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ชื่อโครงการ (ไทย) บทบาทของแลคโตบาซิลลัสและบิฟิโดแบคทีเรียมในการเพิ่มความสามารถกีดขวางของเยื่อบุผิวและต่อต้านการอักเสบที่เกิดจากแบคทีเรียก่อโรคทางเดินอาหาร

(อังกฤษ) **Role of *Lactobacillus* and *Bifidobacterium* in the enhancement of epithelial barrier function and anti-inflammation induced by gastrointestinal bacterial pathogens**

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บทคัดย่อ

บทบาทของแลคโตบาซิลลัสและบิฟิโดแบคทีเรียมในการเพิ่มความสามารถกีดขวางของเยื่อบุผิวและต่อต้านการอักเสบที่เกิดจากแบคทีเรียก่อโรคทางเดินอาหาร

แลคโตบาซิลลัสและบิฟิโดแบคทีเรียมสายพันธุ์จำเพาะสามารถส่งเสริมหน้าที่กีดขวางของเยื่อบุผิวและป้องกันการทำลายส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุผิวและต่อต้านการอักเสบที่เกิดจากแบคทีเรียก่อโรคทางเดินอาหาร ผู้วิจัยได้ทำการคัดกรองแลคโตบาซิลลัสและบิฟิโดแบคทีเรียมสายพันธุ์ไทยที่สามารถเพิ่มความแข็งแรงของส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุผิวโดยวิธีหาค่า transepithelial electrical resistance (TER) ในเซลล์เยื่อบุลำไส้ Caco-2 พบว่ามีแลคโตบาซิลลัส 8 สายพันธุ์ (LF-L12, LO-NL49, LM-B57, LP-XB7, LS-B37, LS-B60, LR-L34 และLC-L39) และบิฟิโดแบคทีเรียม 3 สายพันธุ์ (BA-B24, BB-NB42 และ BP-NB48) สามารถป้องกันการทำลายส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุลำไส้ที่เกิดจาก คลอสทริเดียม ดิฟฟิไซล์ได้อย่างมีนัยสำคัญแต่ไม่พบเชื้อที่สามารถป้องกันการทำลายส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุลำไส้ ที่เกิดจาก เฮลิโคแบคเตอร์ ไพโลไร จากการคัดเลือกแลคโตบาซิลลัสสายพันธุ์ LR-L34 มาศึกษาต่อ พบว่า LR-L34 มีความสามารถในการแก้ไขการทำลายหน้าที่กีดขวางของเยื่อบุผิวจาก คลอสทริเดียม ดิฟฟิไซล์ ด้วย แม้ไม่ดีเท่าการป้องกัน LR-L34 ยังสามารถป้องกันการทำลายหน้าที่กีดขวางของเยื่อบุผิวจาก แคมไพโลแบคเตอร์ เจจูไน แต่ไม่สามารถป้องกันการทำลายที่เกิดจาก วิบริโอ คอเลอเร O1 Inaba และ ซัลโมเนลลา ไทฟีมิวเรียม LR-L34 สามารถเพิ่มระดับของการแสดงออกของโปรตีนที่เป็นองค์ประกอบของส่วนเชื่อมติดกันแน่นได้แก่ claudin-1 และสามารถป้องกันการลดลงของการแสดงออกของ claudin-1 และ occludin ที่ถูกชักนำโดย คลอสทริเดียม ดิฟฟิไซล์ ได้อย่างมีนัยสำคัญ นอกจากนี้พบว่า LR-L34 มีความสามารถในการต้านการอักเสบโดยกดการสร้างอินเตอร์ลิวคิน-8 (IL-8) ที่กระตุ้นด้วย คลอสทริเดียม ดิฟฟิไซล์ จากการคัดเลือกบิฟิโดแบคทีเรียมสายพันธุ์ BB-NB42 มาศึกษาต่อ พบว่า BB-NB42 มีความสามารถป้องกันการทำลายส่วนเชื่อมติดกันแน่นที่มีสาเหตุจาก ซัลโมเนลลา ไทฟีมิวเรียม และ แคมไพโลแบคเตอร์ เจจูไน แต่ไม่สามารถป้องกันการทำลายจากวิบริโอ คอเลอเร O1 Inaba นอกจากนี้ BB- NB42 สามารถเพิ่มระดับของการแสดงออกของโปรตีน occludin และ claudin-1 ได้อย่างมีนัยสำคัญ และสามารถป้องกันการลดลงของการแสดงออกของoccludin, JAM-1 และ claudin-1 ที่ถูกชักนำโดย คลอสทริเดียม ดิฟฟิไซล์ ได้อย่างมีนัยสำคัญ อย่างไรก็ตาม BB-NB42 ไม่มีความสามารถในการกดการสร้างอินเตอร์ลิวคิน-8 (IL-8) ที่กระตุ้นด้วยคลอสทริเดียม ดิฟฟิไซล์

คำสำคัญ : แลคโตบาซิลลัส / บิฟิโดแบคทีเรียม / คลอสตริเดียม ดิฟฟิไซล์ / ส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุผิว/อินเตอร์ลิวคิน-8

Abstract

Role of *Lactobacillus* and *Bifidobacterium* in the enhancement of epithelial barrier function and anti-inflammation induced by gastro-intestinal bacterial pathogens

Specific strains of *Lactobacillus* spp. and *Bifidobacterium* spp. can enhance epithelial barrier function and prevent pathogen-induced damage of epithelial tight junctions (TJs) and anti-inflammation induced by gastrointestinal bacterial pathogens. We screened indigenous *Lactobacillus* and *Bifidobacterium* Thai isolates with the ability to increase the integrity of TJs by transepithelial electrical resistance (TER) assay in Caco-2 cells. Eight *Lactobacillus* isolates (LF-L12, LO-NL49, LM-B57, LP-XB7, LS-B37, LS-B60, LR-L34 and LC-L39) and three *Bifidobacterium* isolates (BA-B24, BB-NB42 and BP-NB48) can prevent the destruction of TJs by *Clostridium difficile*. LR- L34 which was selected for further investigation had the ability to improve the intestinal epithelial barrier destroyed by *C. difficile* although the magnitude of improvement is lower than that of protection. LR-L34 also had protective effect on the destruction of TJs by *Campylobacter jejuni* but not by *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium . LR-L34 was able to increase the expression of claudin-1 significantly and its pretreatment prevented *C. difficile*-induced decrease in the expression of claudin-1 and occludin significantly. Furthermore, LR-L34 has the ability to suppress *C. difficile*-induced interleukin-8 (IL-8) production. Further investigation of BB-NB42 demonstrated that it prevented the damage of TJ integrity by *C. jejuni* and *S.* Typhimurium but not by *V.cholerae* O1 Inaba. BB-NB42 increased the expression of claudin-1and occludin significantly and its pretreatment prevented the decrease in expression of claudin-1, JAM-1 and occludin significantly. However, BB-NB42 does not have the ability to suppress *C. difficile*-induced IL-8 production.

Keywords: *Lactobacillus* / *Bifidobacterium* / *Clostridium difficile* / tight junctions/ IL-8

CONTENTS

Page

LIST OF TABLES

Table **Page**

Table **Page**

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

TNF- α	Tumor necrosis factor alpha
IL	Interleukin
TJs	Tight junctions
JAMs	Junctional adhesion molecules
ZOs	Zonula occludens
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
TEER	Transepithelial electrical resistance
kDa	Kilodalton
CDI	*Clostridium difficile* infection
IBD	Inflammatory bowel disease
CDAD	*Clostridium difficile* -associated diarrhea
AAD	Antibiotic-associated diarrhea
LAB	Lactic acid bacteria
GI	Gastrointestinal tract
MRS	deMan Rogosa Sharp
CO_2	Carbon dioxide
H_2	Hydrogen
N_2	Nitrogen
DMEM	Dulbecco's modified eagle medium
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
CFU	Colony forming unit
°C	Degree Celsius
ATCC	American type culture collection
TSA	Tryptone soya agar
ml	Milliliter
cm^2	Square centimeters
µm	Micrometers
mm	Millimeters
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing tween
Tris	Tris(hydroxymethyl)-aminomethane
SD	Standard deviation
UV	Ultraviolet radiation
DW	Distilled water
Conc	Concentration
et al.	et alii
g	Gram
h	Hour
HCl	Hydrochloric acid
NaCl	Sodium chloride
DTT	Dithiothreitol
APS	Ammonium persulfate
l	Liter
M	Molar
mM	Millimolar
mg	Milligram
rpm	Round per minute
µl	Microliter

INTRODUCTION

The intestinal epithelium is a single layer of cells which has crucial role in not only the absorption of nutrients but also barrier functions that protect foreign agents and bacterial pathogens [1]. An important component for the maintenance of barrier integrity is the junctional complexes which seal paracellular space between epithelial cells. They consist of tight junctions (TJs), gap junctions, adherens junctions and demosomes which regulate the paracellular permeability and the integrity of the epithelial barrier [2].

The tight junctions (TJs) resided in the apical intercellular junctions have two functions, the barrier function and the fence function which regulates the passage of ions, water and various molecules through paracellular space, and maintain cell polarity by forming a fence to prevent intermixing of molecules in the apical membrane with those in the lateral membrane, respectively [3]. TJs are complex structure of several proteins; transmembrane proteins (such as occludin, claudin and junctional adhesion molecules [JAM]) that it is connected with actin cytoskeleton by adaptor proteins (zonula occludens;ZOs). This structure joins intercellular space and stabilizes integrity of the intestinal epithelial barrier [4, 5]. Furthermore, the structure complexes of TJs are the first line of defense against pathogens. However, TJs are destroyed by pathogens and other factors such as cytokines with different mechanism in destruction which lead to various diseases [6].

