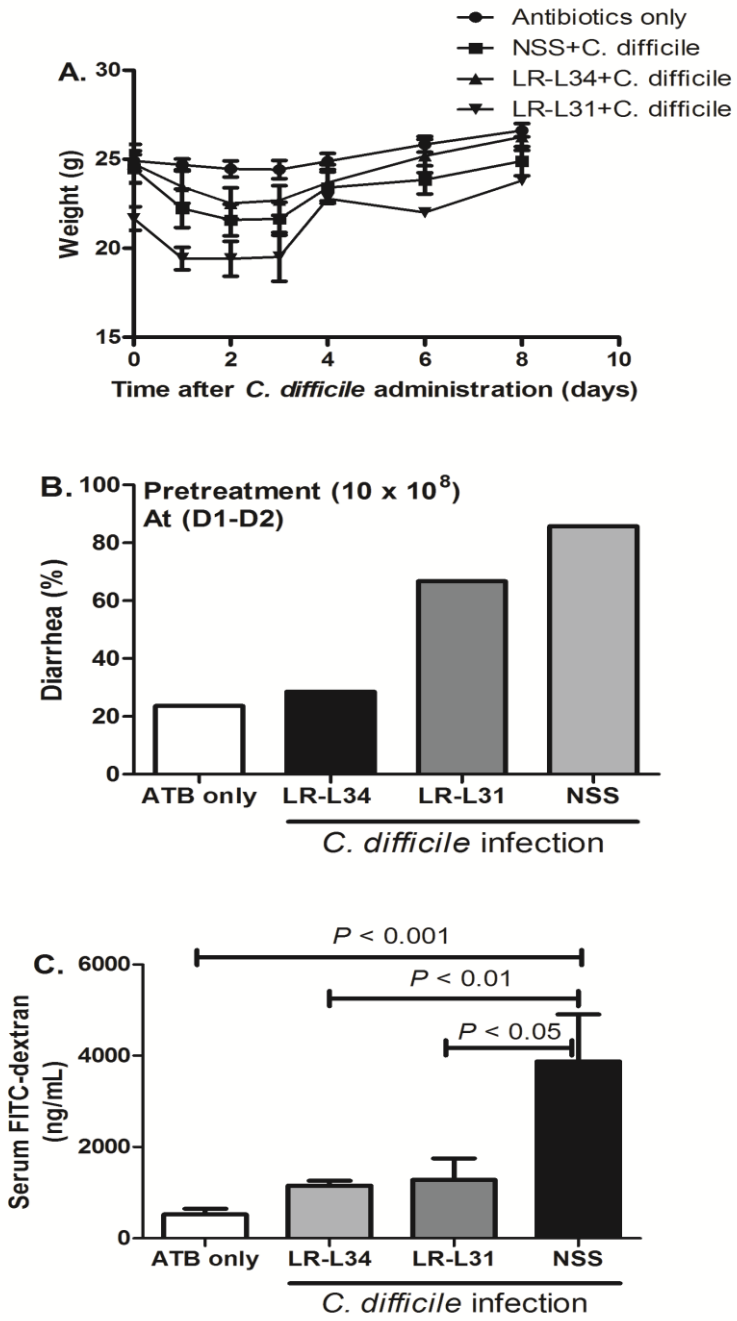
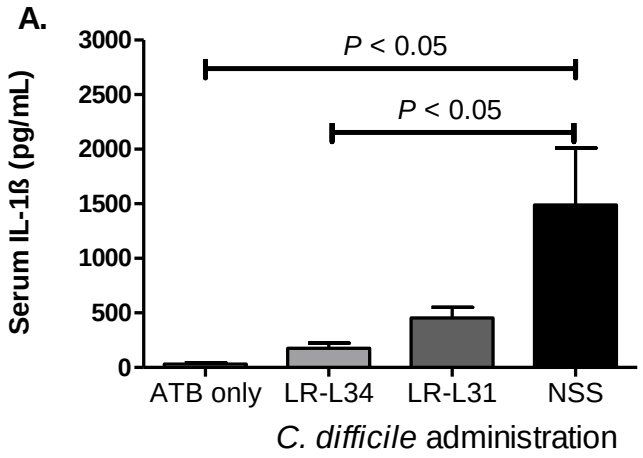
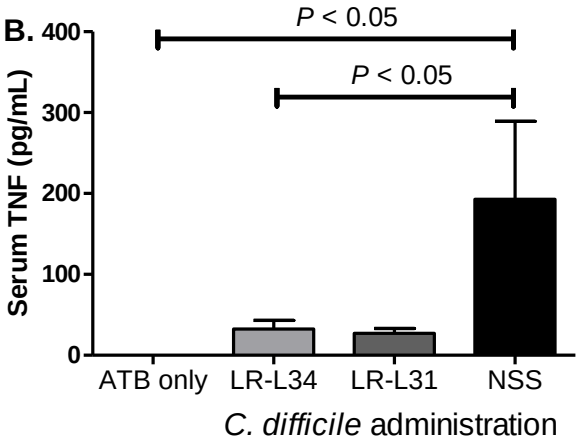
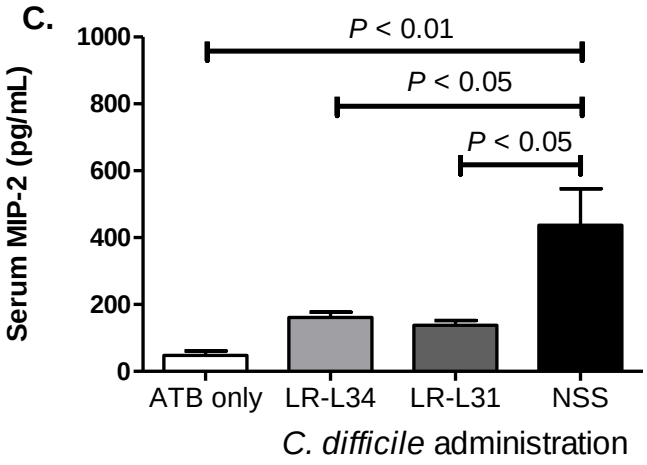


Figure 6. Simultaneous treatment with 1.0 x 10^8 cells of LR-L34 or LR-L31 per mouse (D0-D1). .A; Weight of mice (g). B; Percent diarrhea was observed at D1- D2. C; Serum FITC-dextran was detected at D3.


A.
Serum IL-1ß (pg/mL)
3000
2500
2000
1500
1000
500
0
P < 0.05
P < 0.05
ATB only
LR-L34
LR-L31
NSS
C. difficile administration


B.
Serum TNF (pg/mL)
400
300
200
100
0
P < 0.05
P < 0.05
ATB only
LR-L34
LR-L31
NSS
C. difficile administration


C.
Serum MIP-2 (pg/mL)
1000
800
600
400
200
0
P < 0.01
P < 0.05
P < 0.05
ATB only
LR-L34
LR-L31
NSS
C. difficile administration

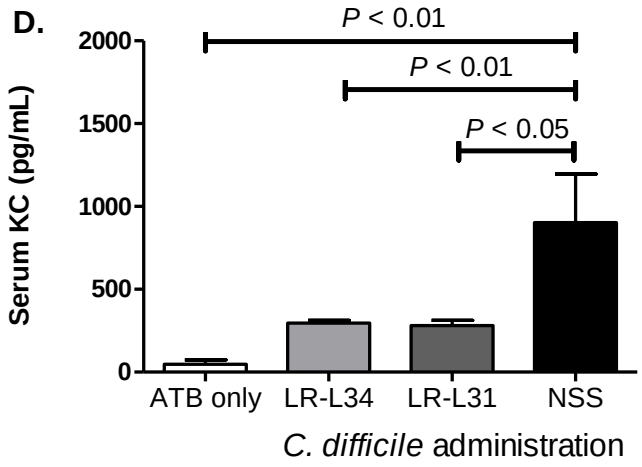
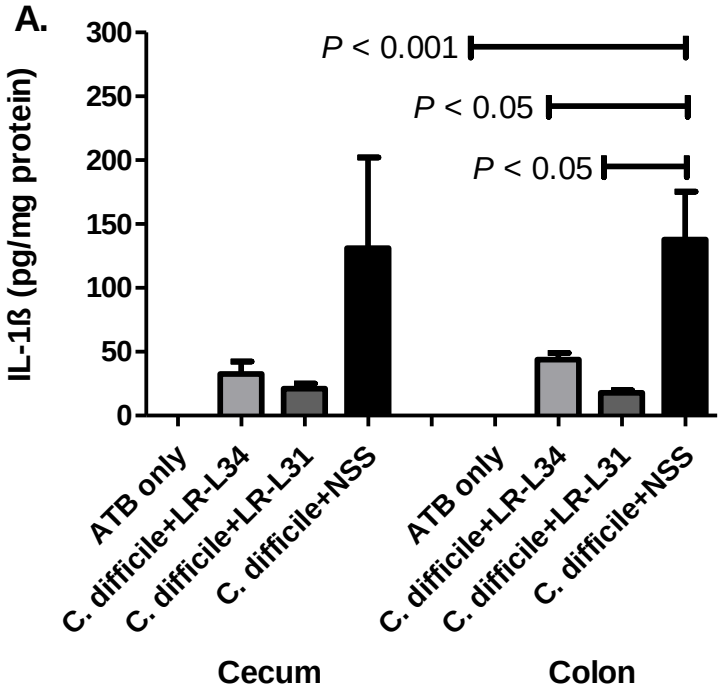
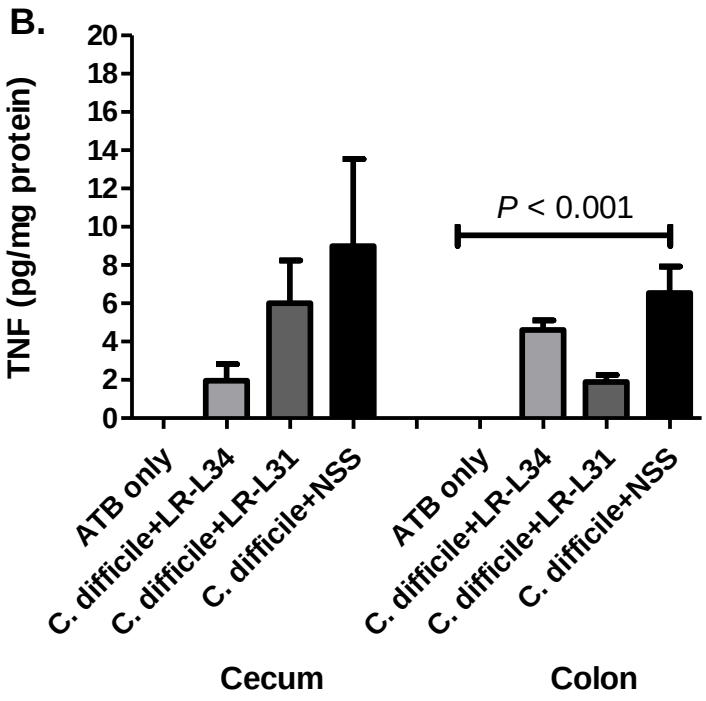
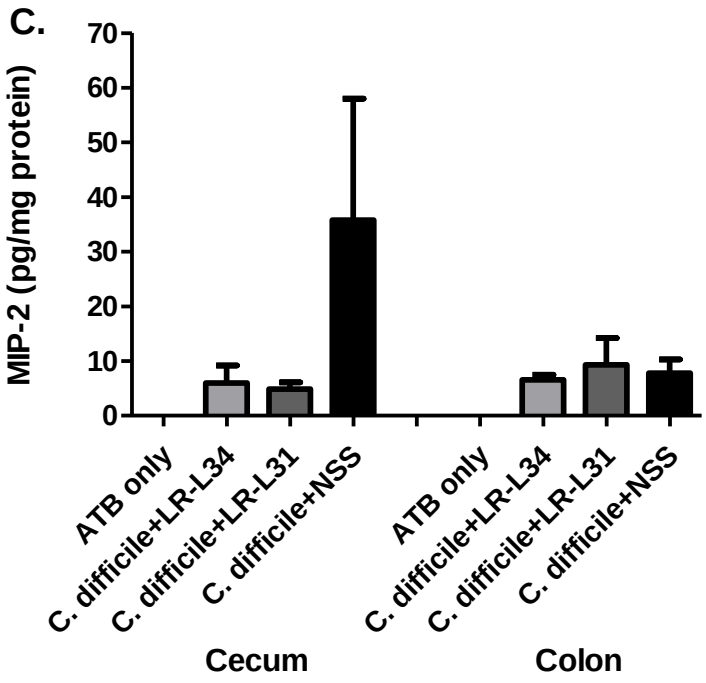


Figure 7. Simultaneous treatment with 1.0 x 10^8 cells of LR-L34 or LR-L31 on the day of *C. difficile* infection (D0-D1). Serum cytokines were analyzed using ELISA assay at day3. A; IL-1β (pg/mL), B; TNF-α (pg/mL), C; MIP-2 (pg/mL), D; KC (pg/mL).




B.
TNF (pg/mg protein)
20
18
16
14
12
10
8
6
4
2
0
P < 0.001
ATB only
C. difficile+LR-L34
C. difficile+LR-L31
C. difficile+NSS
ATB only
C. difficile+LR-L34
C. difficile+LR-L31
C. difficile+NSS
Cecum
Colon


C.
MIP-2 (pg/mg protein)
70
60
50
40
30
20
10
0
ATB only
C. difficile+LR-L34
C. difficile+LR-L31
C. difficile+NSS
ATB only
C. difficile+LR-L34
C. difficile+LR-L31
C. difficile+NSS
Cecum
Colon

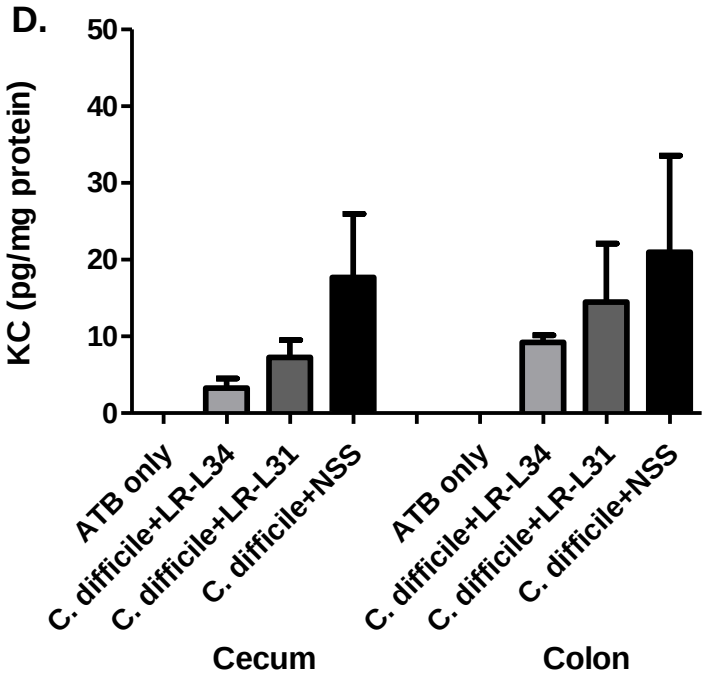


Figure 8. Simultaneous treatment with 1.0 x 10^8 cells of LR-L34 or LR-L31 on the day of *C. difficile* infection (D0-D1). Tissue cytokines were analyzed using ELISA at day3. A; IL-1β (pg/mg protein), B; TNF-α (pg/mg protein), C; MIP-2 (pg/mg protein), D; KC (pg/mg protein).