Clostridium difficile is an anaerobic, gram positive-spore forming bacillus which causes diarrhea and colitis [7]. At present, *C. difficile* is the most important healthcare-associated pathogens in the United States and Europe and the incidences of *C. difficile* infection (CDI) have increased worldwide. The incidence rate of CDI has increased from 82,000 in 1996 to 178,000 in 2003 in the United States [8]. The major risk factors of CDI are antibiotic treatment and hospitalizations. Another risk factors that lead to CDI including age older than 65 years, inflammatory bowel disease (IBD), immunosuppression (such as cancer, steroid treatment, HIV infection, or organ transplant), chronic liver disease, end-stage renal disease and tube feeding [9, 10]. In addition, CDI are reported in non-risk group such as children, pregnant women, community-acquired infection and patients with no previous exposure to antibiotic [11]. The clinical symptom varies from asymptomatic to mild self-limited diarrhea and severe pseudomembranous colitis [12]. 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 [13, 14]. The pathogenic process starts with initial colonization in human intestinal epithelial and the production two toxins of *C. difficile*. Toxin A binds to specific receptor on the surface of the intestinal epithelium and toxin B can move through the basolateral side of cell membrane after tight junctions disruption [15]. Toxin A and toxin B modify and inactivate Rho GTPase proteins via glucosylation resulting in the rearrangement of actin cytoskeleton, disruption of tight junctions, rounding up of cell, cell death and loss of intestinal epithelium barrier function [16-18]. Previous studies showed that *C. difficile* toxins disrupt tight junctions which were observed from the decrease in transepithelial electrical resistance and the increase of paracellular permeability in epithelial cell lines incubated with toxins [19-21]. Disruptions of tight junctions allow the toxins move to basolateral side of epithelial cells and laminar propriae and induce the release of several proinflammatory cytokines such as interleukin (IL)-8, tumor necrosis factor alpha (TNF- α), IL-1 and IL-6, leading to inflammatory response because of neutrophil and lymphocyte influx resulting in pseudomembrane formation and diarrhea [22, 23].

The common agents for CDI treatment are metronidazole and vancomycin [24]. The problem for treatment of CDI is recurrent of disease, approximately 10% to 20% of patients with CDI have recurrent infection of disease in initial episode which occur within 5-8 days after treatment stops [25]. The recurrence of disease occurs in two forms including relapse and reinfection [26]. At present, clinical trials of probiotics like *Saccharomyces boulardii*, *Lactobacillus* GG and *Lactobacillus plantarum* 299V resulted in the reduction of recurrence of disease [27-29].

The FAO/WHO provides the definition of probiotics that are 'live organisms which when administered in adequate amounts confer a health benefit on the host' [30]. The most common probiotics include *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli* (such as *E. coli* Nissle 1917) and yeast (such as *Saccharomyces boulardii*) [30]. These probiotics were used to prevent and treat patients with gastrointestinal disorder, especially antibiotic-associated diarrhea (AAD) and *C. difficile* infection [31, 32]. The treatments with antibiotics disturb and inhibit growth of normal flora in the intestine. In contrast, probiotics can restore and preserve homeostasis of normal flora in the intestine [30]. Furthermore, probiotics have other beneficial effects to host such as the increase in the integrity of tight junction, immunomodulation and competition with pathogen in the adherence to surface of intestinal epithelium [33, 34].

Lactobacilli are lactic acid bacteria (LAB) which are gram-positive, nonspore-forming rods or coccobacilli with low G+C content. They are fermentative bacteria which are classified into three groups including obligately homofermentative, facultatively heterofermentative and obligately heterofermentative group [35, 36]. Lactobacilli were isolated from several sources of human and animal including GI tract, vaginal tract, and oral cavities. Some strains of lactobacilli were used in food industry while some strains are probiotics [35]. It has been reported that specific strains of *Lactobacillus* spp. can prevent the disruption of TJs of intestinal epithelial cells. For examples, *Lactobacillus rhamnosus* strain GG can prevent enterohemorrhagic *Escherichia coli* O157:H7 (EHEC)-induced redistribution of tight junctions [37] and specific strain of *Lactobacillus plantarum* can protect enteroinvasive *Escherichia coli* (EIEC) and enteropathogenic *Escherichia coli* (EPEC)-induced change in intestinal epithelial barrier function [38, 39].

Bifidobacteria are Gram positive, rod shaped, anaerobic bacteria which are inhabitant of the human intestinal tract. They can produce lactic acid and aceticthat arethe main products of glucose utilization.The amount of bifidobacteria decreased with increased age.After birth, the number of bifidobacteria is high in the intestinal tract but in the elderly the number is decreased [40]. Bifidobacteria such as *B. longum, B. bifidum, B. breve* and *B. infantis* have been considered as therapeutic benificial probiotics for human health [41].The role of bifidobacteria in prevention of intestinal infections have been reported that they can antagonise the growth of pathogens [42]. Bifidobacteria have been reported to enhance the intestinal epithelial barrier function and prevent TJ integrity from pathogen-induced damage. For examples, *B. infantis* conditioned medium enhanced epithelial barrier function shown by increased transepithelial electrical resistance (TEER) of T84 cells, and increased expression of ZO-1 and occludin [43] and *B. lactis* 420 cell-free supernatant increased TER of Caco-2 cells and prevented the TJ integrity destroyed by *Escherichia coli*O157:H7 [44].

This study aimed to search for indigenous *Lactobacillus* and *Bifidobacterium* Thai isolates that can enhance the integrity of tight junctions and prevent and/or improve the damage of TJs by *C. difficile* and other important gastrointestinal bacterial pathogens including *Salmonella enterica* subspecies Typhimurium *(Salmonella* Typhimurium*), Vibrio cholerae* O1 Inaba, *Campylobacter jejuni* and *Helicobacter pylori.*

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

A total of 29 *Lactobacillus* spp. and 17 *Bifidobacterium* spp which were previously isolated from infant feces, breast milk and gastric biopsies were used in this study (Table 1). Lactobacilli and bifidobacteria were inoculated on de Man, Rogosa and Sharpe agar (MRS, Oxoid, Oxoid Ltd., Basingstoke, Hampshire, England)and MC (Modified Columbia) agar (Oxoid, Basingstoke, Hampshire, England), respectively. They were incubated at 37 ^{0}C in anaerobic condition (The AnaeroPack system, Mitsubishi Gas Chemical, H_2: 5%, CO_2: 10%, N_2: 85%) for 24 hours. After incubation, they were suspended in Dulbecco's modified eagle media (DMEM; containing 20% fetal bovine serum and 2.5% HEPES) to obtain a final concentration of $1.0x10^9$ CFU/mL for further use in the experiment.

C. difficile B2-CU-0001-54 was obtained from feces of an infected patient positive for *C. difficile* toxins A and B by VIDAS® *Clostridium difficile* A & B (Biomérieux, France) at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. This strain is positive for TcdA and TcdB as determined by the reactivity with mouse anti-TcdA and anti-TcdB monoclonal antibodies (Meridian Life Science, Inc.). *C. difficile* was inoculated on Brucella agar (Becton, Dickinson, France), *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium ATCC 13311 were inoculated on tryptone soya agar [TSA] (Oxoid Ltd., Basingstoke, Hampshire, England), and *Campylobacter jejuni* was inoculated on Brucella agar (Becton, Dickinson, France) with 5% sheep blood. They were incubated at 37 ^{0}C in anaerobic condition (The AnaeroPack system, Mitsubishi Gas Chemical, H_2: 5%, CO_2: 10%, N_2: 85%) for 24 hours.

H. pylori ATCC 43504 (ATCC, Manassas, VA) was cultured on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco New Zealand Ltd, Auckland, New Zealand) and 7% (v/v) sheep blood under microaerophilic conditions (6-12%O_2, 5-8% CO_2) at 37°C for 48-72 hours.

They were suspended in Dulbecco's modified eagle media (DMEM; containing 20% fetal bovine serum and 2.5% HEPES) to obtain a final concentration of $1.0x10^8$ CFU/mL for further use in the experiment.

Table 1. *Lactobacillus* and *Bifidobacterium* used in this study [45-47]

***Lactobacillus* and *Bifidobacterium* isolated from infant feces**	***Lactobacillus* and *Bifidobacterium* isolated from breast milk**	***Lactobacillus* isolated from gastric biopsy**
L. gasseri L2 (LG-L2)	*L. fermentum* Lac31 (LF-Lac31)	*L. plantarum* XB7(LP-XB7)
L. gasseri L3 (LG-L3)	*L. rhamnosus* Lac43(LR-Lac43)	*L. salivarius* B37(LS-B37)
L. fermentum L7 (LF-L7)	*L. casei* Lac44(LC-Lac44)	*L. murinus* B57(LM-B57)
L. fermentum L12 (LF-L12)	*L. salivarius* NL3(LS-NL3)	*L. salivarius* B60(LS-B60)
L. ruminis L13 (LRU-L13)	*L. gasseri* NL8(LG-NL8)	*L. salivarius* B73(LS-B73)
L. mucosae L15 (LM-L15)	*L. mucosae* NL45(LM-NL45)	*L. plantarum* B90(LP-B90)
L. gasseri L29 (LG-L29)	*L. oris* NL49 (LO-NL49)	*L. salivarius* B101(LS-B101)
L. rhamnosus L31(LR-L31)	*L. plantarum* NL61(LP-NL61)	*L. casei* B103(LC-B103)
L. rhamnosus L33(LR-L33)	*B. bifidum* NB13 (BB-NB13)	*L. casei* B106(LC-B106)
L. rhamnosus L34(LR-L34)	*B. bifidum* NB42 (BB-NB42)	
L. rhamnosus L35(LR-L35)	*B. breve* Bif29 (BB-Bif29)	
L. casei L39 (LC-L39)	*B. catenulatum* NB38 (BC-NB38)	
B. adolescentis B14 (BA-B14)	*B. dentium* NB11 (BD-NB11)	
B. adolescentis B24 (BA-B24)	*B. dentium* NB14 (BD-NB14)	
B. catenulatum B38 (BA-B38)	*B. pseudocatenulatum* NB2	
B. longum B9 (BL-B9)	(BP-NB2)	
B. longum B36 (BL-B36)	*B. pseudocatenulatum* NB45	
B. longum B103 (BL-B103)	(BP-NB45	
B. pseudocatenulatum B11	*B. pseudocatenulatum* NB48	
(BP-B11)	(BP-NB48)	
B. pseudocatenulatum B57		
(BP-B57)		