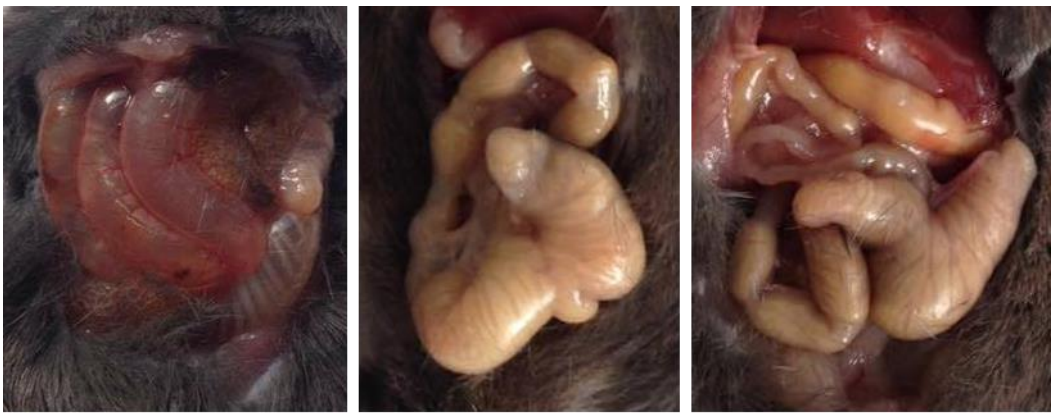


Figure 9. The swelling of intestine of *C. difficile*-infected mice without and with treatment of LR-L34 or LR-L31

Although the results of the above experiments demonstrated that 1 x 10^8 cells of LR-L34 given simultaneously with *C. difficile* were able to attenuate the disease in mice, we investigated the dose and timing for appropriate application of LR-L34. First, we pretreated mice with LR-L34 or LR-L31 (1 x 10^8 cells) for 7 days before antibiotics administration to the day of *C. difficile* infection (D-13 to D0). The results indicated that pretreatment with *L. rhamnosus* LR-L34 or LR-L31 diminished percent diarrhea but did not improve the mortality rate and body weight change in mice. (data not shown).

We then tried to give LR-L34 at the dose of 1 x 10^8 cells or 1 x 10^6 cells for 4 days starting at the day of clindamycin injection to the day before sacrifice (D-1 to D2) (Figure 10). The application of LR-L34 (1 x 10^6 cells) resulted in 100 percent survival of mice while the group received LR-L34 (1 x 10^8 cells) resulted in 50 percent survival (Figure 11A). Body weight change of the group received *L. rhamnosus* LR-L34 (1 x 10^6 cells) was significantly different (p <0.05) as compared with *C. difficile*-infected group while that of the group received LR-L34 (1 x 10^8 cells) was not significantly different (Figure 11B). Percent diarrhea of groups treated with *L. rhamnosus* LR-L34 (1 x 10^6 cells), (1 x 10^8 cells) and *C. difficile*-infected group was 12.50, 66.67, and 87.50, respectively (Figure 11C).

Treatment with LR-L34 (1 x 10^6 cells) for 4 days from (D-1)-(D2) suppressed serum IL-1β, TNF-α, MIP-2, and KC as compared with *C. difficile*-infected group (Figure 12A-D) and attenuated proinflammatory cytokine IL-1β, TNF-α and chemokines MIP-2, and KC in both cecum and colon tissue as compared with *C. difficile*-infected group (Figure 13A-D). Treatment with LR-L34 (1 x 10^8 cells) resulted in the decrease of some cytokines (Figures 13 and 14). Mice infected with *C. difficile* showed the swelling of ileum, cecum and colon with small amount of intestinal content. Treatment with *L. rhamnosus* LR-L34 reduced swelling of the intestine and also increased cecum and colon content. Treatment with *L. rhamnosus* LR-L34 (1 x 10^8 cells) showed more swelling of intestine form colon than *L. rhamnosus* LR-L34 1 x 10^6 cells. These results were shown in Figure 14.

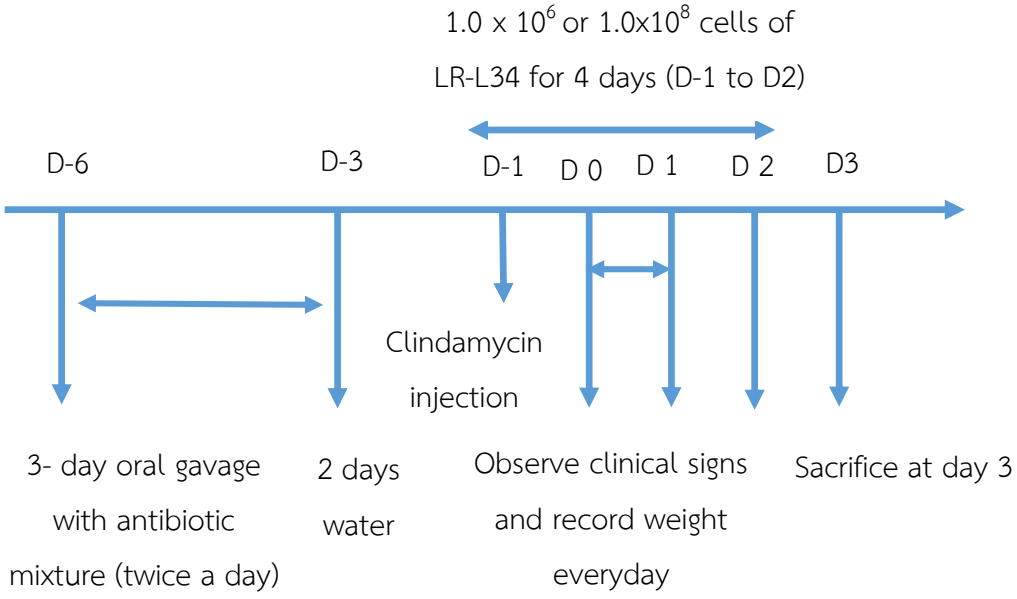
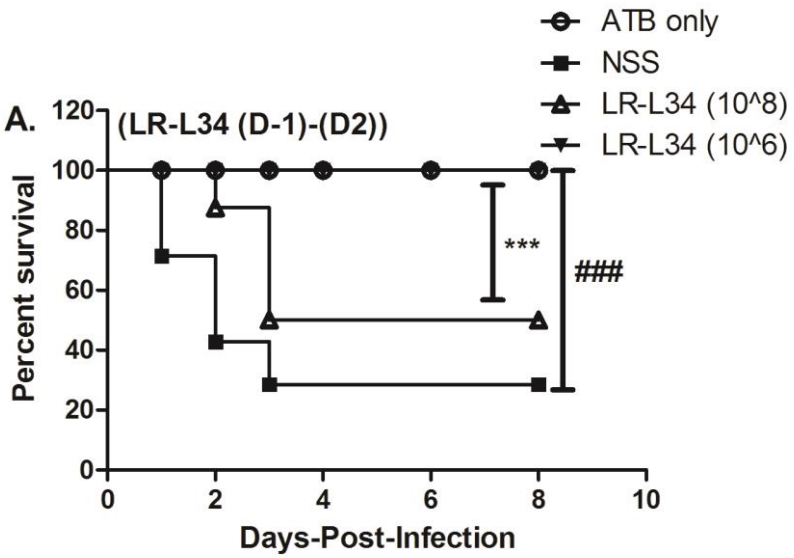


Figure 10. Experimental scheme for the application of *L. rhamnosus* L34 ($1.0x10^6$ or $1.0x10^8$ cells/mouse) for 4 days (D -1 to D2) in *C. difficile* – infected mice.



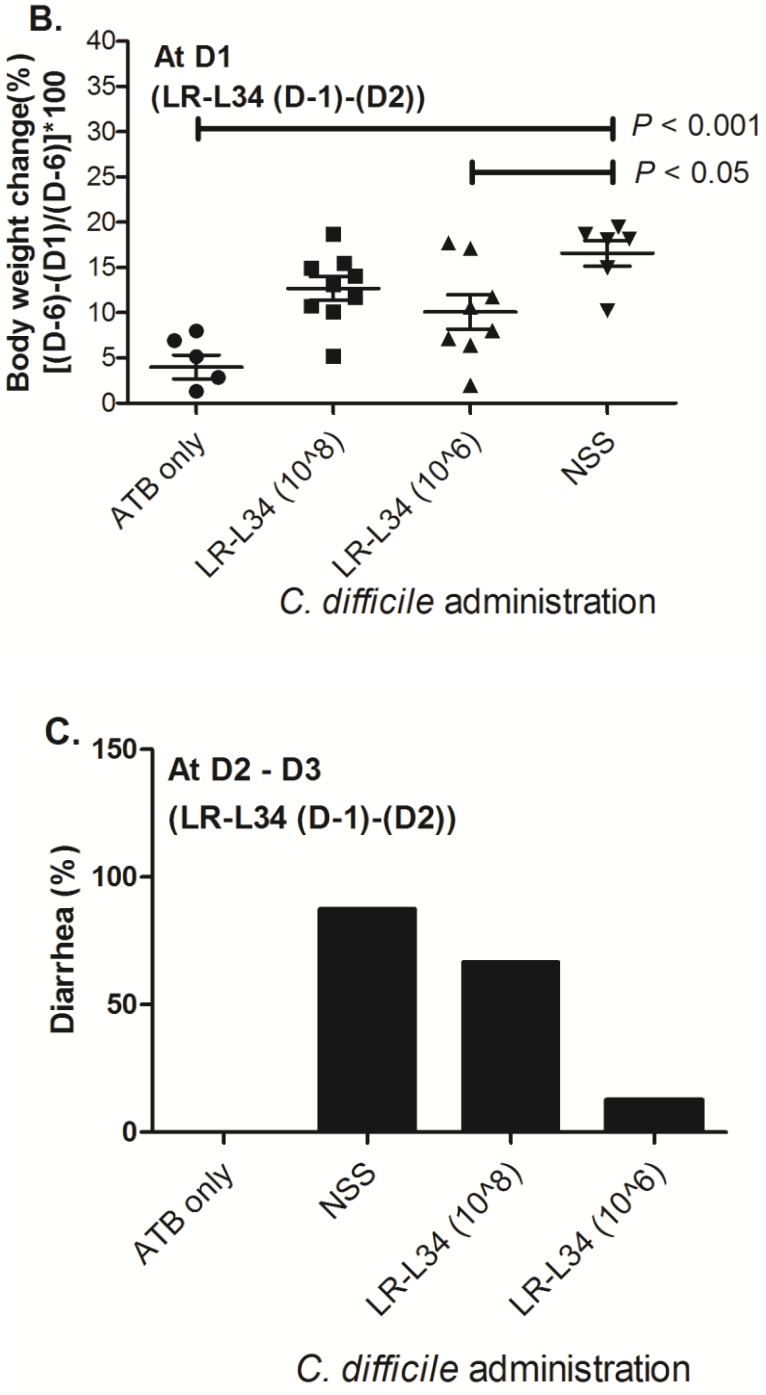
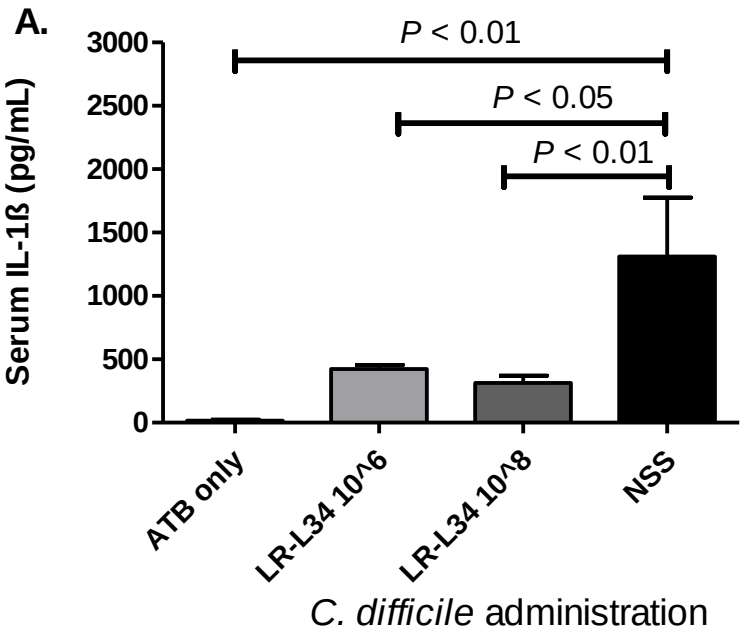
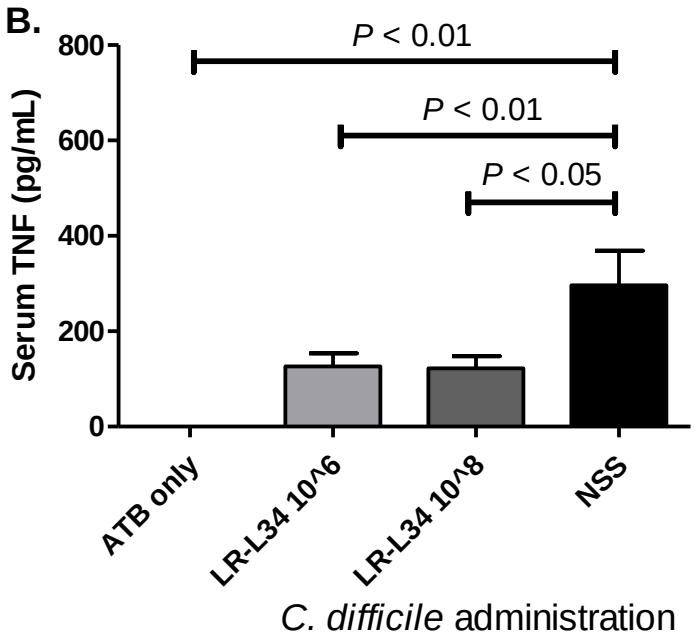


Figure 11. Application of *L. rhamnosus* (1 x 10^6 or 1 x 10^8 cells/mouse) for 4 days (D-1 to D2). A; Percent survival was observed at D0 – D8, B; Weight change (%) was measured at D1 and D2, C; Percent diarrhea was observed at D1- D2.