2. Transepithelial electrical resistance (TEER) assay for *Lactobacillus* / *Bifidobacterium*

Caco-2 human colorectal adenocarcinoma cells (ATCC HTB-37) were grown as previously described by Anderson *et al.* [48] with modification. Cells were grown in 75 cm^2 flasks with DMEM supplemented with 20% fetal bovine serum and 2.5% HEPES at 37 ^0C under 5% CO_2 for 48 hours and seeded on transwell insert (6.5 mm diameter, 0.4 μm pore size, 0.33 cm^2 surface area, Collagen membrane insert, Costar/Corning, NY, U.S.A.) at a density of $5x10^4$ cells/well. Transwell was incubated at 37 ^0C in a humidified atmosphere with 5% CO_2. Culture medium was changed every second day. Cells were

grown for 18 days and added at the apical side with 100 µL of $1.0x10^9$ CFU/mL of *Lactobacillus* spp. or *Bifidobacterium* spp. After incubation for 24 h, TER was measured by using a voltohmmeter (EVOM^2 Epithelial Tissue Voltohmmeter, WorldPrecision Instruments, FL). Blank control contained only Caco-2 cells and media. The electrical resistance was recorded and calculated by the following formula:

TEER ($\Omega.cm^2$) = (Total resistance – Blank resistance) (Ω) x Area (cm^2).

3. *Lactobacillus / Bifidobacterium* and pathogens coculture

Lactobacillus spp. or *Bifidobacterium* spp. and pathogens were prepared as described above. *Lactobacillus/Bifidobacterium* was incubated alone with 18 days old Caco-2 cells or pretreated for 3 hours before the addition of pathogens. In case of *C. difficile*, 18 days old Caco-2 cells were infected with *C. difficile* for 3 hours before the addition of *Lactobacillus/ Bifidobacterium*. Each pathogen was also tested for its effect on TEER. One hundred microlitres of $1.0x10^8$ -$1.0x10^9$ CFU/mL *Lactobacillus* or *Bifidobacterium* and 100 µL of $1.0x10^7$ -$1.0x10^8$ CFU/mL of each pathogen were added at the apical side. After incubation for 24 h, TEER was measured as described above.

4. The effect of cell viability on TEER

Selected *Lactobacillus* or *Bifidobacterium* was killed by 254-nm ultraviolet irradiation for 45-60 min. in a biological safety carbinet (Model ATC 1200 N, Astec Microflow, Science Tech co.,Ltd.) and used as described above. The viability of irradiated cells was checked by culture on MRS or MC agar as appropriate. The plates were incubated at 37 ^{0}C in anaerobic condition (The AnaeroPack system, Mitsubishi Gas Chemical, H_2: 5%, CO_2: 10%, N_2: 85%) for 24 hours. Untreated bacterial suspension was also plated on MRS or MC agar as a control.

5. Western blotting for determining the distribution and expression of tight junction proteins

Caco-2 cells were grown in 75 cm^2 flasks with DMEM supplemented with 20% fetal bovine serum and 2.5% HEPES at 37 ^{0}C under 5% CO_2 for 48 hours. Cells were seeded on 6-well plate (Nunclon®Δ, Roskilde, Denmark) at a density of $5x10^4$ cells/well and incubated at 37 ^{0}C in a humidified atmosphere with 5% CO_2. Culture medium was changed every second day. Polarized Caco-2 cells were treated as described above. The

protein samples from Caco-2 cells were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cells were washed two times in PBS and lysed in cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS). After centrifugation at 13000xg for 10 min at 4°C, the supernatant was collected and assayed for protein content with the Pierce® BCA protein assay kit (Pierce Biotechnology, Illinois, USA). Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels and then transferred to a PVDF membrane (Bio-Rad, Philadelphia, USA). After blocking overnight in Tris-buffered saline (TBS) containing 0.05% Tween (TBS-T) and 10% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and incubated for 1 hour at room temperature in 1: 50 primary antibody (rabbit anti-Claudin-1, or rabbit anti-occludin, or rabbit anti-JAM, or rabbit anti-ZO-1, both from Cell Signaling, USA). After three washes with TBS-T, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody. Following three washes with TBS-T, the membranes were developed for visualization of protein by the addition of enhanced chemiluminescence reagent and signal was detected on X-ray film. Peroxidase signals were detected and analyzed by ImageJ 1.46 program.

6. Effect of *Lactobacillus* and *Bifidobacterium* on the suppression of IL-8 production

Lactobacillus conditioned media (LCM) or *Bifidobacterium* conditioned media (BCM) were prepared as follow. Briefly, 24 h cultures were adjusted to an OD_{600} 0.1 and incubated anaerobically for 48 h. Supernatants were collected, filtered with 0.22 μm Millex-GV Filter Units (Millipore, USA) and concentrated by Eppendorf Vacufuge® vacuum concentrator (Eppendorf North America, USA) at 60°C for 2.5 h. Pellets of *Lactobacillus* and *Bifidobacterium* were resuspended in McCoy's 5a modified medium (Gibco-Invitrogen, Carlsbad, CA, USA) and stored at -20°C until further analysis. LCM or BCM was coculture with *C. difficile* on HT-29 colonic epithelial cells (ATCC HTB-38). Supernatants from co-culture assays were tested for the effects of *Lactobacillus* or *Bifidobacterium* on IL-8 production. IL-8 concentrations were measured using a Human CXCL8/IL-8 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

7. Statistical analysis

All experiments, except the screening test, were done in biological replicates as stated in the tables and data represented standard error. The data were analyzed using the Student's t-test with one-tailed distribution.

RESULTS

1. *Lactobacillus* spp. can increase the integrity of tight junctions on human colorectal adenocarcinoma cell line (Caco-2 cells)

Lactobacillus spp. were tested for the ability to enhance human intestinal epithelial barrier function by transepithelial electrical resistance (TEER) assay. This method is to test the integrity of tight junctions by measurement of TEER of Caco-2 cells. Twenty-nine *Lactobacillus* spp. were co-cultured with Caco-2 cells in transwell and TEER was measured at 24 hours. The results of TEER were shown in Tables 2-5 and Figures 1-4. Three isolates of *Lactobacillus* spp. including *L. fermentum* L12 (LF-L12), *L. oris* NL49 (LO-NL49) and *L. murinus* (LM-B57) increased TEER significantly. The enhancement effect is strain- specific since only *L. fermentum* L12 increased TEER whereas *L. fermentum* L7 and Lac31 did not. LF-L12, LO-NL49 and LM-B57 were selected for further investigation. In addition, four isolates of *Lactobacillus* spp. including *L. gasseri* L3 (LG-L3), *L. plantarum* XB7 (LP-XB7), *L. salivarius* B37 (LS-B37) and *L. salivarius* B60 (LS-B60) which did not increase TEER significantly were selected for further study because they were previously shown to suppress *C. difficile*-induced IL-8 production[49]. Furthermore, three isolates of *Lactobacillus* spp. including *L. rhamnosus* L34 (LR-L34), *L. casei* L39 (LC-L39) and *L. plantarum* B90 (LP-B90) which were previously shown to suppress *C. difficile*-induced IL-8 production[49] were selected for further study although they decreased TEER. Summary of selected *Lactobacillus* spp. for further investigation was shown in Table 6.

2. Destruction of intestinal epithelial barrier functions by *Clostridium difficile* and other intestinal bacterial pathogens on Caco-2 cells

The intestinal epithelial barrier functions can be destroyed by *Clostridium difficile* and other intestinal bacterial pathogens including *Vibrio cholera*e O1 Inaba, *Salmonella*

Typhimurium ATCC 13311 and *Campylobacter jejuni*. These pathogens destroy the integrity of tight junctions by the disruption of tight junctions. *C. difficile* and other intestinal bacterial pathogens were co-cultured with Caco-2 cells ($1x10^7$-$1x10^9$ CFU/ml). TEER was measured at 24 hours. *C. difficile* and other intestinal bacterial pathogens decreased TEER significantly on Caco-2 cells. In addition, the decrease of TEER was dose-dependent as shown in Figures 5 and 6, respectively. However, only *C. jejuni* decreased TEER at 48 hours, it was then co-cultured with Caco-2 cells and measured TEER at 48 hours.

3. *Lactobacillus* spp. prevent the disruption of tight junctions by *Clostridium difficile*

All ten selected *Lactobacillus* spp. including LF-L12, LO-NL49, LM-B57, LG-L3, LP-XB7, LS-B37, LS-B60, LR-L34, LC-L39 and LP-B90 were tested for the ability to prevent the disruption of tight junctions by *C. difficile*. Caco-2 cells were pretreated with *Lactobacillus* spp. ($1x10^9$ CFU/ml) for 3 hours and infected with *C. difficile* ($1x10^8$ CFU/ml) in transwell. TEER was measured at 24 hours. Pretreatment of 9 *Lactobacillus* spp. on Caco-2 cells could increase TEER significantly as compared with that of cells infected with *C. difficile*. Only LP-B90 pretreatment did not result in significantly increased TEER. The results were shown in Table 7 and Figure 7.

LR-L34, LC-L39, LP-XB7 and LS-B37 were selected for further investigation because they could suppress *C. difficile*-induced IL-8 productions. LF-L12, LO-NL49, LM-B57 and LS-B60 which increased TEER significantly in the same assay were kept as stock culture for further study. LG-L3 was excluded from this study as it is vancomycin-susceptible.

4. Effect of the proportion of *Lactobacillus* spp. and *Clostridium difficile* on TEER

Since the enhancement of TEER as described above was not high, the proportions of *Lactobacillus* spp. and *C. difficile* in co-culture assay were adjusted and investigated for the effect on TEER. LC-L39 was selected for this study. Suspension of $1x10^8$-$1x10^{10}$ CFU/mL LC-L39 was co-cultured with $1x10^7$-$1x10^9$ CFU/mL *C. difficile* and TEER was determined in each combination. The result in Figure 8 indicated that the proportion of

$1.0x10^8$ CFU/mL LC-L39 and $1.0x10^7$ CFU/mL *C. difficile* was appropriate and used in further experiment.

5. Effect of four *Lactobacillus* spp. on TEER when added before and after *Clostridium difficile* in co-culture assay

LR-L34, LC-L39 and LS-B37 increased TEER when cells were treated with *Lactobacillus* spp. alone (Tables 8-11), whereas LF-XB7 alone decreased TEER (Table 11). In addition, when cells were pretreated with all four *Lactobacillus* spp. followed with *C. difficile*, TEER increased significantly compared with cells were infected *C. difficile* alone (Tables 8-11 and Figures 9-12). When cells were treated with *C. difficile* before and *Lactobacillus* was added later, LR-L34 and LC-L39 increased TEER significantly. In addition, LR-L34 could increase TEER more significantly than LC-L39, LS-B37 and LF-XB7. LR-L34 was thus selected for further investigation.

6. Effect of live LR-L34 and UV-irradiated LR-L34 on the prevention of tight junction disruption by *C. difficile* and other bacterial pathogens except *H. pylori*

Live and UV-treated LR-L34was tested for the effect on the disruption of tight junctions by *C. difficile* and other bacterial pathogens including *Vibrio cholerae* O1 Inaba, *Salmonella* Typhimurium and *Campylobacter jejuni*. The results showed that live LR-L34 could increase TEER significantly more than UV-treated LR-L34 when cells were pretreated with *Lactobacillus* species for 3 hours before the addition of *C. difficile*. In contrast, UV-treated LR-L34 could increase TEER significantly more than live LR-L34 when cells were pretreated with *Lactobacillus* species for 3 hours before the addition of *C. jejuni*. However, both live LR-L34 and UV-treated LR-L34 not prevent the intestinal integrity destroyed by *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium. The result was shown in Tables 12-15 and Figures 13-16. In addition, the effect of LR-L34 to prevent the integrity of tight junctions that disrupted by *C. difficile* were observed the expression of TJs proteins by western blot assay.

7. *Lactobacillus* spp. prevent the disruption of tight junctions by *Helicobacter pylori*

All five selected *Lactobacillus* spp. including LP-XB7, LS-B37, LM-B57, LS-B60 and LS-B78 were tested for the ability to prevent the disruption of tight junctions by *H. pylori*. Caco-2 cells were pretreated with *Lactobacillus* spp. ($1x10^9$ CFU/ml) for 3 hours and infected with *H. pylori* ($1x10^8$ CFU/ml) in transwell. TEER was measured at 48 hours. Pretreatment of three *Lactobacillus* spp. on Caco-2 cells could not significantly increase TEER when compared with that of cells infected with *H. pylori*. The results were shown in Table 16 and Figure 17.

8. The effect of LR-L34 on the expression of tight junctions proteins

Western blot analyses were performed to determine the relative proteins expression of JAM-1, claudin-1 and occludin in Caco-2 cells. The expression of JAM-1 and occludin non-significantly increased when cells were treated with LR-L34 alone as compared with control while the expression of claudin-1 increased significantly. In contrast, the expression of JAM-1, claudin-1 and occludin non-significantly decreased when cells were infected *C. difficile* alone as compared with control. Furthermore, the expression of claudin-1 and occludin increased significantly when cells were pretreated with LR-L34 for 3 hours followed by the infection with *C. difficile* as compared with cells infected with *C. difficile*, whereas the expression of JAM-1 non-significanly increasedas compared with cells infected *C. difficile* alone (Figure 18).

9. *Bifidobacterium* spp. can enhance intestinal epithelial resistance of human colorectal adenocarcinoma cell line (Caco-2 cells)

Bifidobacterium spp. were screened in TEER assay to evaluate their effect on the tight junction integrity between the differentiated Caco-2 monolayers. Seventeen *Bifidobacterium* spp. previously isolated from breast milk [50] and infant feces [51] were used in this study (Table 1). They were added on apical side of Caco-2 cells and incubated for 24 h. After incubation, the resistance across each monolayer was measured by voltohmmeter and TEER was calculated from the resistance. The effects of *Bifidobacterium* spp. on TEER were shown in Tables 17-19 and Figures 19-21. Five *Bifidobacterium* isolates increased TEER when compared their effects with the control media. *B. psedocatenulatum* NB48 (BP-NB48) and *B. bifidum* NB42 (BB-NB42) increased TEER significantly while *B. catenulatum* NB38 (BC-NB38), *B. longum* B103

(BL-B103) and *B. adolescentis* B24 (BA-B24) non-significantly increased TEER. *Bifidobacterium* spp. which increased TEER and chosen for investigation were shown in Table 20.

10. Destruction of tight junctions integrity of human intestinal epithelial cells by *Clostridium difficile*

C. difficile can disrupt tight junctions which leads to decreased intestinal epithelial barrier function. To investigate the effect of *C. difficile* on human colorectal adenocarcinoma cell line (Caco-2 cells), *C. difficile* (100 µl) at concentration of $1x10^7$-$1x10^9$ CFU/ml were incubated with Caco-2 cells for 24 h. After incubation, the integrity of tight junctions was measured by TEER assay. As seen in Figure 22, the addition of *C. difficile* resulted in decrease of TEER significantly on Caco-2 cells when compared with the control media. Furthermore, the decrease of TEER was dose-dependent.

11. *Bifidobacterium* spp. prevent *Clostridium difficile*-induced damage of the integrity of tight junctions

Five *Bifidobacterium* spp. which increased TEER including BA-B24, BB-NB42, BC- NB38, BL-B103 and BP-NB48 and used in this experiment. They were investigated for the ability in prevention of tight junctions integrity damage by *C. difficile*. Caco-2 cells were pretreated with *Bifidobacterium* spp. at concentration of $1x10^9$ CFU/ml for 3 h before the addition of *C. difficile* at concentration of $1x10^8$ CFU/ml. The integrity of tight junctions was measured by TEER assay at 24 h after incubation. The results in Table 21 and Figure 23 showed that three *Bifidobacterium* spp. including BA-B24, BB-NB42 and BP-NB48 significantly prevented *C.difficile*-induced damage of the integrity of tight junctions. BB-NB42 had highest effect on the prevention of the damage of tight junction integrity. These three bifidobacteria were selected for further investigation.

12. Effects of three *Bifidobacterium* spp. on TEER when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells

To investigate the ability of *Bifidobacterium* spp. in the restoration of the integrity of tight junctions disrupted by *C. difficile*, Caco-2 cells were treated with *C. difficile*

before the addition of bifidobacteria. As shown in Tables 22-24 and Figures 24-26, when Caco-2 cells were treated with *C. difficile* before and bifidobacteria were added later, none of the three bifidobacteria could restore the tight junction integrity. Effect of bifidobacteria on tight junction integrity was tested only for the prevention in further experiment.

13. Effect of different proportion of *Bifidobacterium* spp. and *Clostridium difficile* on TEER

Since the effect of *Bifidobacterium* spp. on the enhancement of tight junction integrity was not high as shown above, the effect of different proportions of *Bifidobacterium* spp. and *C. difficile* on TEER was investigated. *Bifidobacterium* spp. at $1x10^8$ - $1x10^{10}$ CFU/ml was added on apical side of Caco-2 cells for 3 h before the addition of *C. difficile* at $1x10^7$-$1x10^9$ CFU/ml. TEER was measured at 24 h in each combination. In this experiment, BB-NB42 and BA-B24 were selected for testing. The result in Figures 27 and 28 showed that the optimal concentration of *Bifidobacterium* spp. and *C. difficile* was$1x10^8$ CFU/ml and $1x10^7$ CFU/ml, respectively. The proportion of *Bifidobacterium* spp. and *C. difficile* by these concentrations was selected for further investigation.

14. Effect of live or UV-treated *Bifidobacterium* spp. on the prevention of *C.difficile*-induced damage of tight junctions

Caco-2 cells pretreated with live or UV-treated of *Bifidobacterium* spp., including BB-NB42, BA-B24 and BP-NB48, at $1x10^8$ CFU/ml for 3 h prior to infection of *C. difficile* at $1x10^7$ CFU/ml. TEER was measured at incubation for 24 h. The results in Table 25 and Figure 29 indicated that live BB-NB42 alone increased TEER significantly compared with control media and UV-treated BB-NB42 alone non-significantly increased TEER. In the pretreatment assay, it was found that live and UV- treated BB-NB42 increased TEER significantly compared with cells infected with *C. difficile* alone. However, the TEER increasing by live BB-NB42 was higher than that of UV-treated BB-NB42.