A.
Serum IL-1ß (pg/mL)
3000
2500
2000
1500
1000
500
0
P < 0.01
P < 0.05
P < 0.01
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
C. difficile administration


B.
Serum TNF (pg/mL)
800
600
400
200
0
P < 0.01
P < 0.01
P < 0.05
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
C. difficile administration

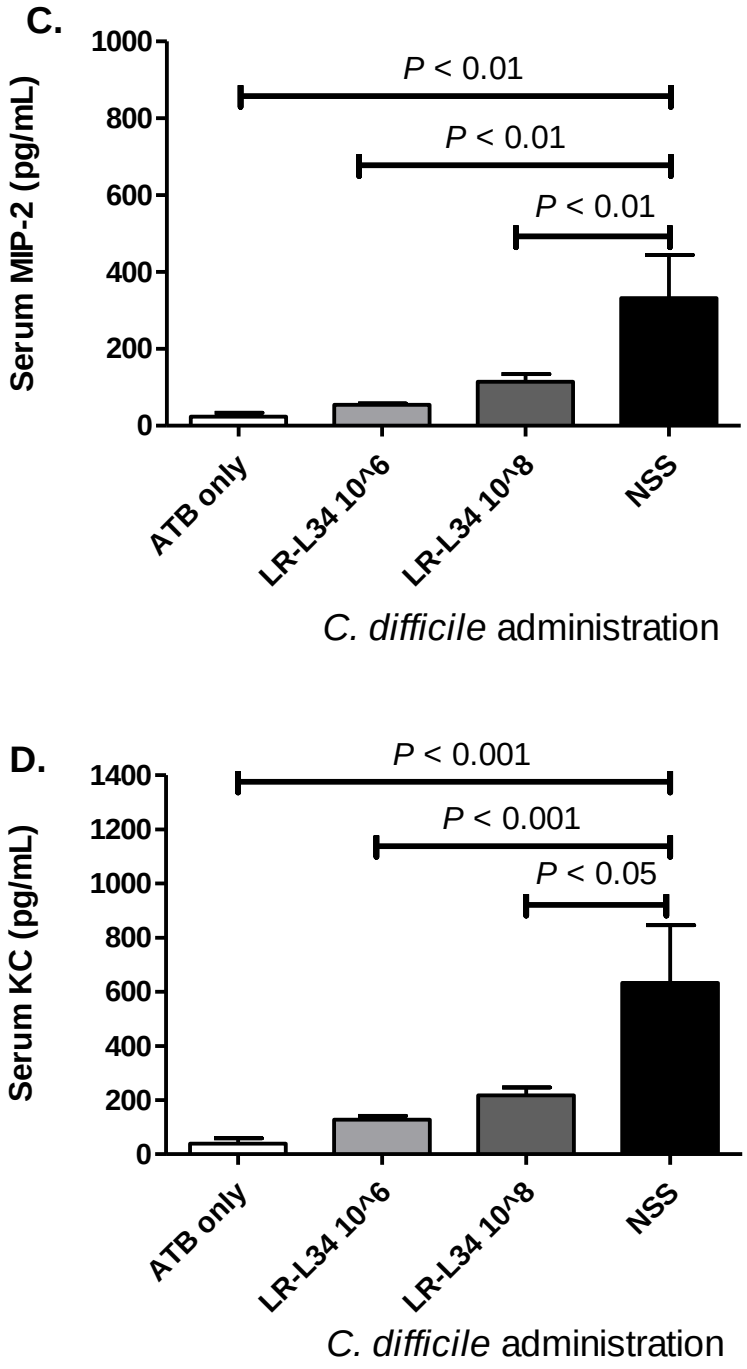
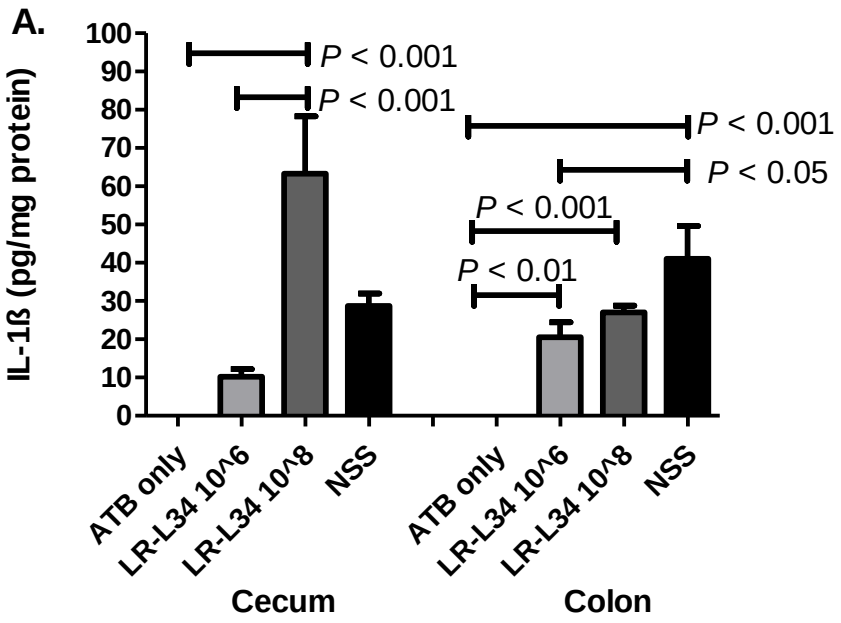
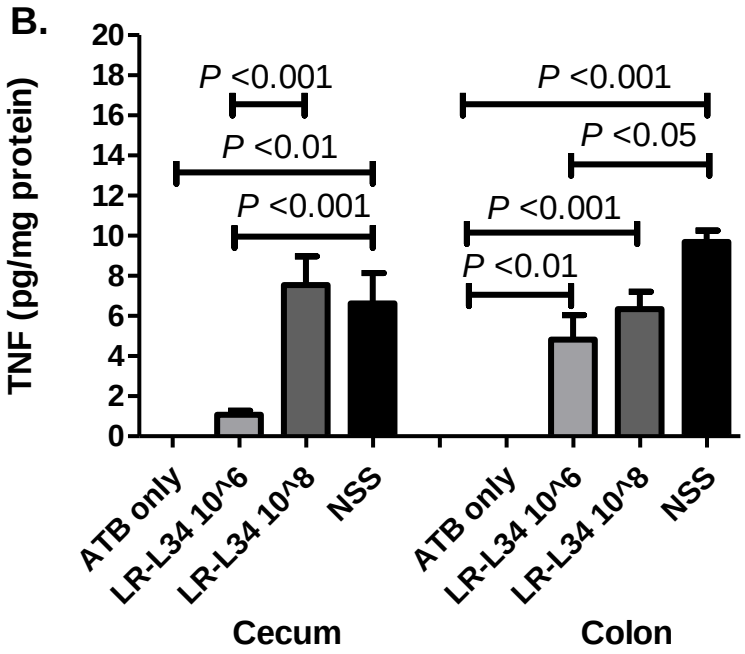


Figure 12. Treatment with *Lactobacillus rhamnosus* L34 (1 x 10^6 or 1 x 10^8 CFU) for 4 days (D-1- D2). Serum cytokines were analyzed using ELISA at day3. A; IL-1β (pg/mL), B; TNF-α (pg/mL), C; MIP-2 (pg/mL), D; KC (pg/mL).


A.
IL-1ß (pg/mg protein)
100
90
80
70
60
50
40
30
20
10
0
P < 0.001
P < 0.001
P < 0.001
P < 0.05
P < 0.001
P < 0.01
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
Cecum
Colon


B.
TNF (pg/mg protein)
20
18
16
14
12
10
8
6
4
2
0
P <0.001
P <0.01
P <0.001
P <0.001
P <0.05
P <0.001
P <0.01
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
ATB only
LR-L34 10^6
LR-L34 10^8
NSS
Cecum
Colon

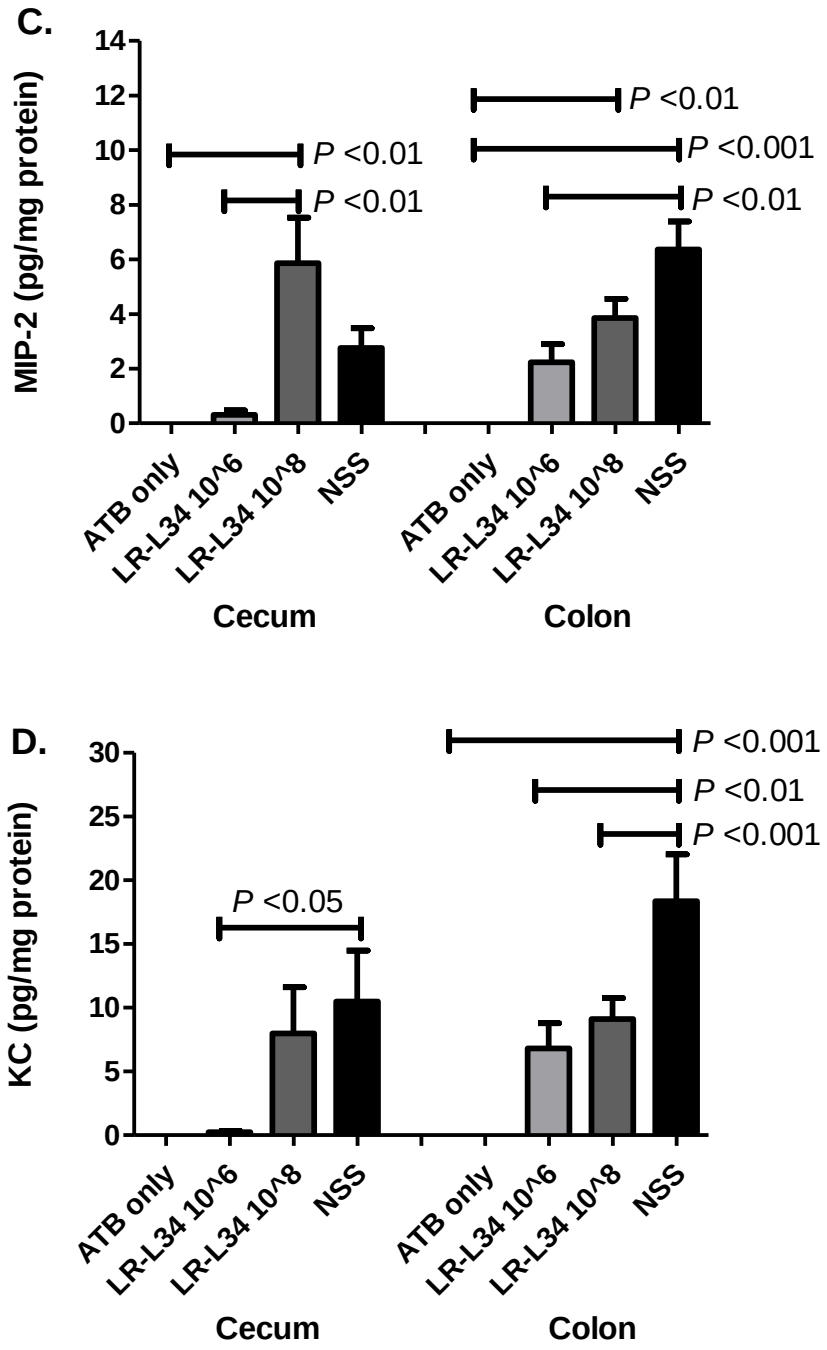


Figure 13. Treatment with *Lactobacillus rhamnosus* (1 x 10^6 or 1 x 10^8 CFU) for 4 days from clindamycin injection to sacrifice (D-1- D2). Tissue cytokines were analyzed using ELISA assay at day3. A; IL-1β (pg/mg protein), B; TNF-α pg/mg protein), C; MIP-2 pg/mg protein), D; KC pg/mg protein).

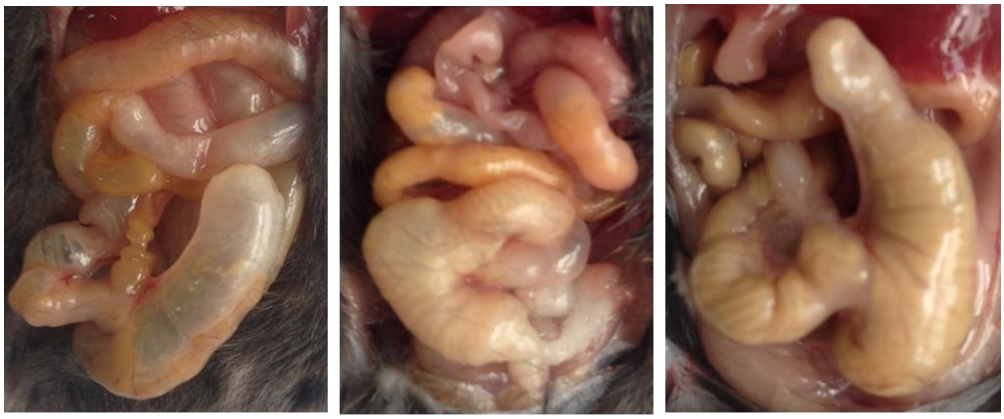


Figure 14. The swelling of intestine of *C. difficile*-infected mice without and with treatment of LR-L34 (10^8 and 10^6 cells) for 4 days (D-1 to D2).

3. Effect of *Lactobacillus casei* treatment on *C. difficile*-infected mice

We tested three *L. casei* strains, *L. casei* 39 (LC-L39), *L. casei* B13 (LC-B13) and *L. casei* B106 (LC-B106). Based on the results from *L. rhamnosus* L34, we used the same protocol as described above. It was found that the application of $1.0x10^6$ and $1.0x10^8$ cells of LC-L39 per mouse resulted in 100 percent survival in mice whereas the survival rate of *C. difficile*-infected group was 50% (Figure 15A). The average body weight of mice in LC-L39-treated group at $1.0x10^8$ cells/mouse was not significantly increased, whereas treatment of $1.0x10^6$ cells LC-L39 did not improve the body weight change as compared with *C. difficile*-infected group (Figure 15B). The application of LC-L39 ($1.0x10^8$ cells/mouse) reduced *C. difficile*-associated diarrhea in mice as compared with group received LC-L39 ($1.0x10^6$ cells/mouse) and *C. difficile*-infected group (7.14%, 42.68% and 71.63%, respectively) (Figure 15C).