Similarly, the results in Table 26 and Figure 30 showed that treatment of Caco-2 cells with live BA-B24 alone increased TEER significantly and UV-treated BA-B24 non-

significantly increased TEER when compared with control media. When Caco-2 cells were pretreated with live or UV- treated BA-B24, it was found that TEER increased significantly compared with cells treated with *C. difficile* alone. However, live BA-B24 increased TEER higher than UV-treated BA-B24.

As shown in Table 27 and Figure 31, live BP-NB48 alone non- significantly increased TEER but UV-treated BP-NB48 alone non- significantly decreased TEER when compared with control media. In addition, pretreatment with live or UV- treated BP-NB48 increased TEER significantly when compared with cells treated with *C. difficile*. However, live BP-NB48 increased TEER was higher than UV-treated BP-NB48.

The above results indicated that BB-NB42, BA-B24 and BP-NB48 prevent integrity of tight junctions disrupted by *C. difficile*. Since BB-NB42 had the greatest effect on TEER when Caco-2 cells were infected with *C. difficile*, it was thus chosen for further investigation.

15. The effect of BB-NB42 in the prevention of the damage of tight junctions integrity caused by *Vibrio cholerae*, *Salmonella* Typhimurium and *Campylobacter jejuni*

Since *V. cholerae*, *S.* Typhimurium and *C. jejuni* are important enteropathogens which cause the disruption of tight junctions, this experiment investigated the ability of BB-NB42 to prevent the damage of tight junction integrity caused by these pathogens. Caco-2 cells were pretreated with BB-NB42 for 3 h and infected with *V. cholerae*, *S.* Typhimurium for 24 h or *C. jejuni* for 48 h [52]. TEER was measured after each incubation. The result in Table 28 and Figure 32 showed that Caco-2 cell treated with *V. cholerae* O1 Inaba resulted in the significant decrease in TEER compared with control media. This indicated that *V. cholerae* O1 Inaba disrupted the tight junctions integrity. However, pretreatment of Caco-2 cells with BB-NB42 did not significantly increase TEER compared with cells infected with *V. cholerae* O1 Inaba alone. This indicated that BB-NB42 did not prevent the tight junctions integrity damage by *V. cholerae*.

In contrast with the effect on *V. cholerae*, the result in Tables 29-30 and Figures 33-34 showed that pretreatment of BB-NB42 on Caco-2 cells for 3 h before infection with *S.* Typhimurium or *C. jejuni* resulted in a significant increase of TEER compared with cells infected with *S.* Typhimurium or *C. jejuni* alone. BB-NB42 can thus prevent the damage of the integrity of tight junctions by *S.* Typhimurium and *C. jejuni*.

16. The effect of BB-NB42 on the expression of tight junction proteins

The relative protein expression of tight junction proteins which include ZO-1, Occludin, JAM-1 and Claudin-1 in Caco-2 cells in each condition was determined by western blot analysis. The result in Figure 35 showed that *C. difficile* infection on Caco-2 cells decreased the expression of tight junction proteins. In the expression of ZO-1, it was found that Caco-2 cells were co-cultured with BB-NB42 alone non-significantly increased ZO-1 expression when compared to the expression in untreated cells. Pretreatment of Caco-2 cells with BB-NB42 before infection with *C. difficile* non-significantly increased ZO-1 expression when compared with cells infected with *C. difficile*.

Caco-2 cells treated with BB-NB42 alone increased occludin expression significantly when compared with untreated cells. When BB-NB42 were pretreated on Caco-2 cells for 3 h prior to *C. difficile* infection, it was found that occludin expression increased significantly compared with cells infected with *C. difficile*. BB-NB42 thus prevented *C. difficile*-induced decrease in expression of occludin protein.

For JAM-1 expression of Caco-2 cells treated with BB-NB42 alone, it was non-significantly increased compared with untreated cells. When Caco-2 cells were pretreated with BB-NB42 before infection with *C. difficile* for 3 h, it was found that the expression of JAM-1 was increased significantly compared with cells infected with *C. difficile* alone. This indicated that BB-NB42 prevents *C. difficile*-induced decrease in expression of JAM-1 protein.

Claudin-1 expression in BB-NB42 -treated cells alone and pretreated cells prior to *C. difficile* infection increased significantly compared with control cells and cells infected with *C. difficile* alone, respectively. These indicated that BB-NB42 prevented *C. difficile*-induced claudin-1 protein expression.

17. Effect of LR-L34, BB-NB42, BA-B24 and BP-NB48 on the suppression of *C. difficile*-induced IL-8 production

LCM of LR-L34 significantly inhibited *C. difficile*-induced IL-8 production as shown in Table 31 and Figure 36. However, BCM of BB-NB42, BA-B24 and BP-NB48 did not inhibit *C. difficile*-induced IL-8 production as shown in Table 32 and Figure 37.

Table 2. The effects of *Lactobacillus* spp. isolated from infant feces on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	146.63	9.11	100	
L3 (*L. gasseri*)	156.09	17.18	106.45	0.22338
L33 (*L. rhamnosus*)	74.36	3.65	50.71	0.00011
L34 (*L. rhamnosus*)	97.90	8.25	66.77	0.00118
L35 (*L. rhamnosus*)	93.50	5.92	63.77	0.00053
L39 (*L. casei*)	125.07	5.97	85.30	0.01327

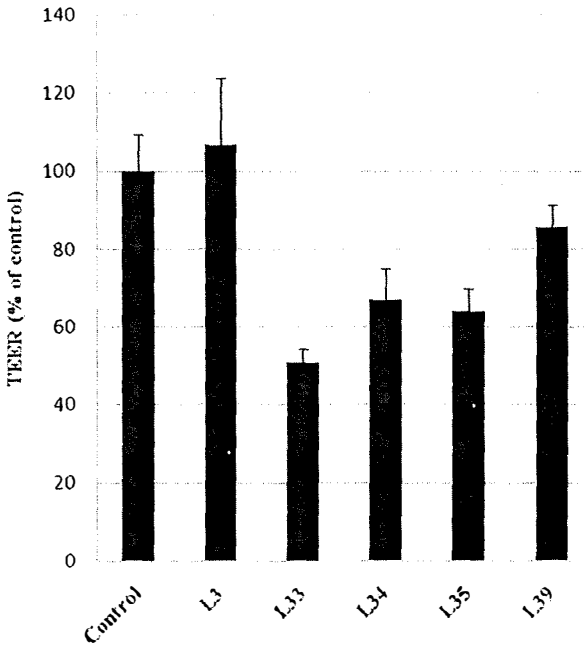


Figure 1. Change in the TEER by *Lactobacillus* spp. isolated from infant feces across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 3. The effects of *Lactobacillus* spp. isolated from infant feces or gastric biopsy on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	114.51	2.29	100	
L2 (*L. gasseri*)	99.33	7.05	86.74	0.01195
L7 (*L. fermentum*)	105.93	3.67	92.51	0.01322
L12 (*L. fermentum*)	146.52	17.35	127.95	0.01696 *
L13 (*L. ruminis*)	131.12	19.38	114.51	0.10722
L15 (*L. mucosae*)	122.43	9.60	106.92	0.11851
L29 (*L. gasseri*)	100.98	6.44	88.18	0.01328
L31 (*L. rhamnosus*)	102.63	0.33	89.63	0.00044
XB7 (*L. plantarum*)	116.71	4.20	101.92	0.23525

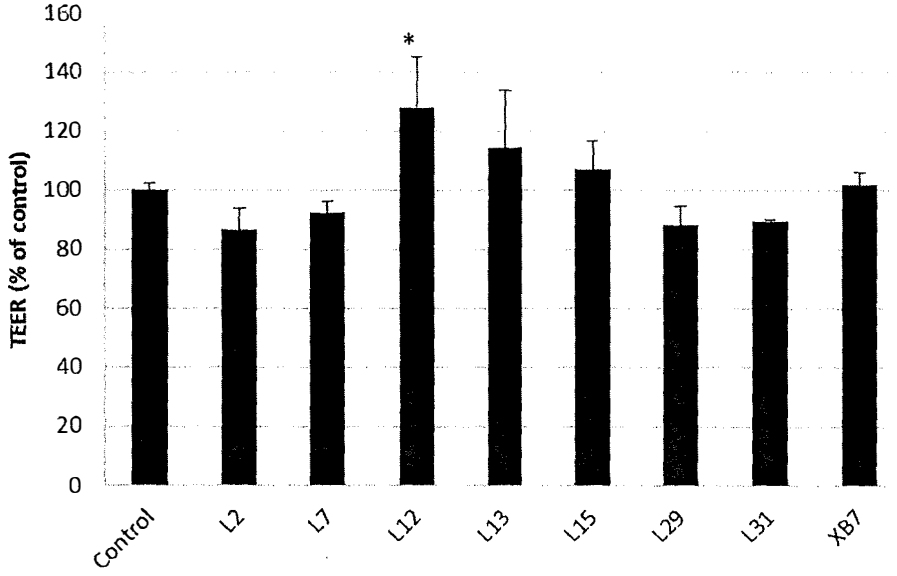


Figure 2. Change in the TEER by *Lactobacillus* spp. isolated from infant feces and gastric biopsies across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 4. The effects of *Lactobacillus* spp. isolated from breast milk on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	136.40	7.69	100	
Lac31 (*L. fermentum*)	100.65	15.77	73.79	0.0121
Lac43 (*L. rhamnosus*)	123.75	11.73	90.73	0.0966
Lac44 (*L. casei*)	140.58	1.75	103.06	0.2051
NL3 (*L. salivarius*)	138.60	18.15	101.61	0.4281
NL8 (*L. gasseri*)	97.02	12.12	71.13	0.0045
NL46 (*L. mucosae*)	107.25	4.00	78.63	0.0022
NL49 (*L. oris*)	162.58	9.81	119.19	0.0110 *
NL61 (*L. plantarum*)	148.50	21.09	108.87	0.2017