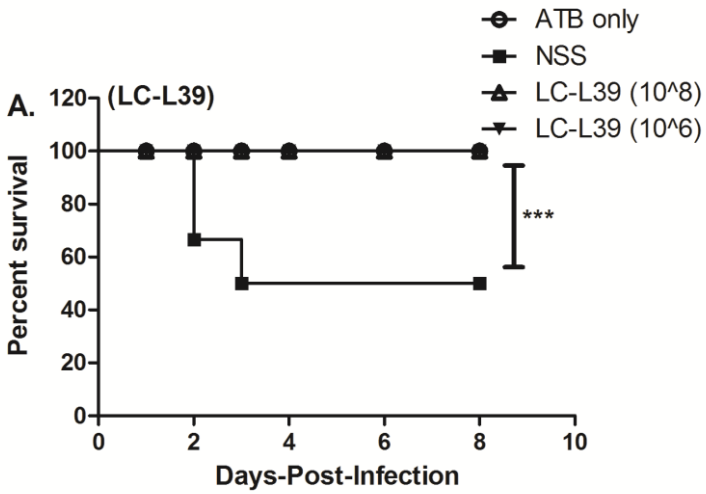
For *L. casei* B13 (LC-B13), it was found that the application of $1.0x10^6$ and $1.0x10^8$ cells of LC-B13 per mouse resulted in 100 percent survival in mice whereas the survival rate of *C. difficile*-infected group was 58% (Figure 16A). Treatment of $1.0x10^6$ or $1.0x10^8$ cells of LC-B13 showed improvement of the body weight as compared with *C. difficile*-infected group (Figure 16B). The application of LC-B13 either $1.0x10^6$ or $1.0x10^8$ cells/mouse reduced *C. difficile*-associated diarrhea as compared with *C. difficile*-infected group (Percent diarrhea was 9.09%, 9.09% and 55.33%, respectively) (Figure 16C).

L. casei B106 (LC-B106), it was found that the application of $1.0x10^6$ and $1.0x10^8$ cells of LC-B106 per mouse resulted in 96 and 100 % survival in mice, respectively whereas the rate of *C. difficile*-infected group was 58% (Figure 17A). Treatment of $1.0x10^6$ or $1.0x10^8$ cells of LC-B106

showed improvement the body weight as compared with *C. difficile*-infected group (Figure 17B). The application of LC-B106 ($1.0x10^8$ cells/mouse) reduced *C. difficile*-associated diarrhea as compared with group received LC-B106 ($1.0x10^6$ cells/mouse) and *C. difficile*-infected group (Percent diarrhea was 9.09%, 27.07% and 60.00%, respectively) (Figure 17C).

Treatment with LC-B106 (1×10^6 cells) for 4 days (D-1 to D2) suppressed the levels of serum IL-1β, MIP-2, and KC as compared with *C. difficile*-infected group while LC-B13 suppressed the levels of all serum cytokines and LC-B39 suppressed only serum IL-1β (Figure 18A-D). Treatment with higher dose of LC-B13 and LC-B106 (1×10^8 cells) suppressed the levels of all tested serum cytokines while LC-L39 could not suppress all of these cytokines (Figure 18A-D)

For the suppression of tissue cytokines, treatment with LC-B106 (1×10^8 CFU) decreased the levels of proinflammatory cytokines IL-1β, TNF-α and chemokines MIP-2, and KC in colon tissue but treatment with LC-L39 or LC-B13 (1×10^8 CFU) decreased the levels of IL-1β, TNF-α and MIP-2 in colon tissue as compared with *C. difficile*-infected group (Figure 19A-D). Treatment with LC-B13, LC-B106 (1×10^8 cells) decreased only the levels of MIP-2 and KC in cecum tissue as compared with *C. difficile*-infected group (Figure 19A-D). Treatment with a lower dose ($1x10^6$) of *L. casei* strains had less effect in the suppression of cytokine production. The finding indicated that **pretreatment with *L. casei* (1×10^8 CFU) at the day of clindamycin injection to the day before sacrifice (D-1)-(D2)** was appropriate dose and proper time to attenuate disease severity and increase survival rate in *C. difficile*-infected mice



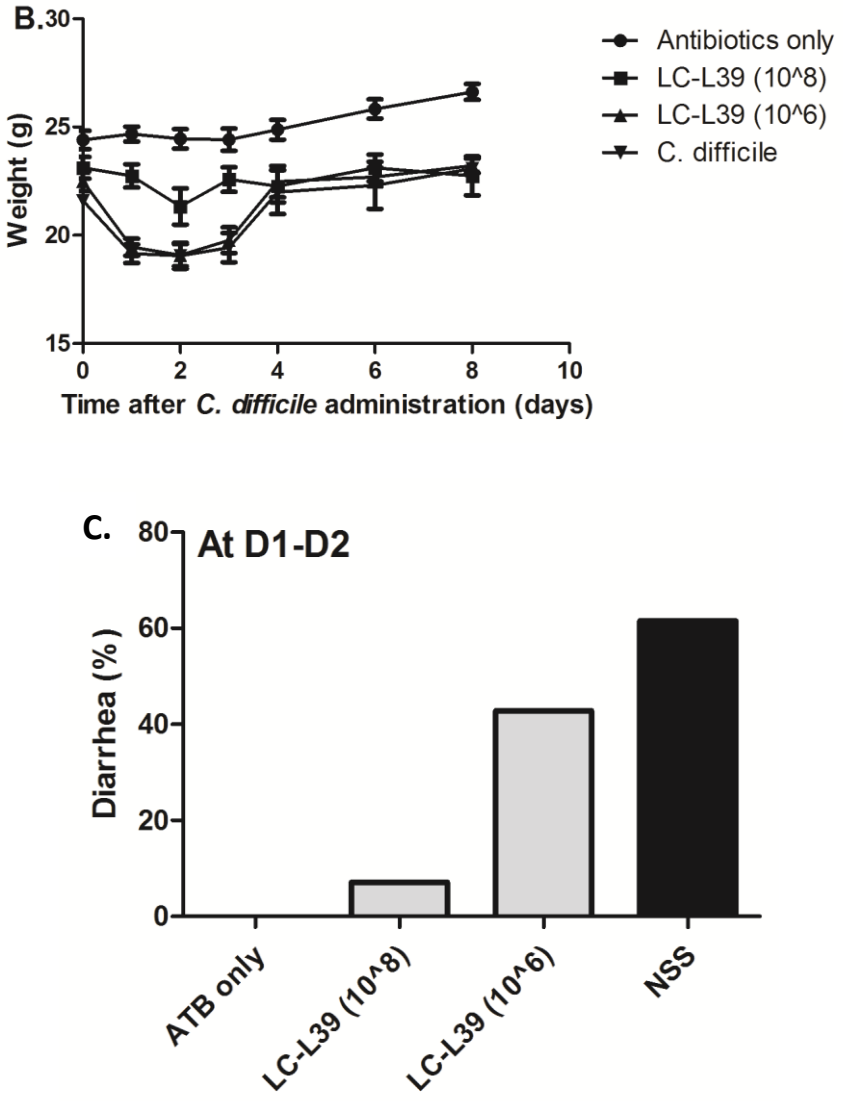
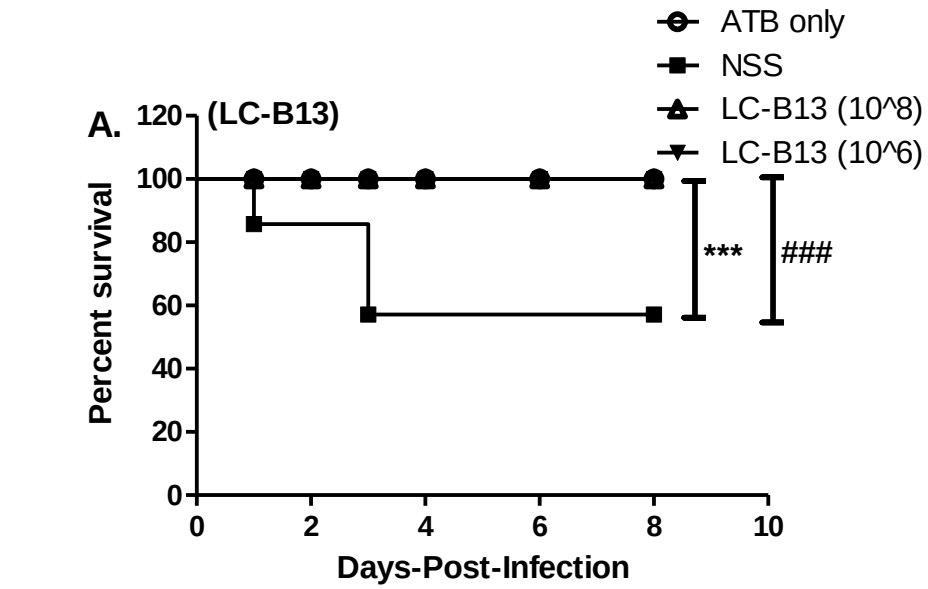
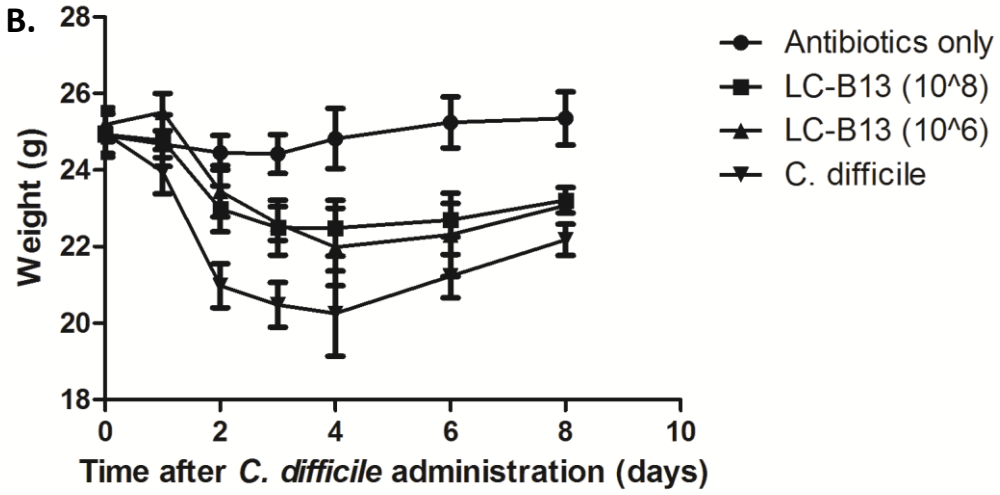


Figure 15. Treatment with *L. casei* (LC-L39) (1 x 10^6 or 1 x 10^8 cells/mouse) for 4 days from CLIN to Sacrifice (D-1 – D2). A; Percent survival was observed at D0 – D8, B; Weight (g), C; Percent diarrhea was observed at D1- D2.


A.
(LC-B13)
ATB only
NSS
LC-B13 (10^8)
LC-B13 (10^6)
Percent survival
120
100
80
60
40
20
0
0
2
4
6
8
10
Days-Post-Infection
***
###


B.
Antibiotics only
LC-B13 (10^8)
LC-B13 (10^6)
C. difficile
Weight (g)
28
26
24
22
20
18
0
2
4
6
8
10
Time after C. difficile administration (days)

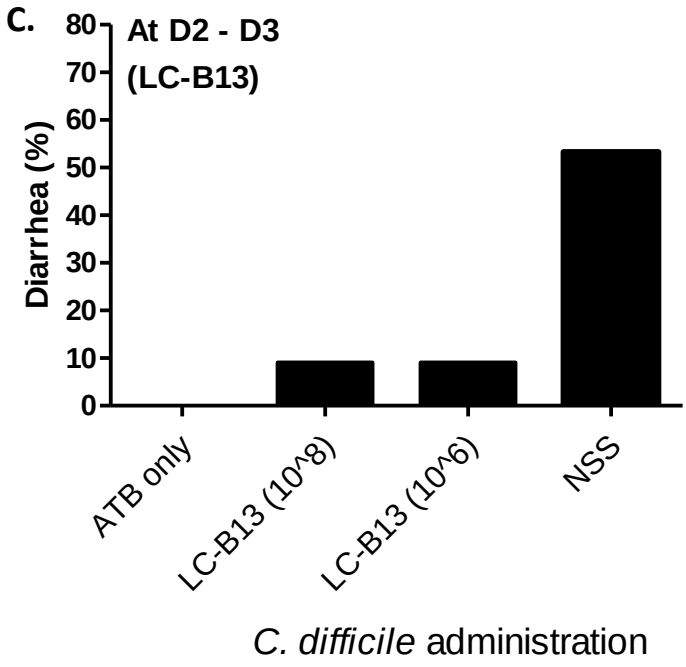
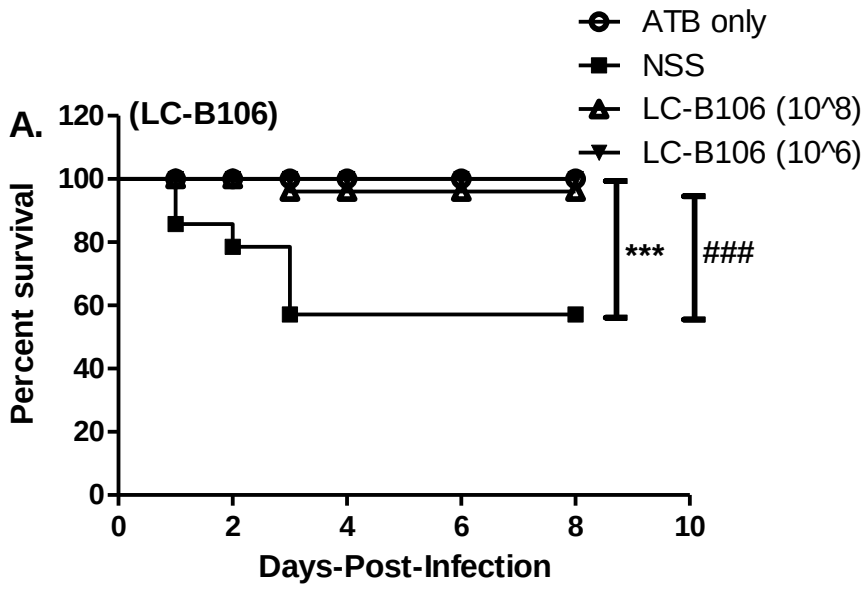


Figure 16. Pretreatment with *L. casei* (LC-B13) (1 x 10^6 and 1 x 10^8 cells/mouse) for 4 days from CLIN to Sacrifice (D-1 – D2). A. Percent survival was observed at D0 – D8; B-C. Weight change (%) was measured at D1 and D2; D. Percent diarrhea was observed at D1- D2.



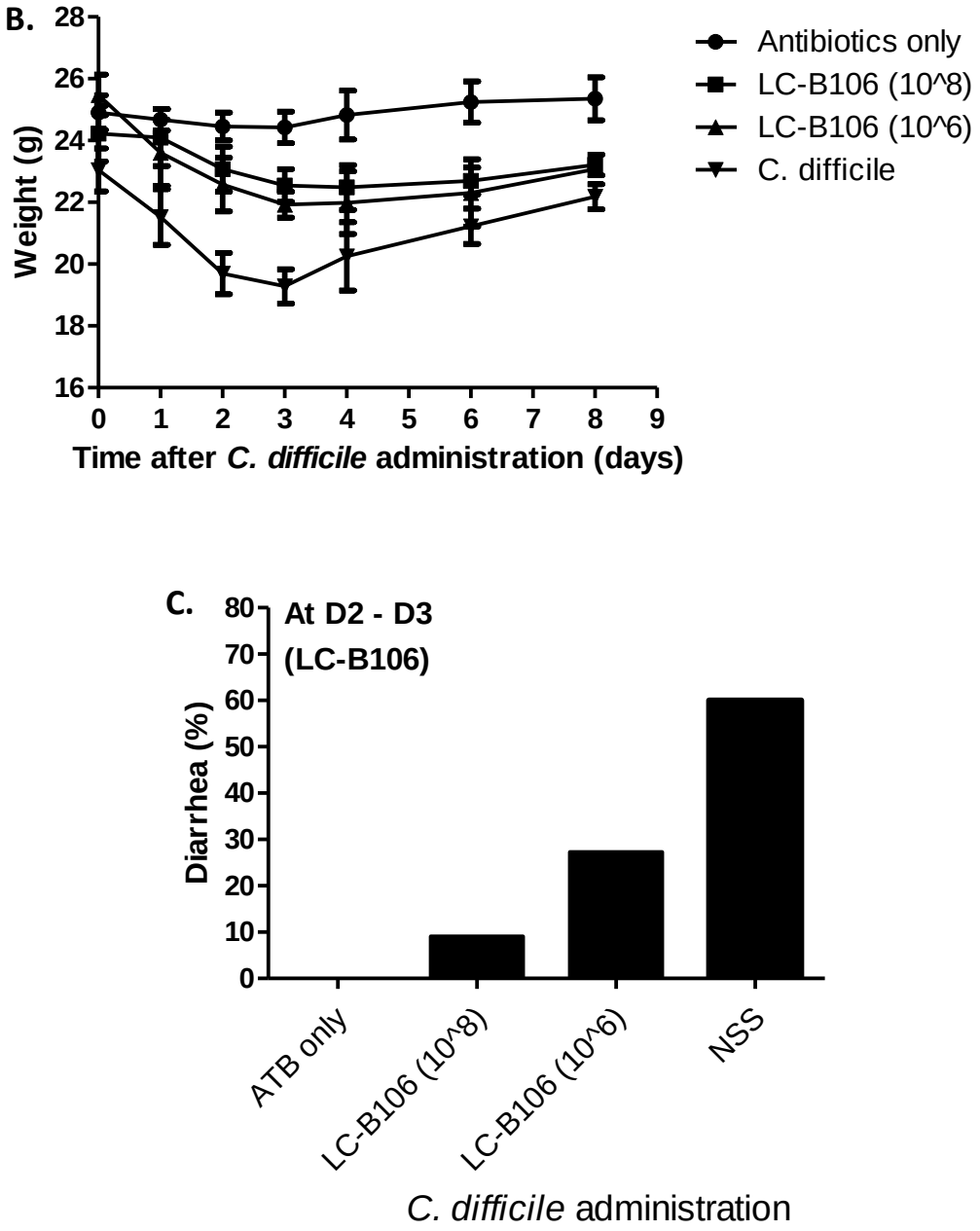


Figure 17. Pretreatment with *L. casei* (LC-B106) (1 x 10^6 and 1 x 10^8 cells/mouse) for 4 days from CLIN to Sacrifice (D-1 – D2). A; Percent survival was observed at D0 – D8, B; Weight (g), C; Percent diarrhea was observed at D1- D2.

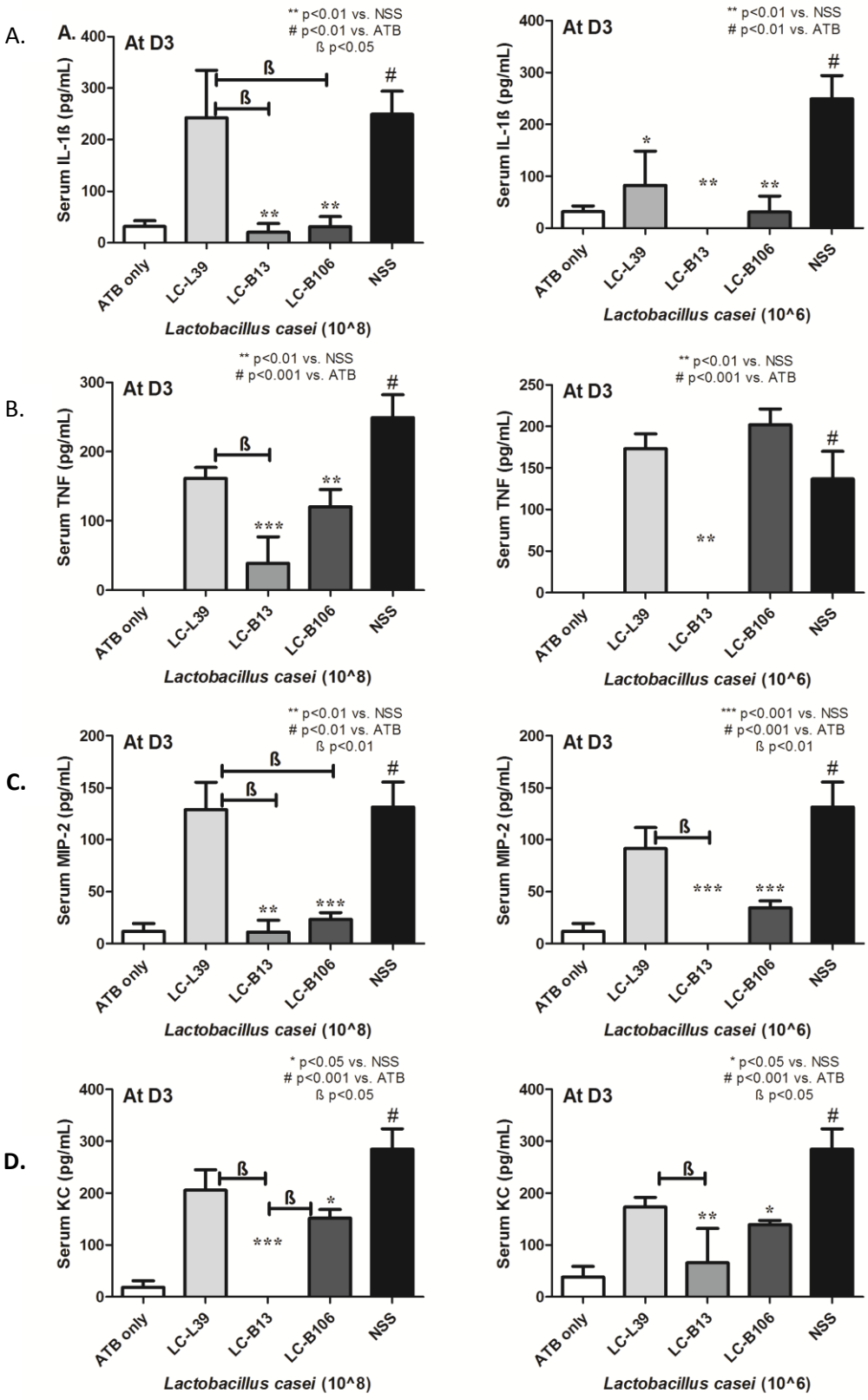


Figure 18. Pretreatment with *L. casei* (1 x 10^6 and 1 x 10^8 cells/mouse) for 4 days from CLIN to Sacrifice (D-1 – D2). A. IL-1β (pg/mL); B. TNF-α (pg/mL); C. MIP-2 (pg/mL); D. KC (pg/mL).

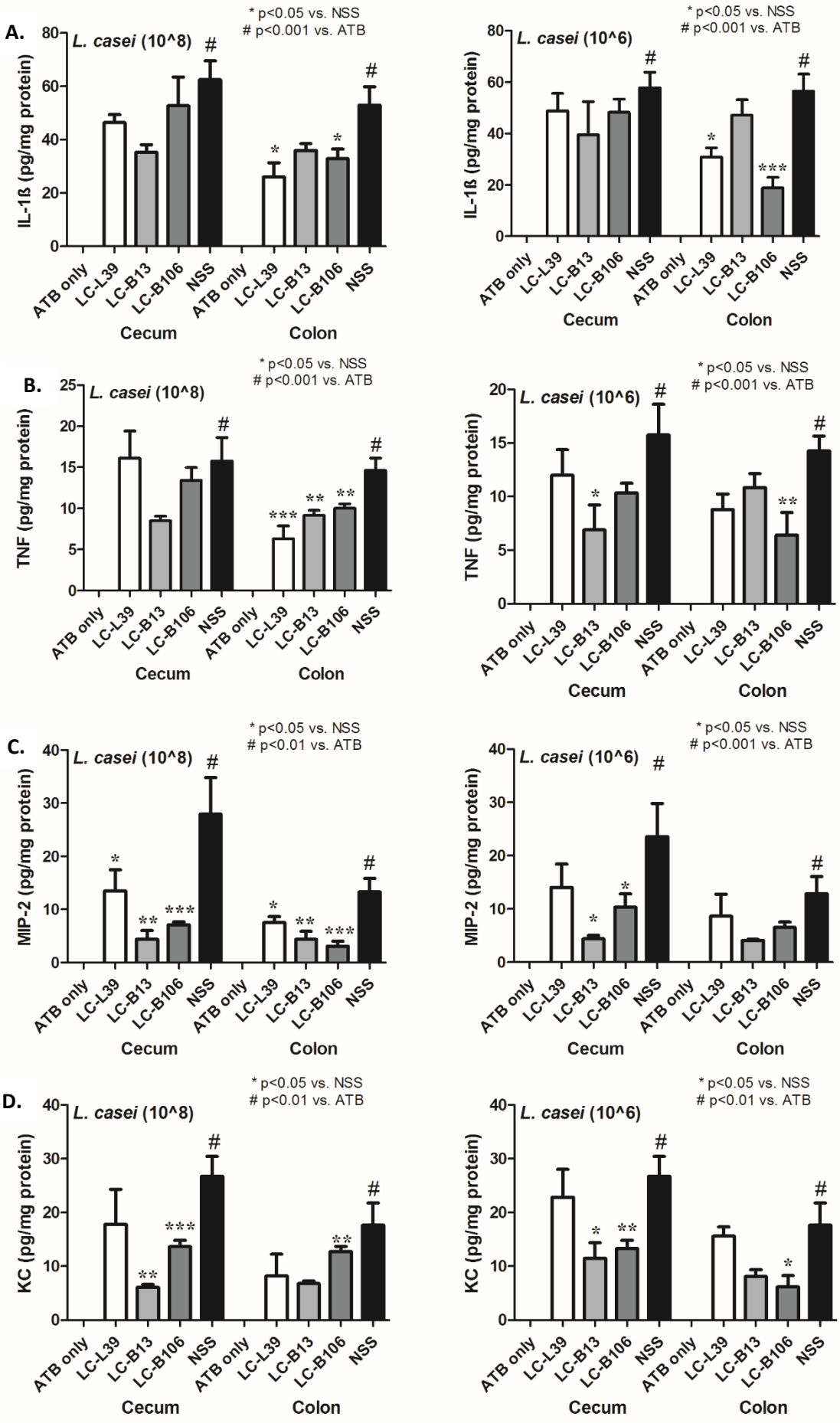

A.
L. casei (10^8)
* p<0.05 vs. NSS
# p<0.001 vs. ATB
IL-1ß (pg/mg protein)
L. casei (10^6)
B.
TNF (pg/mg protein)
C.
* p<0.05 vs. NSS
# p<0.01 vs. ATB
MIP-2 (pg/mg protein)
D.
KC (pg/mg protein)
ATB only
LC-L39
LC-B13
LC-B106
NSS
Cecum
Colon

Figure 19. Treatment with *L. casei* (1 x 10^6 and 1 x 10^8 cells/mouse) for 4 days (D-1 to D2). Cytokine was measured in cecum and colon. A. IL-1β (pg/mg tissue); B. TNF-α (pg/mg tissue); C. MIP-2 (pg/mg tissue); D. KC (pg/mg tissue).

4. Histopathology of *C. difficile*-infected mice treated with *L. rhamnosus* L34, *L. casei* L39, *L. casei* B13 and *L. casei* B106

Mice infected with *C. difficile* showed higher histology scores than uninfected group. For the 4-day treatment (D-1 to D2), *L. rhamnosus* L34 (LR-L34), *L. casei* L39 (LC-L39), *L. casei* B13 (LC-B13) and *L. casei* B106 (LC-B106) reduced histology score when compared with *C. difficile*-infected mice group while *L. rhamnosus* L31 a strain without the capability of suppressing IL-8 did not reduce histology score. *L. casei* B13 reduced histology score the most (Figure 20).

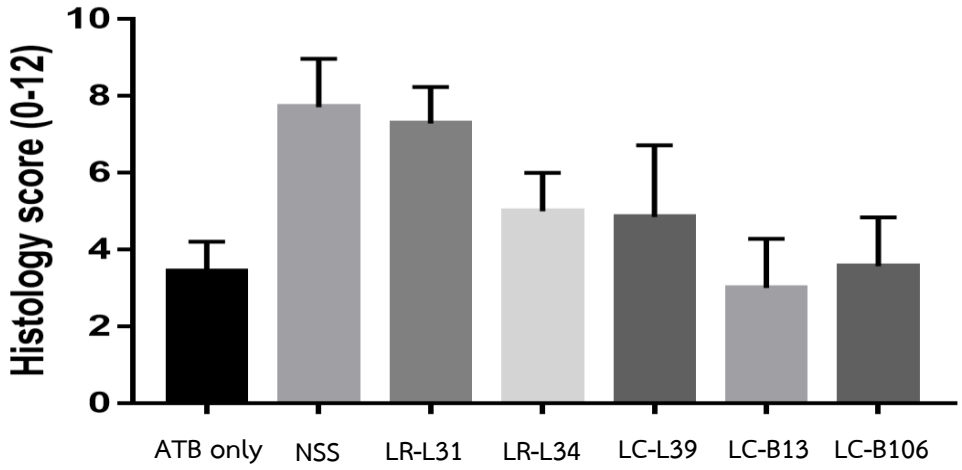


Figure 20. Treatment with *L. rhamnosus* L34, *L. casei* L39, B13 and B106 decreased disease severity in *C. difficile*-infected mice determined by histopathology.

5. Quantitation of *C. difficile* in mice by real-time PCR

To investigate the effect of *Lactobacillus* on the quantity of *C. difficile* in cecum and colon of mice, real-time PCR was performed to quantitate *C. difficile*. *L. rhamnosus* L34 was first tested. The data showed that *L. rhamnosus* L34 non-significantly reduced the number of *C. difficile* as shown in Figure 21.