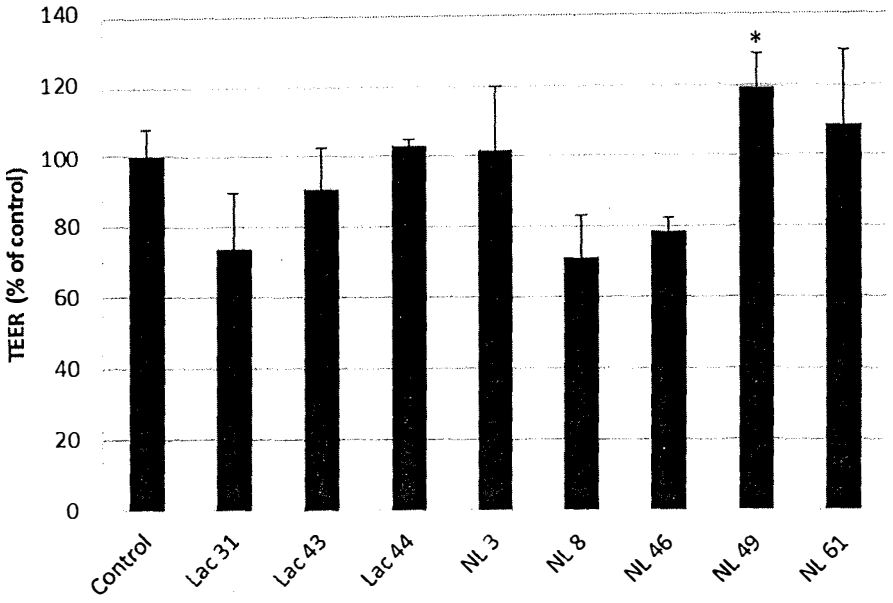


Figure 3. Change in the TEER by *Lactobacillus* spp. isolated from breast milk across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 5. The effect of *Lactobacillus* spp. isolated from gastric biopsy on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	103.07	18.08	100	
B37 (*L. salivarius*)	107.25	10.04	104.06	0.3720
B57 (*L. murinus*)	135.74	1.82	131.70	0.0179 *
B60 (*L. salivarius*)	114.62	5.99	111.21	0.1764
B74 (*L. salivarius*)	98.67	13.41	95.73	0.3760
B90 (*L. plantarum*)	94.93	8.30	92.10	0.2588
B101 (*L. salivarius*)	123.97	18.50	120.28	0.1171
B103 (*L. casei*)	120.89	14.12	117.29	0.1248
B106 (*L. casei*)	123.53	16.03	119.85	0.1082

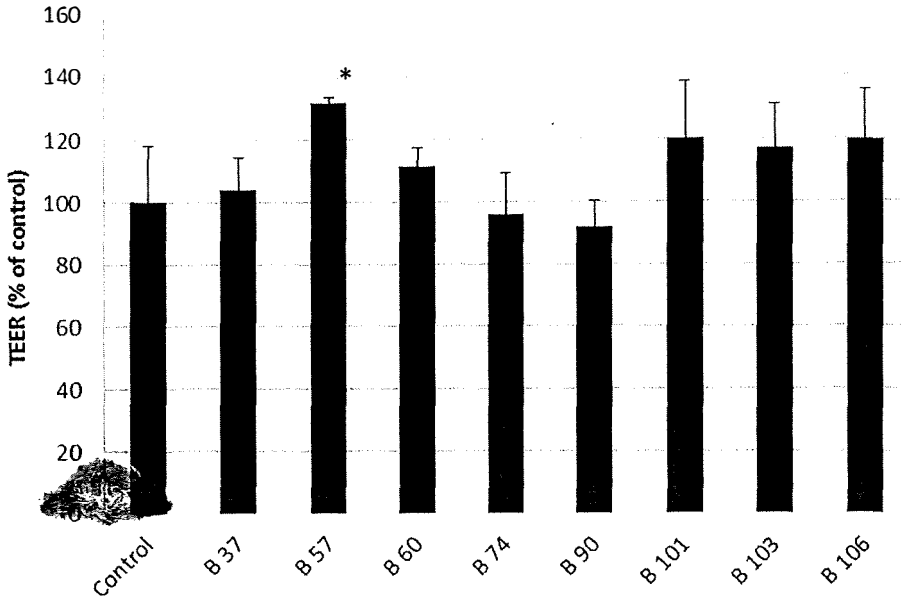


Figure 4. Change in the TEER by *Lactobacillus* spp. isolated from gastric biopsies across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 6. Selected *Lactobacillus* spp. for further study

***Lactobacillus* spp. which increased TEER significantly**	***Lactobacillus* spp. which non-significantly increased TEER and suppressed *C. difficile*-induced IL-8 production**	***Lactobacillus* spp. which decreased TEER and suppressed *C. difficile*-induced IL-8 production**
L. fermentum L12 (LF-L12)	*L. gasseri* L3 (LG-L3)	*L. rhamnosus* L34 (LR-L34)
L. oris NL49 (LO-NL49)	*L. plantarum* XB7 (LP-XB7)	*L. casei* L39 (LC-L39)
L. murinus B57 (LM-B57)	*L. salivarius* B37 (LS-B37)	*L. plantarum* B90 (LP-B90)
	L. salivarius B60 (LS-B60)	

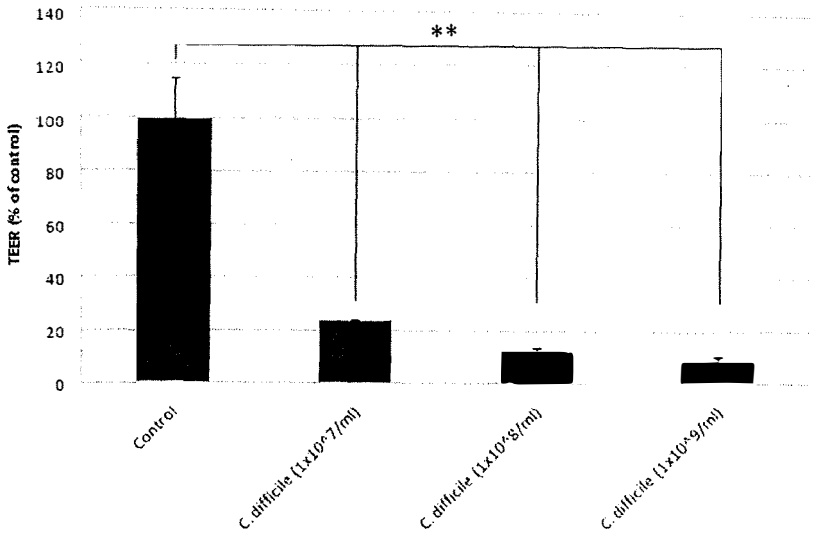


Figure 5. The effects of *Clostridium difficile* on tight junctions in Caco-2 human colorectal adenocarcinoma cells. SD, standard deviation; significantly lower than the control (DMEM, Caco-2 media control), ** $p<0.01$. The experiments were performed once in duplicate.

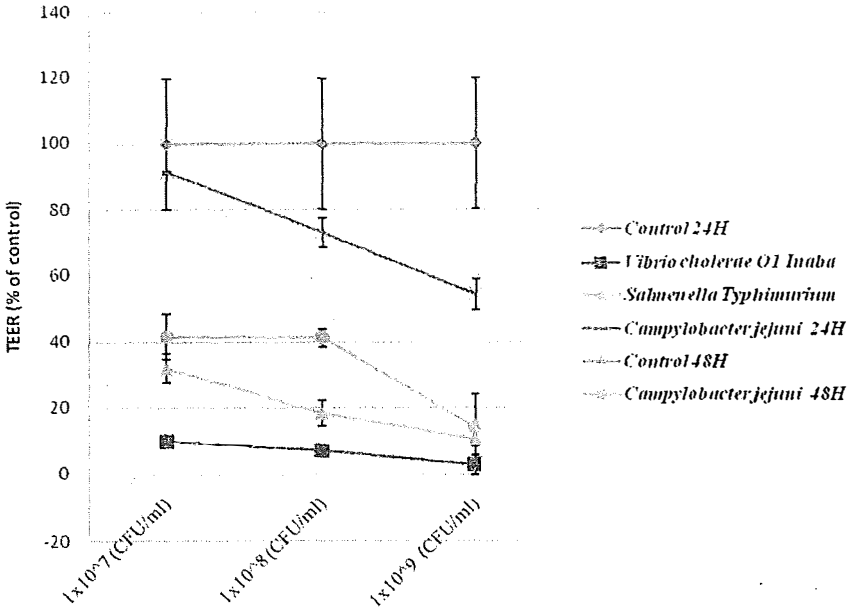


Figure 6. The effects of *Vibrio cholerae* 01 Inaba, *Salmonella* Typhimurium ATCC 13311 and *Campylobacter jejuni* on tight junctions in Caco-2 human colorectal adenocarcinoma cells. SD, standard deviation; significantly lower than the control (DMEM, Caco-2 media control), * $p<0.05$, ** $p<0.01$. The experiments were performed once in duplicate.

Table 7. The enhancement effects of *Lactobacillus* spp. on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed three times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	138.77	2.57	100	
L12+*C. difficile*	16.17	0.47	11.65	0.0016**
NL49+*C. difficile*	16.34	0.23	11.77	0.0006***
XB7+*C. difficile*	17.82	1.40	12.84	0.0075**
B37+*C. difficile*	16.34	0.23	11.77	0.0006***
B57+*C. difficile*	19.14	0.47	13.79	0.0008***
B60+*C. difficile*	14.85	1.87	10.70	0.0307*
B90+*C. difficile*	13.53	3.73	9.75	0.1439
L3+ *C. difficile*	16.34	0.23	11.77	0.0006***
L34+ *C. difficile*	14.03	0.23	10.11	0.0015**
L39+ *C. difficile*	14.03	0.23	10.11	0.0015**
C. difficile	9.74	0.23	7.02	1.0E-04

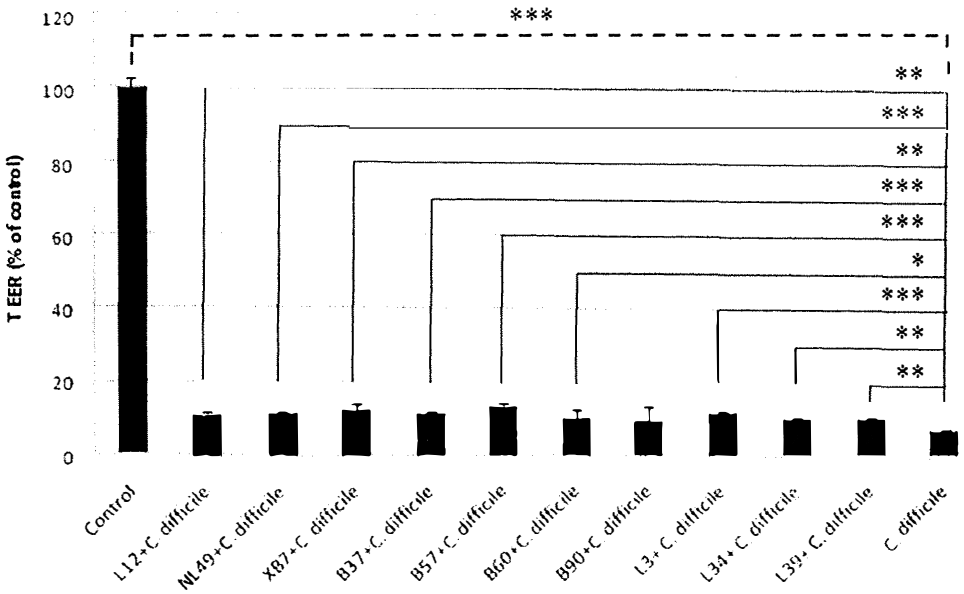


Figure 7. The enhancement effects of *Lactobacillus* spp. on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *p<0.05, **p<0.01, ***p<0.001

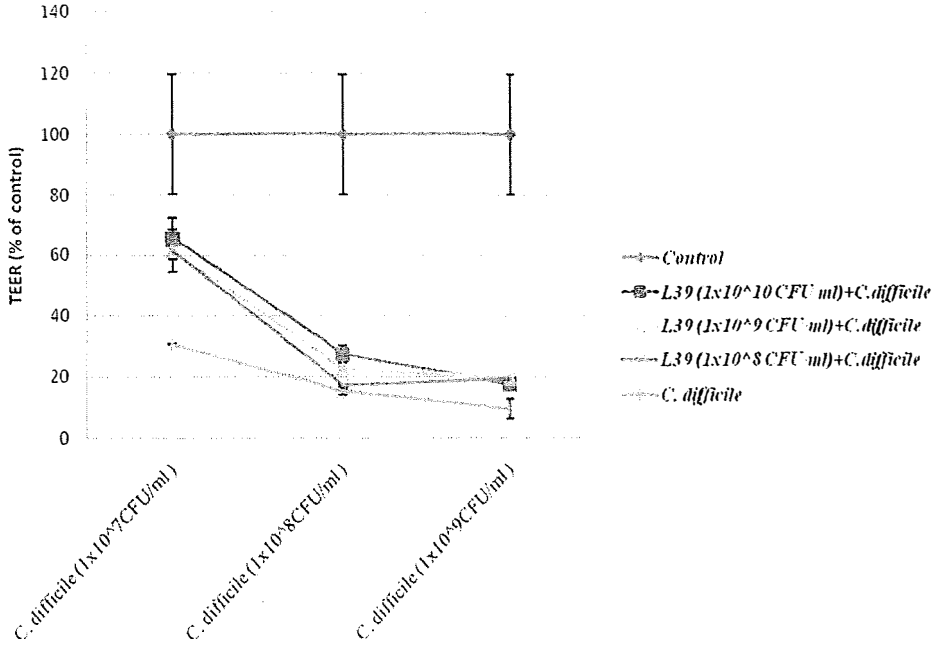


Figure 8. TEER from different proportion of *Lactobacillus casei*-L39 and *Clostridium difficile*. The experiments were performed once in duplicate.

Table 8. The enhancement effects of *Lactobacillus rhamnosus* L34 on transepithelial electrical resistance (TEER) when added before *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average (Ω.cm2)	**SD**		
Control	104.78	10.31	100	
C. difficile	23.72	12.17	22.64	4.475E-10***
L34 ($1x10^8$)	115.95	21.24	110.67	0.1009489
L34($1x10^8$)+ *C. difficile*($1x10^7$)	67.77	25.45	64.69	0.0002926***
C. difficile($1x10^7$)+ L34($1x10^8$)	38.53	18.83	36.77	0.0413885*

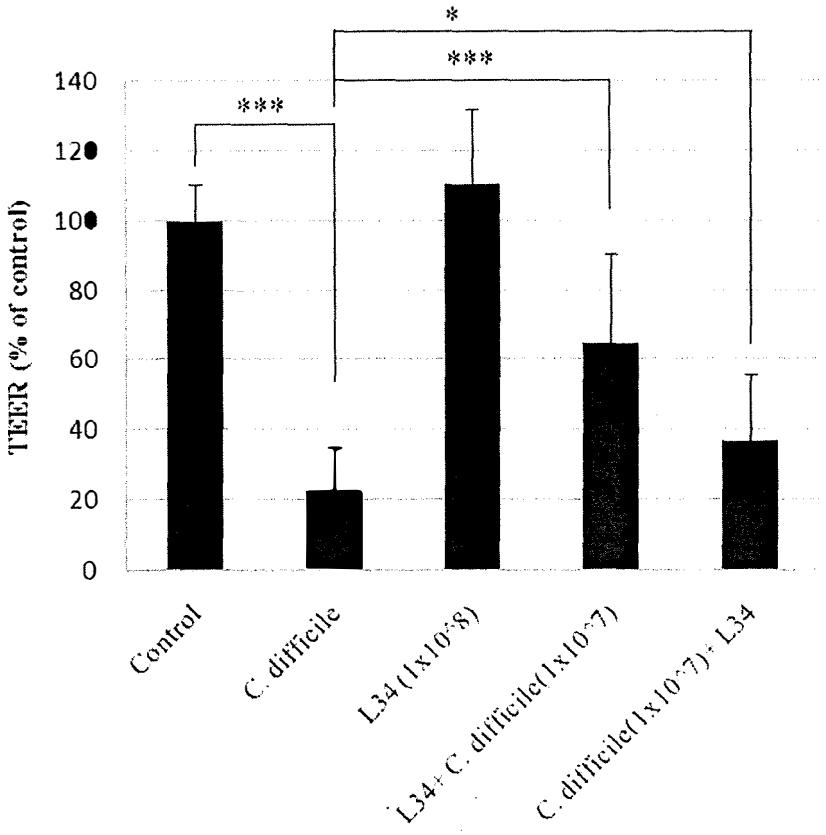


Figure 9. The enhancement effects of LR-L34 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 9. The effects of *Lactobacillus casei* L39 on transepithelial electrical resistance (TEER) when added before and after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	80.19	4.20	100	
C. difficile	11.88	2.80	14.81	0.0014**
L39 ($1x10^8$)	90.42	12.13	112.76	0.1884
L39($1x10^8$)+*C.difficile*($1x10^7$)	18.65	0.23	23.25	0.0382*
C. difficile($1x10^7$)+ L39($1x10^8$)	19.14	0.47	23.87	0.0343*

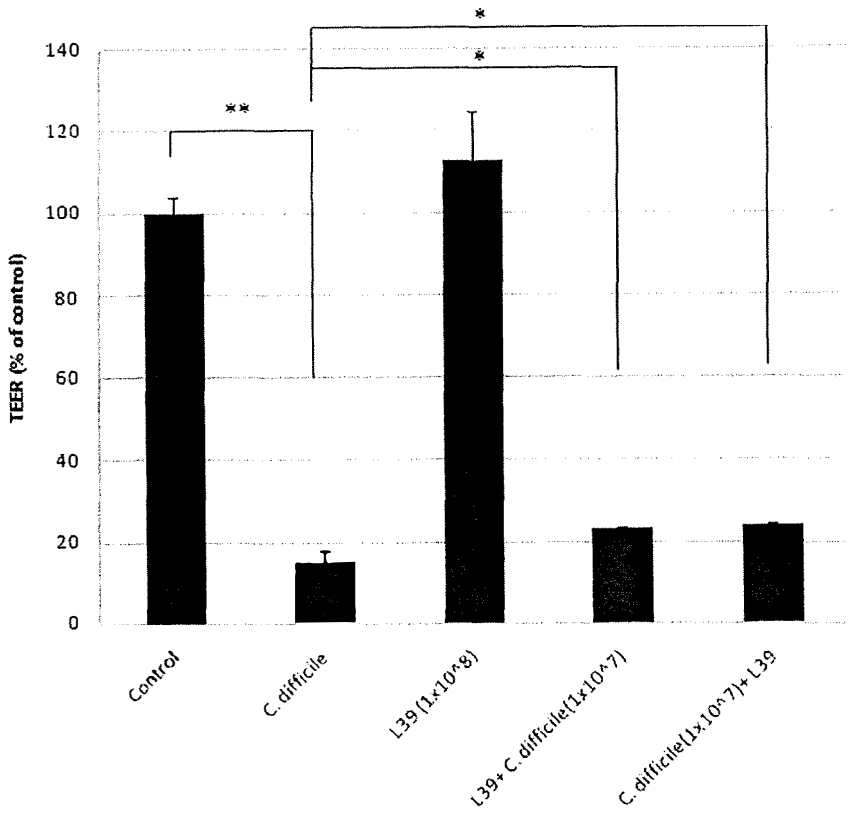


Figure 10. The enhancement effects of LC-L39 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, $*p<0.05$, $**p<0.01$