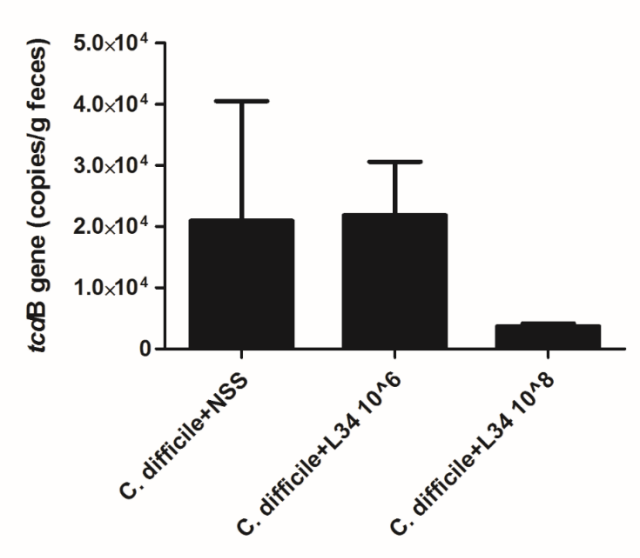


Figure 21. Real-time PCR analysis of *C. difficile* in cecum and colon content in pretreatment with *L. rhamnosus* LR-L34.

6. Fecal microbiome analysis of mice treated with *Lactobacillus* spp.

To investigate the ability of *Lactobacillus* spp. to restore (change) microbiota in *C. difficile*-infected mice, microbiome analysis was performed. A total of 68 fecal samples at specific days as shown in Tables 3 were collected and subjected to DNA extraction. The libraries were created but still waited for sequencing. Moreover, we plan to study the effect of *L. casei* to restore (change) microbiota in *C. difficile*- infected mice within a next few months as shown in Table 4. Microbiome analysis takes a long time to perform and also needs specialist to analyze the results.

Table 3. Fecal samples of mice in *L. rhamnosus* treatment

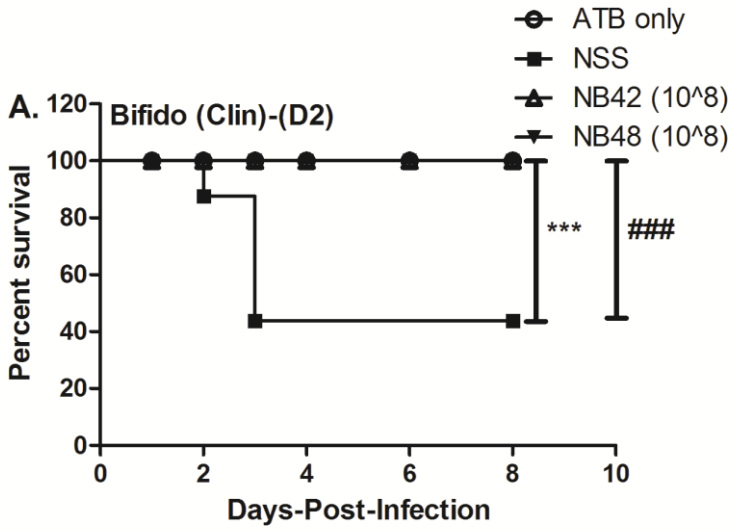
Group	D-7	D-3	D3
Control	2	2	8
C. difficile	2	2	8
L34	8	8	8
L31	2	2	8
Total	14	14	40

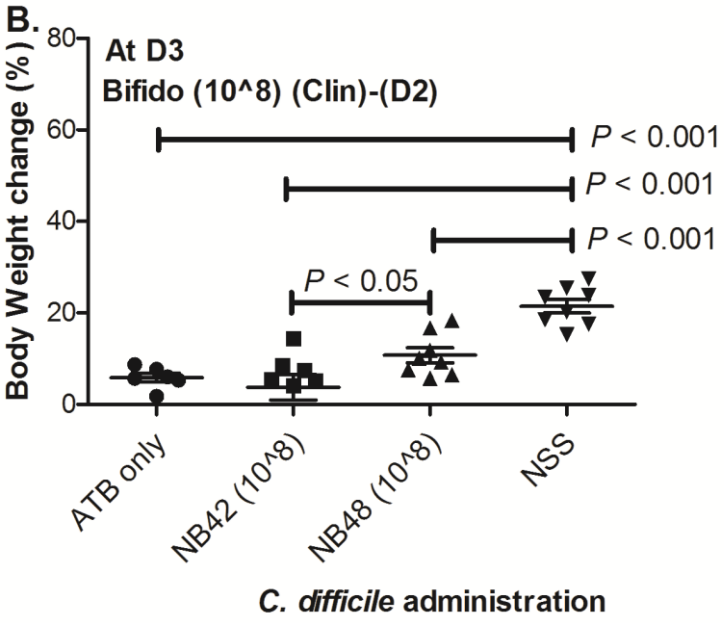
Table 4. Fecal samples of mice in *C. difficile* in *L. casei* treatment

Group	D-7	D-3	D3
Control	2	2	8
C. difficile	2	2	8
L39	8	8	8
L13	8	8	8
B106	8	8	8
Total	28	28	28

7. Effect of *Bifidobacterium* spp. treatment on *C. difficile*-infected mice

We tested two *Bifidobacterium* strains, *Bifidobacterium bifidum* NB42 (BB-NB42) and *Bifidobacterium pseudocatenulatum*-NB48 (BP-NB48) with the same protocol as described above. It was found that the application of $1.0x10^8$cells of BP-NB42 and BP-NB48 per mouse resulted in 100 percent survival in mice whereas the rate of *C. difficile*-infected group was 50% (Figure 22A). The average body weights in the groups of BP-NB42- or BP-NB48-treated mice was not significantly changed when compared with those before *C. difficile* infection (Figure 22B). The average body weights of BP-NB42- and BP-NB48-treated groups were significantly different (p=0.001) as compared with *C. difficile*-infected group (Figure 22B). The application of BP-NB42 and BP-NB48 reduced *C. difficile*-associated diarrhea to 0 and 10%, respectively.(Figure 22C).





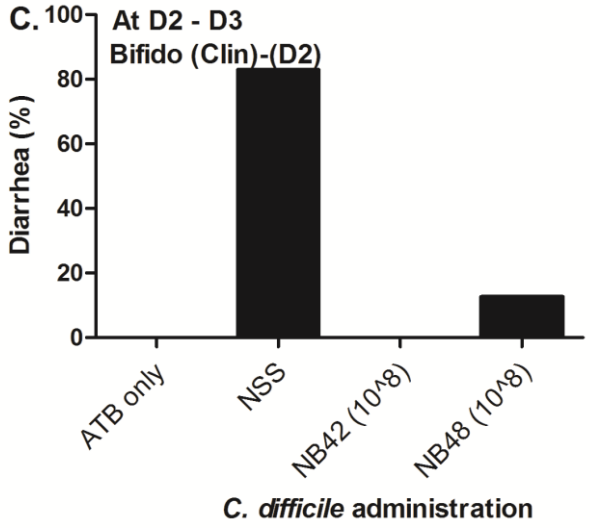


Figure 22. Treatment with *Bifidobacterium* spp. (1 x 10^8 cells/mouse) for 4 days from CLIN to Sacrifice (D-1 – D2). A; Percent survival was observed at D0 – D8, B; body weight loss, C; Percent diarrhea was observed at D1- D2.

8. Treatment with Lacto cocktail (LR-L34 and LC-L39), Bifido cocktail (BB-NB42 and BP-NB48) and Lacto-Bifido cocktail (LR-L34, LC-L39, BB-NB42 and BP-NB48)

We investigated the combination of *Lactobacillus* and/or *Bifdobaterium* to modulate disease severity in *C. difficile*-infected mice. Mice received Lacto cocktail (LR-L34 and LC-L39; total of 1 x 10^6 CFU), Bifido cocktail (BB-NB42 and BP-NB48; total of 1 x 10^6 cells) and Lacto-Bifido cocktail (LR-L34, LC-L39, BB-NB42 and BP-NB48, total of 1 x 10^6 cells) for 4 days (D-1 to D2). Mice of Lacto-cocktail group showed 100 % survival, whereas *C. difficile*-infected group, Bifido-cocktail and Lacto-Bifido cocktail groups showed 50.00, 83.33 and 57.13%, respectively (Figure 23A). Body weights (BW) of the mice were shown in the Figure 23B. The BW change of Lacto cocktail-treated group was less than that of *C. difficile* -infected group (Figure 23C-E). The BW change of either Bifido cocktail- or Lacto-Bifido cocktail -treated group was not significantly different when compared with *C. difficile*-infected group (Figure 23C-E). Only Lacto cocktail treatment reduced percent diarrhea to14.28% (Figure 23F). Percent diarrhea of *C. difficile*-infected group, Bifido-cocktail, and Lacto-Bifido cocktail was 85.71, 71.43 and 100%, respectively (Figure 23F). The results indicated that **treatment with Lacto-** could increase survival rate and also reduced diarrhea in *C. difficile*-infected mice while Bifido-cocktail or Lacto-Bifido cocktail could not.

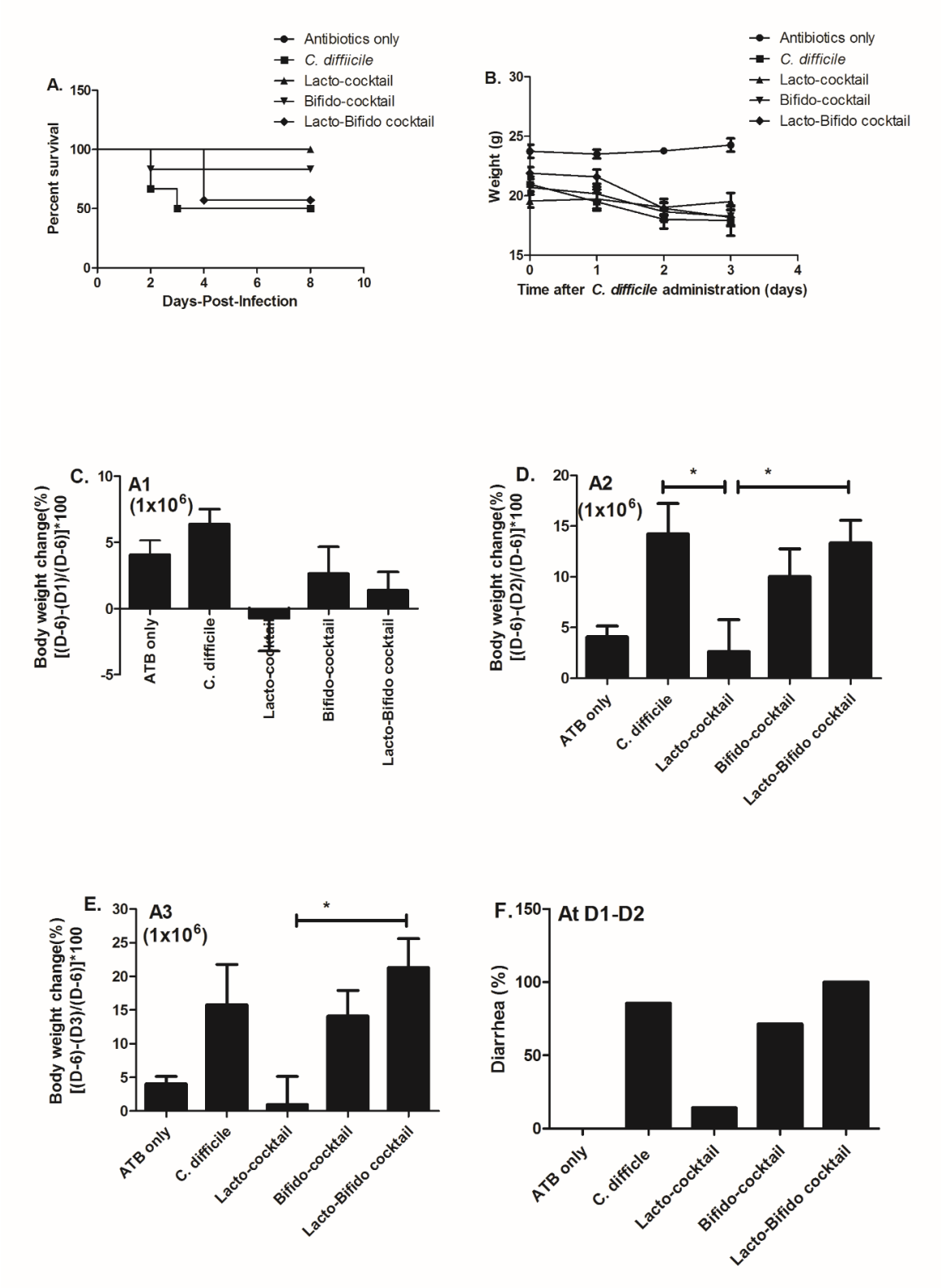


Figure 23. Treatment with Lacto cocktail, Bifido cocktail and Lacto-Bifido cocktail (total of 1 x 10^6 cells) for 4 days (D-1 to D2). A. percent survival; B. Weight (g), C-E. Body weight change; H. percent diarrhea at D1-D2.

Discussion

Animal models have been developed to study the pathogenesis and novel therapeutics of *C. difficile* [25]. Some models use antibiotic cocktail to destroy normal microbiota in gastrointestinal tracts of mice [17, 18] and some use a single antibiotic for stimulating *C. difficile* infection [26, 27]. In an attempt to test the ability of specific strains of *Lactobacillus* and *Bifidobacterium* for the protection of *C. difficile* infection, a C57BL/6 mouse model previously developed [28, 29] was applied with minor modification. We first tried the administration of spores of a hypervirulent strain *C. difficile* BAA 1870 to mice. It was found that it took very long time to prepare the desired amount of spores for the experiment. We then performed oro-gastric infection of mice with various amount of vegetative cells of *C. difficile*. Antibiotic cocktails was applied to disrupt intestinal microbiota before *C. difficile* infection. It was found that giving *C. difficile* dose of 1.0×10^{10} cells for 2 days resulted in the development of clinical signs characterized by diarrhea (wet tail and traces of liquid stool), weight loss, hunched posture and the death of about half of the mice.

We first started with testing various amounts of *L. rhamnosus* L34 (LR-L34) at different time points and found that the administration of LR-L34 (1×10^6 cells) for 4 days at the day of clindamycin injection to the day before sacrifice (D-1 to D2) reduced mortality, body weight change, diarrhea, gut leakage and pathology of mice and also ameliorated tissue and systemic inflammatory responses. As summarized in Table 5. We are waiting for the result of microbiome analysis to find whether this strain is able to restore gut microbiota. Testing other strains of *Lactobacillus* and *Bifidobacterium* either alone or in combination showed satisfactory results when each of the test strain was applied alone. Only Lacto cocktail gave satisfactory result (Table 5).

Although more tests need to be performed for most strains, we have shown the capability of these specific strains in the protection of *C. difficile* infection.

Table 5. Summary of the suppression of clinical symptoms and inflammation of *C. difficile*-infected mice by treatment with *Lactobacillus* and *Bifidobacterium* for 4 days (D-1 to D2)

	Antibiotic only	*C. difficile*	LR-L34	LC-L39	LC-B13	LC-B106	BB-NB42	BP-NB48	Lacto-cocktail
% Survival	100	47.3	100.00	100.00	100.00	100.00	100.00	100.00	100.00
% BW change (D2)	5.19	18.06	10.85	8.57	12.94	11.22	3.37	10.74	2.64
% Diarrhea	0	71.45	12.50	7.14	9.09	9.09	0	12.50	14.28
Serum FITC-dextran	904.17	3875.58	1156.91**	ND	ND	ND	ND	ND	ND
Reduction of serum cytokines									
IL-1b			(+)*	(+)*	(+)**	(+)**	ND	ND	ND
TNF-α			(+)**	(-)	(+)***	(+)**	ND	ND	ND
MIP-2			(+)**	(-)	(+)**	(+)***	ND	ND	ND
KC			(+)***	(-)	(+)***	(+)*	ND	ND	ND
Reduction of cecum cytokines									
IL-1b			(-)	(-)	(-)	(-)	ND	ND	ND
TNF-α			(+)***	(-)	(-)	(-)	ND	ND	ND
MIP-2			(-)	(+)*	(+)**	(+)***	ND	ND	ND
KC			(+)*	(-)	(+)**	(+)***	ND	ND	ND
Reduction of colon cytokines									
IL-1b			(+)*	(+)*	(-)	(+)*	ND	ND	ND
TNF-α			(+)*	(+)***	(+)**	(+)**	ND	ND	ND
MIP-2			(+)**	(+)*	(+)**	(+)***	ND	ND	ND
KC			(+)**	(-)	(-)	(+)**	ND	ND	ND
Histology score	3.50	7.80	5.20	4.85	3.06	3.58	ND	ND	ND

(+), Positive; (-), Negative; ND, not determined and will be performed further; *Significantly different at $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Conclusion

We have identified six strains of *Lactobacillus* and *Bifidobacterium* with the ability to protect against *C. difficile infection*. Our result demonstrated that the preventive effect of *L. rhamnosus* L34 was mediated via improving gut leakage and down-regulating both local and systemic inflammatory responses. More experiments are still ongoing to define the functional properties contributing to the protective effects of these strains.

(Associate Professor Dr. Somying Tumwasorn)
Principle Investigator
29 / 06 / 2561

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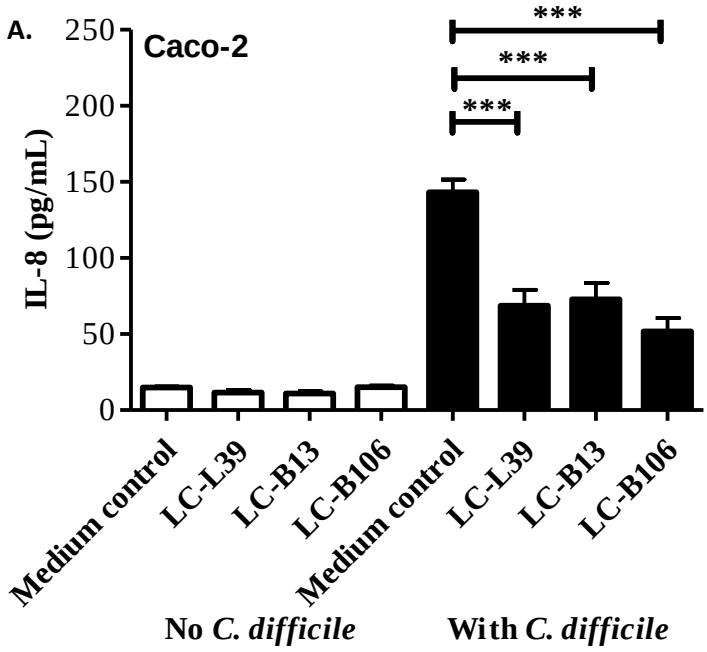
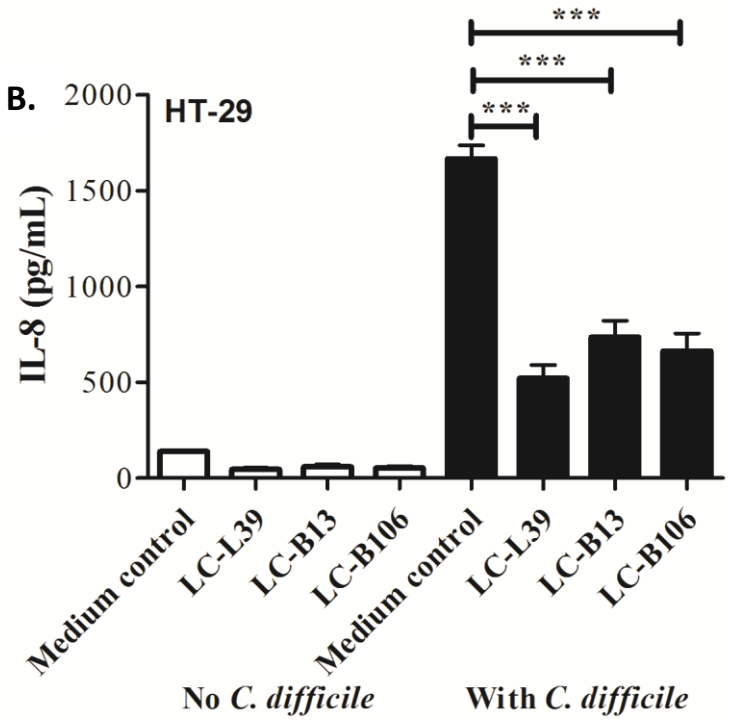
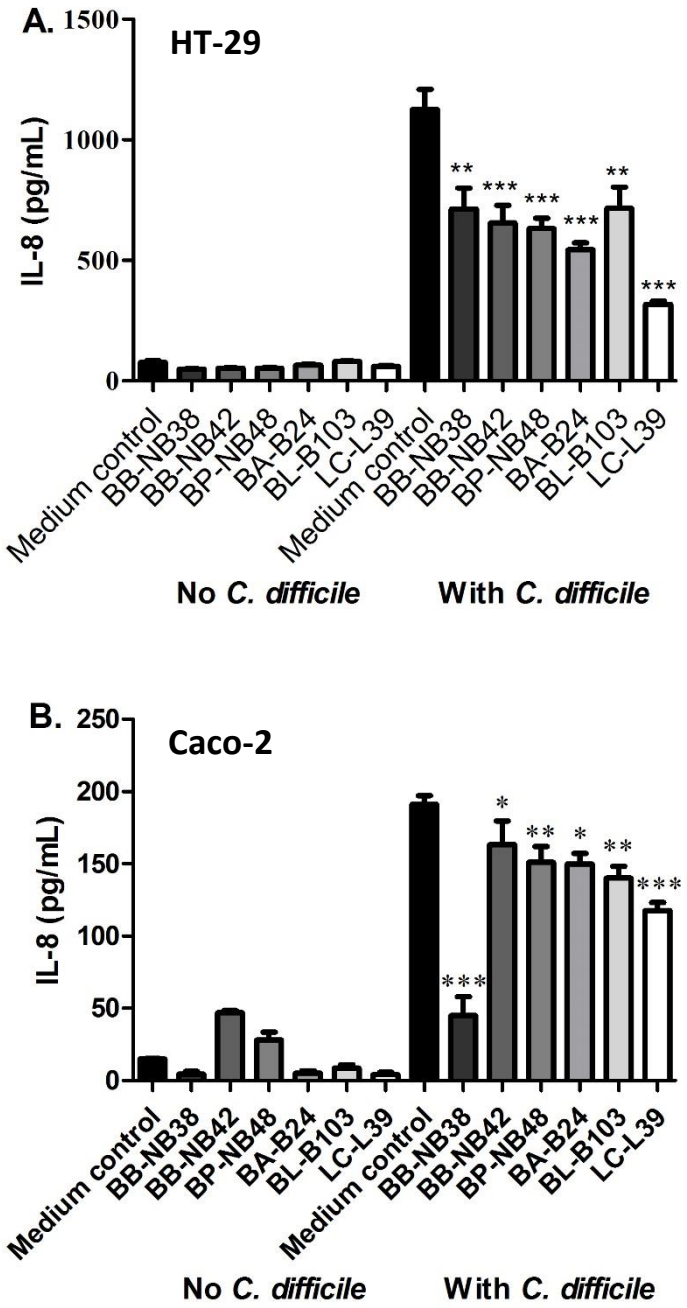
Supplementary Data





Supplement data 1. Human-derived *Lactobacillus* spp. produce factors suppressing *C. difficile*-induced pro-inflammatory cytokine production in Caco-2 or HT-29 intestinal epithelial cells. LCM from human-derived lactobacilli were tested for the ability to suppress IL-8 production in Caco-2 (A) or HT-29 (B) cells stimulated with *C. difficile*. Cells were incubated with 5% LCM in the presence or absence of *C. difficile* ATCC BAA1870 for 24 h and IL-8 production was measured by ELISA. The results were from three independent experiments in triplicate and are expressed as the mean ± SEM, ****p*-value <0.001 as compared to medium control.



Supplement data 2**.** Human-derived *Bifidobacterium* spp. produce factors suppressing *C. difficile*-induced pro-inflammatory cytokine production in Caco-2 or HT-29 intestinal epithelial cells. BCM from human-derived *Bifidobacterium* were tested for the ability to suppress IL-8 production in Caco-2 or HT-29 cells stimulated with *C. difficile*. Cells were incubated with 5% BCM in the presence or absence of *C. difficile* ATCC BAA1870 for 24 h and IL-8 production was measured by ELISA. LCM of LC-L39 were used as positive control. The results were from three independent experiments in triplicate and are expressed as the mean ± SEM, ****p*-value <0.001 as compared to medium control.

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EDUCATION

B.S. (Hons) in Food Science & Technology, Kasetsert University, Bangkok, Thailand, 1975

M.S. in Microbiology, Kasetsart University, Bangkok, Thailand, 1977

Ph.D. in Immunology & Me dical Microbiology, University of Florida, College of Medicine, Gainesville, Florida, USA, 1988

TRAINING: Computer Applications in Molecular Biology, International Center for Genetic Engineering and Biotechnology, Trieste, Italy, 1991

POSITIONS

Associate Professor and Head of Bacteriology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand

Head**,** Bacteriology Unit and Diagnostic Molecular Bacteriology Lab, Department of Microbiology, King Chulalongkorn Memorial Hospital, Thailand

ACADEMIC APPOINTMENTS

Instructor of Microbiology, Faculty of Medicine, Chulalongkorn University, 1997-1980

Assistant Professor of Microbiology, Faculty of Medicine, Chulalongkorn University, 1980-1996

Associate Professor of Microbiology, Faculty of Medicine, Chulalongkorn University, 1996 **–** present

Graduate Coordinator, Interdepartmental Program of Medical Microbiology, Graduate School, Chulalongkorn University, 1988-1997

Head, Diagnostic Molecular Bacteriology Lab, Department of Microbiology, King Chulalongkorn Memorial Hospital, 1990-present

Head, Bacteriology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, 2001-present

PROFESSIONAL ACTIVITIES

Invited speaker, Global Engage's 2nd Microbiome R&D and Business Collaboration Congress and Probiotics Congress : Asia on **Gastric *Lactobacillus* with Specific Activity against *Helicobacter pylori***, 29 February– 1March 2016; Kuala Lumpur, Malaysia.

Invited speaker, Global Engage's 4th Microbiome R&D and Business Collaboration Congress and 3rd Probiotics Congress: Asia on **Leaky gut-associated diseases and Probiotics,** 5-6 March 2018, Singapore.

PUBLICATIONS (In reverse chronological order)

Surawut S, Panpetch W, Makjaroen J, Tangtanatakul P, Thim-uam A, Wongphoom J, **Tumwasorn S**, Leelahavanichkul A. *Helicobacter pylori* infection increased anti-dsDNA and enhanced lupus severity in symptomatic FcγRIIb-deficient lupus mice. Manuscript ID: 363133. Frontiers in Microbiology 2018 (accepted for publication).

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Boonma P, Spinler JK, Venable SF, Versalovic J, **Tumwasorn S**. *Lactobacillus rhamnosus* L34 and *Lactobacillus casei* L39 suppress *Clostridium difficile*-induced IL-8 production by colonic epithelial cells. BMC Microbiol 2014; 14:177 (doi: 10.1186/1471-2180-14-177).Boonma P, Spinler JK, Qin X, Jittaprasatsin C, Muzny DM, Doddapaneni H, Gibbs R, Petrosino J, **Tumwasorn S**, Versalovic J. Draft

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Panpetch W, Thiraworawong T, **Tumwasorn S**. Human gastric-biopsy derived lactobacilli suppress *Helicobacter pylori*-induced interleukin-8 production from gastric epithelial cells *in vitro*. International Journal of Interferon, Cytokine and Mediator Research 2011; 3:43-49

Vilaichone RK, Mahacahai V, **Tumwasorn S**, Kachintorn U. *CagA* genotype and metronidazole resistant strain of *Helicobacter pylori* in functional dyspepsia in Thailand. J Gastroenterol Hepatol. 2011; 26 Suppl 3:46-48.

Mahachai V, Sirimontaporn N, **Tumwasorn S**, Thong-Ngam D, Vilaichone RK. Sequential therapy in clarithromycin-sensitive and -resistant *Helicobacter pylori* based on polymerase chain reaction molecular test. J Gastroenterol Hepatol 2011; 26(5):825-828.

Supiyaphuna P, **Tumwasorn S**, Udomsantisuk N, Keelawat S, Songsrisanga W, Prasurthsin P, Sawatpanich A**.** Diagnostic tests for tuberculous lymphadenitis: fine needle aspirations using tissue culture in mycobacteria growth indicator tube and tissue PCR. Asian Biomedicine 2010; 4(5):787-792.

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Taweechotipatr M, Iyer C, Spinler JK, Versalovic J, **Tumwasorn S**. *Lactobacillus saerimneri* and *Lactobacillus ruminis*: novel human-derived probiotic strains with immunomodulatory activity. FEMS Microbiol Lett. 2009;293(1):65-72.

Spinler JK, Taweechotipatr M, Rognerud CL, **Tumwasorn S**, Versalovic J. Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe. 2008; 14(3): 166-171.

Phowthongkum P, Puengchitprapai A, Udomsantisook N, **Tumwasorn S**, Suankratay C. Spindle cell pseudotumor of the brain associated with Mycobacterium *haemophilum* and *Mycobacterium simiae* mixed infection in a patient with AIDS: the first case report. Int J Infect Dis. 2008; 12(4): 421-424.

Vilaichone RK, Mahachai V, **Tumwasorn S**, Wu JY, Graham DY, Yamaoka Y. Gastric mucosa cytokine levels in relation to host interleukin-1 polymorphisms and *Helicobacter pylori cag A* genotype. Scand J Gastroenterol 2005; 40: 530- 9

Vilaichone RK, Mahachai V, **Tumwasorn S**, Wu JY, Graham DY, Yamaoka Y. Molecular epidemiology and outcome of *Helicobacter pylori* infection in Thailand : a cultural cross roads. Helicobacter 2004; 9: 453-459.

BOOK CHAPTERS

Tumwasorn S. 1998. Molecular Bacteriology: Melioidosis. In: Molecular Biology in Medicine (eds. Sukcharoen N, Mutirangura M, Poovorawan P). Text and Journal Publication Ltd. Co., Bangkok, Thailand.

Tumwasorn S. 2000. Molecular Methods for Antituberculous Testing. In: Antimycobacterial Susceptibility Testing: Clinical Application and Development of New Drugs (ed. Palittapongarnpim P) Training, Publication and Public Relation Unit, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.

BOOK

***Lactobacillus*: Basics to Medical Applications**. Srimuang Printing Co. Ltd. Bangkok. 2018.

PATENT

"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. 5830710, 5834791
Inventors: Ann Prokulske-Fox, **Somying Tumwasorn**, Guylaine Lepine, Naiming Han, Marilyn Lantz, Joseph M. Patti

"Product for anti-inflammation induced by *Helicobacter pylori* in stomach"

Patent applied to Department of Intellectual Property, Ministry of Commerce by Thailand Research Fund. No.1301007136 on April 5, 2013
Inventors: **Somying Tumwasorn**, Thien Thiraworawong, Duangporn Werawatganon, Naruemon Klaikaew

CURRENT RESEARCH

Tumwasorn S (PI)

1 March 2018-29 February 2020
National Science and Technology Development Agency (NSTDA)

Probiotic *Lactobacillus* and *Bifidobacterium* for the intervention against type 2 diabetes
Tumwasorn S (Collaborator)
(Nilaratanakul V, PI)
1 February 2018- 31January 2021
National Science and Technology Development Agency (NSTDA)
Shortening the duration of carbapenem-resistant Enterobacteriaceae (CRE) colonization in the rectum of inpatients with fecal microbiota transplantation from unrelated healthy donors: The randomized controlled trial in King Chulalongkorn Memorial Hospital

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Educations:

1990-1996 M.D. (honors), Chulalongkorn University, Thailand

2002 Board of Internal Medicine, Thailand

2003 Board of Family Medicine, Thailand

2004 Board of Nephrology, Thailand

Master of Science, Chulalongkorn University, Thailand

2005-2010 Visiting fellow at National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, USA

2011 PhD (Biomedical Science), Chulalongkorn University, Thailand

Honors:

2017 DMsc award from department of medical service of Thailand (กรมการแพทย์) with the study of *Lactobacillus rhamnosus* on *C. difficile* infection in mouse model

2016 Cerebos Award from Cerebos incorporation (BRAND's) with the study of *Lactobacillus rhamnosus* derived from Thai infant for sepsis prevention

2016 National Outstanding Researcher Award on Using Animals for Scientific Purpose Development (IAD) from National Research Council of Thailand (NRCT) (รางวัลนักวิจัยดีเด่นที่ใช้สัตว์เพื่องานทางวิทยาศาสตร์ รางวัล สพสว. (IAD Award) ประจำปี พศ. 2559 โดยสถาบันพัฒนาการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์ (สพสว) สำนักงานคณะกรรมการวิจัยแห่งชาติ (วช.))

2005 Visiting fellow award, Kidney Disease Branch, National Institute of Diabetes and Digestive and Kidney Disease

Publications (selected from 51 publication on Pubmed data base):

1. Leelahavanichkul A, Areepium N, Vadcharavivad S, Praditpornsilpa K, Avihingsanon Y, Karnjanabuchmd T, Eiam-Ong S and Tungsanga K. Pharmacokinetics of Sirolimus in Thai Healthy Volunteers. J Med Assoc Thai 2005: 88 Suppl 4: S157-62.

2. Dear JW, Leelahavanichkul A, Aponte A, Hu X, Constant SL, Hewitt SM, Yuen PS and Star RA. Liver proteomics for therapeutic drug discovery: inhibition of the cyclophilin receptor CD147 attenuates sepsis-induced acute renal failure. Crit Care Med 2007 Oct; 35(10):2319-28. (impact factor 6.5)

3. Yasuda H*, Leelahavanichkul A*, Tsunoda S, Dear JW, Takahashi Y, Ito S, Hu X, Zhou H, Doi K, Childs RW, Klinman DM, Yuen PS and Star RA. Chloroquine and inhibition of Toll-like receptor 9 protect from sepsis-induced acute kidney injury. Am J Physiol Renal Physiol. 2008 May; 294(5):F1050-8. (* distribution equally) (impact factor 4.6-5)

4. Doi K, Leelahavanichkul A, Hu X, Yuen PS and Star RA. Pre-existing renal disease promotes sepsis-induced acute kidney injury and worsens outcome. Kidney Int. 2008 Oct; 74(8):1017-25. (impact factor 6.4)

5. Leelahavanichkul A, Yasuda H, Doi K, Yuen PS, Hu X, Zhou H, Yuen PS and Star RA. Methyl-2-acetamidoacrylate decrease sepsis induced acute kidney injury in CD-1 mice. Am J Physiol Renal Physiol. 2008 Dec; 295(6):F1825-35. (impact factor 4.6-5)

6. Nemeth K*, Leelahavanichkul A *, Yuen PS, Mayer B, Parmelee B, Doi K, Robey P.G., Leelahavanichkul K, Hu X, Star RA and Mezey E. Bone marrow stromal cells significantly attenuate

mouse poly-microbial sepsis via monocyte/macrophage derived IL-10. Nature Medicine. 2009 Jan; 15(1):42-9. (* distribution equally) (impact factor 30.1)

7. Doi K, Leelahavanichkul A, Yuen PS, Star RA. Animal models of sepsis and sepsis- induced kidney injury (review). J Clin Invest 2009 Oct; 119(10):2868-78. (impact factor 16)

8. Leelahavanichkul A, Yan Q, Hu X, Eisner C, Huang Y, Chen R, Mizel D, Zhou H, Wright EC, Kopp JB, Schnermann J, Yuen PS, Star RA. Angiotensin II overcomes strain-dependent resistance of rapid CKD progression in a new remnant kidney mouse model. Kidney Int 2010 Dec; 78(11): 1136-53. (impact factor 6.4)

9. Leelahavanichkul A, Huang Y, Hu X, Zhou H, Tsuji T, Kopp JB., Schnermann J, Yuen ST, Star RA. Chronic kidney disease-induced splenic apoptosis and HMGB1 elevation worsens sepsis and sepsis-induced acute kidney injury Kidney Int 2011; Dec; 80(11):1198-211. (impact factor 6.4)

10. Leelahavanichkul A, Bocharov AV, Kurlander R, Baranova IN, Vishnyakova TG, Hu X, Doi K, Csako G, Yuen ST, Star RA, Eggerman TL. Class B Scavenger Receptor Types I and II and CD36 Targeting Improves Sepsis Survival and Acute Outcomes in Mice. J Immunol. 2012 Mar 15;188(6):2749-58. (impact factor 5.74)

11. Seujange Y*, Leelahavanichkul A*, Yisarakun W, Khawsuk W, Meepool A, Phamonleatmongkol P, Saechau W, Onlamul W, Tantiwarattanatikul P, Oonsook W, Eiam-Ong S, Eiam-Ong S. Hibiscus Sabdariffa Linnaeus Aqueous Extracts Attenuate the Progression of Renal Injury in 5/6 Nephrectomy Rats. Ren Fail. 2012 Nov 19 (* distribution equally) (impact factor 0.87)

12. Leelahavanichkul A, Sauza A, Hsu V, Zhou H, Eisner C, Li L, Hu X, Tsuji T, Kent D, Schnermann J, Yuen ST, Star RA. Serum Cystatin C as the marker of sepsis induce acute kidney injury in CD-1 mice; the usefulness and the limitation. Am J Physiol Renal Physiol. 2014 Aug; F939-F948. (impact factor 3.79)

13. Leelahavanichkul A, Somparn P, Panich T, Chancharoenthana W, Wongphom J, Pisitkun T, Hirankarn N, Eiam-Ong S. The levels of serum microRNA-122 in acute liver injury induced by kidney injury and sepsis in CD-1 mouse models. Hepatol Res. 2015 Dec; 45 (13): 1341-52. (impact factor 2.28)

14. Worasilchai N*, Leelahavanichkul A*, Kanjanabuch T, Thongbor N, Lorvinitnun P, Sukhontasing K, Finkelman M, Chindamporn A. (1⟶3)-β-D-glucan and galactomannan testing for the diagnosis of

fungal peritonitis in peritoneal dialysis patients, a pilot study. Med Mycol. 2015 May 1;53(4):338-46. (* distribution equally) (impact factor 2.34)

15. Leelahavanichkul A, Somparn P, Bootprapan T, Tu H, Tangtanatakul P, Nuengjumnong R, Worasilchai N, Tiranathanagul K, Eiam-Ong S, Levine M, Chinampon A, Srisawat N. High-dose ascorbate with low-dose amphotericin B attenuates severity of disease in a model of re-appearance of candidemia during sepsis in the mouse. Am J Physiol Regul Integr Comp Physiol. 2015 Aug 1; 309(3): R223-34 (impact factor 3.66)

16. Leelahavanichkul A, Pongpirul K, Thongbor N, Worasilchai N, Petphuak K, Thongsawang B, Towannang P, Lorvinitnun P, Sukhontasing K, Katavetin P, Praditpornsilpa K, Eiam-Ong S, Chindamporn A, Kanjanabuch T. (1⟶3)-β-D-glucan and galactomannan for a differentiation of chemical "black particles" and fungal particles inside peritoneal dialysis tubing. Perit Dial Int. 2015 Nov 2 (impact factor 2.2)

17. Leelahavanichkul A, Somparn P, Issara-Amphorn J, Eiam-ong S, Avihingsanon Y, Hirankarn N, Srisawat N. Advantage of serum neutrophil gelatinase associated lipocalin in sepsis, impact of bilateral nephrectomy and bilateral ureter obstruction mouse models. Shock. 2016 May; 45 (5): 570-6 (impact factor 3.2)

18. Leelahavanichkul A, Worasilchai N, Wannalerdsakun S, Jutivorakool K, Somparn P, Issara-Amphorn J, Tachaboon S, Srisawat N, Finkelman M, Chindamporn A. Gastrointestinal Leakage Detected by Serum (1⟶3)-β-D-Glucan in Mouse Models and a Pilot Study in Patients with Sepsis. Shock. 2016 May 11. (impact factor 3.2)

19. Leelahavanichkul A, Panpetch W, Worasilchai N, Somparn P, Chancharoenthana W, Nilgate S, Finkelman M, Chindamporn A, Tumwasorn S. Evaluation of gastrointestinal leakage using serum (1⟶3)-β-D-glucan in a Clostridium difficile murine model. FEMS Microbiol Lett. 2016 Aug 28

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20. Ondee T, Surawut S, Taratummarat S, Hirankan N, Palaga T, Pisitkun P, Pisitkun T, Leelahavanichkul A*. Fc gamma receptor IIb deficient mice: A lupus model with increased endotoxin tolerance-related sepsis susceptibility. Shock. 2016 (Accepted) (impact factor 3.2))(* correspondence author)

21. Surawut S, Ondee T, Taratummarat S, Palaga T, Pisitkun P, Chindamporn A, Leelahavanichkul A*. The role of macrophages in the susceptibility of Fc gamma receptor IIb deficient mice to Cryptococcus neoformans. Sci Report. 2016 (Accepted) (impact factor 5.5)(* correspondence author)

22. Rattanasinganchan P, Sopitthummakhun K, Doi K, Hu X, Payne MD, Pisitkun T, Leelahavanichkul A*. Folic acid-induced rat kidney fibrosis: preliminary evaluation as a potential model for identification of fibrosis biomarkers from urinary exosomes. Asian BioMed 2016 (Accepted) (impact factor 0.134))(* correspondence author)

23. Leelahavanichkul A, Chancharoenthana W, Wongphom J, Eiam-Ong S, Srisawat N. Mouse chronic renal ischemic reperfusion injury model of chronic tubulointerstitial kidney disease, suitable model for urinary exosome experiments. Asian BioMed 2016 (Accepted) (impact factor 0.134)

24. Panich T, Chancharoenthana W, Somparn P, Issara-Amphorn J, Hirankarn N, Leelahavanichkul A*. Urinary Exosomal Activating Transcriptional Factor 3 as the Early Diagnostic Biomarker for Sepsis-Induced Acute Kidney Injury. BMC Nephrology. 2016 (Accepted) (impact factor 2.2))(* correspondence author)

25. Cannizzaro L, Rossoni G, Savi F, Altomare A, Marinello C, Saethang T, Carini M, Payne MD, Pisitkun T , Aldini G, Leeelahavanichkul A*. Regulatory landscape of AGE-RAGE-oxidative stress axis and its modulation by PPARγ activation in high fructose diet-induced metabolic syndrome. Nutrition & Metabolism. 2016 (Accepted) (impact factor 3.2)(* correspondence author)

26. Ondee T, Surawut S, Taratummarat S, Hirankan N, Palaga T, Pisitkun P, Pisitkun T, Leelahavanichkul A*. Fc gamma receptor IIb deficient mice: A lupus model with increased endotoxin tolerance-related sepsis susceptibility. Shock. 2016 Jun;47(6):743-752. (impact factor 3.2))(* correspondence author)

27. Surawut S, Ondee T, Taratummarat S, Palaga T, Pisitkun P, Chindamporn A, Leelahavanichkul A*. The role of macrophages in the susceptibility of Fc gamma receptor IIb deficient mice to Cryptococcus neoformans. Sci Report. 2016 Jan 11;7:40006. (impact factor 5.5)(* correspondence author)

28. Rattanasinganchan P, Sopitthummakhun K, Doi K, Hu X, Payne MD, Pisitkun T, Leelahavanichkul A*. Folic acid-induced rat kidney fibrosis: preliminary evaluation as a potential model for identification of fibrosis biomarkers from urinary exosomes. Asian BioMed 2016 (Accepted) (impact factor 0.134))(* correspondence author)

29. Leelahavanichkul A, Chancharoenthana W, Wongphom J, Eiam-Ong S, Srisawat N. Mouse chronic renal ischemic reperfusion injury model of chronic tubulointerstitial kidney disease, suitable model for urinary exosome experiments. Asian BioMed 2016 (Accepted) (impact factor 0.134)

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