Table 10. The effects of *Lactobacillusi salivarius* B37 on transepithelial electrical resistance (TEER) when added before and after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	80.19	4.20	100	
C. difficile	11.88	2.80	14.81	0.0014**
B37 ($1x10^8$)	102.14	4.43	127.37	0.0183*
B37($1x10^8$)+ *C. difficile*($1x10^7$)	20.63	2.10	25.72	0.0358*
C. difficile($1x10^7$)+ B37($1x10^8$)	15.35	0.23	19.14	0.1116

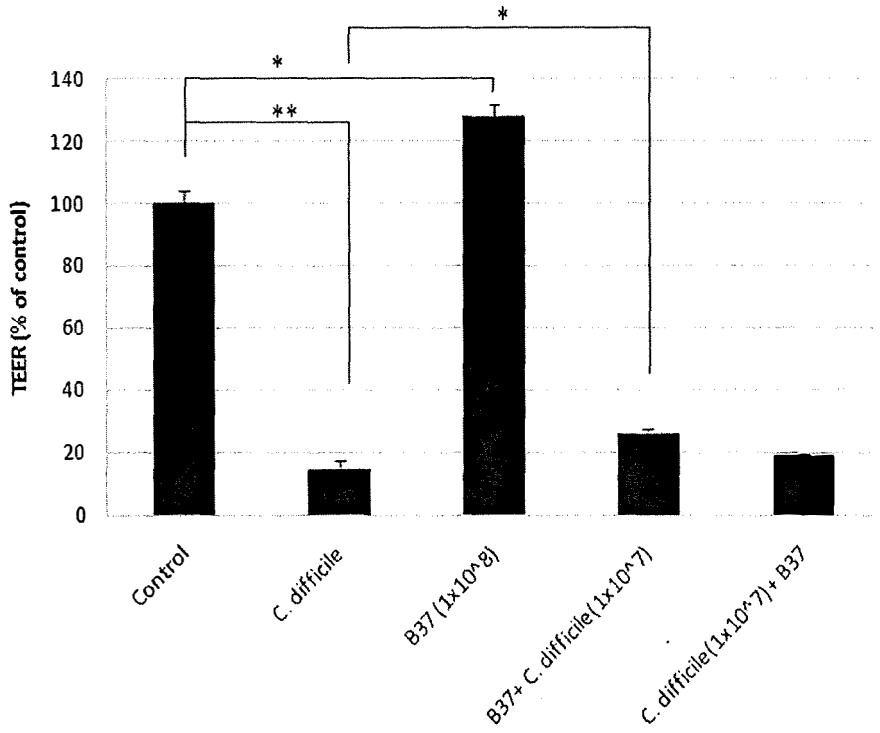


Figure 11. The enhancement effects of LS-B37 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, $*p<0.05$, $**p<0.01$

Table 11. The effects of *Lactobacillusi fermentum* XB7 on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	80.19	4.20	100	
C. difficile	11.88	2.80	14.81	0.0014**
XB7 ($1x10^8$)	53.79	1.87	67.08	0.0074
XB7($1x10^8$)+ *C. difficile*($1x10^7$)	20.30	0.23	25.31	0.0257*
C. difficile($1x10^7$)+ XB7($1x10^8$)	13.53	3.27	16.87	0.3210

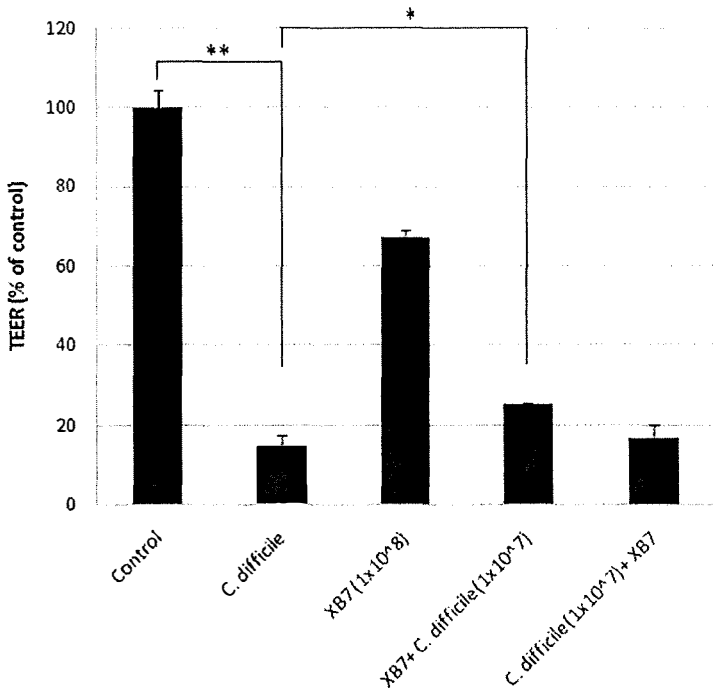


Figure 12. The enhancement effects of LF-XB7 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, $*p<0.05$, $**p<0.01$

Table 12. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average (Ω.cm2)	SD		
Control	105.91	12.76	100	
C. difficile	24.05	11.10	22.71	8.39E-19***
Live L34 ($1x10^8$)	115.95	21.24	109.48	0.0801
UV-treated L34 ($1x10^8$)	86.63	16.24	81.79	0.0021
Live L34($1x10^8$) + *C. difficile*($1x10^7$)	67.77	25.45	63.99	2.88E-06***
UV-treated L34 ($1x10^8$) + *C. difficile*	36.18	12.89	34.16	0.0128*
C. difficile($1x10^7$)+ LiveL34($1x10^8$)	38.53	18.83	36.38	0.0131*

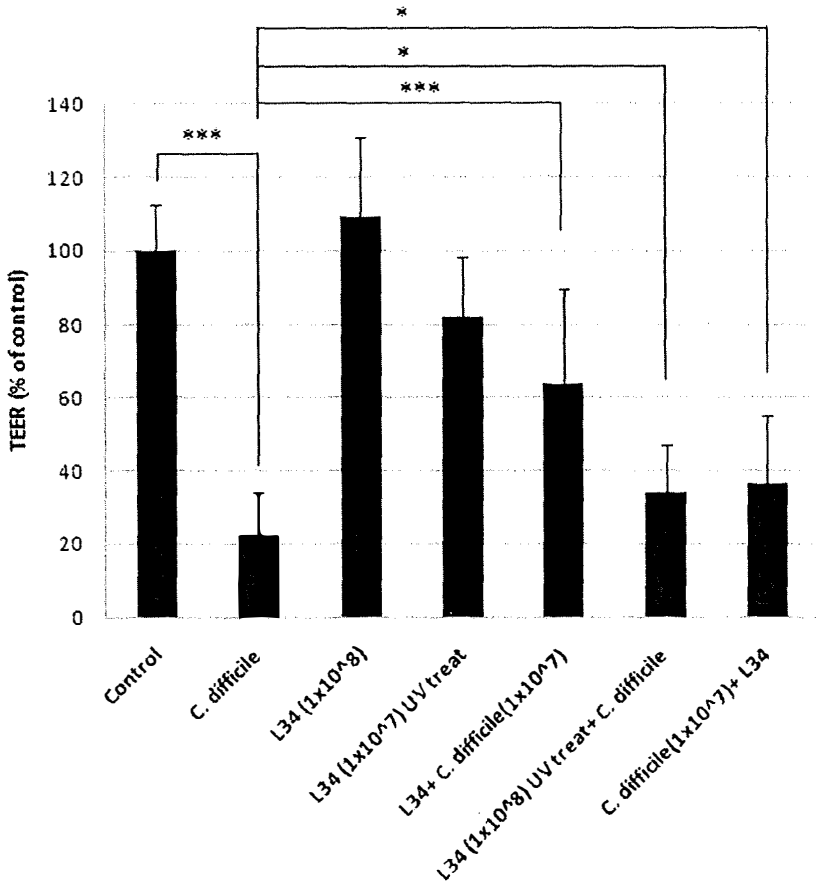


Figure 13. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate. $*p<0.05$, $***p<0.001$

Table 13. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Vibrio cholerae* O1 Inaba in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average (Ω.cm2)	SD		
Control	111.54	6.53	100	
V. cholerae	3.30	0.47	2.96	0.00091***
Live L34 ($1x10^8$)	116.00	17.03	103.99	0.38139
UV-treated L34 ($1x10^8$)	77.55	20.07	69.53	0.07522
Live L34($1x10^8$)+ *V. cholerae*($1x10^7$)	2.64	0.47	2.37	0.14645
UV-treated L34 ($1x10^8$) + *V. cholerae*($1x10^7$)	2.48	0.23	2.22	0.07742

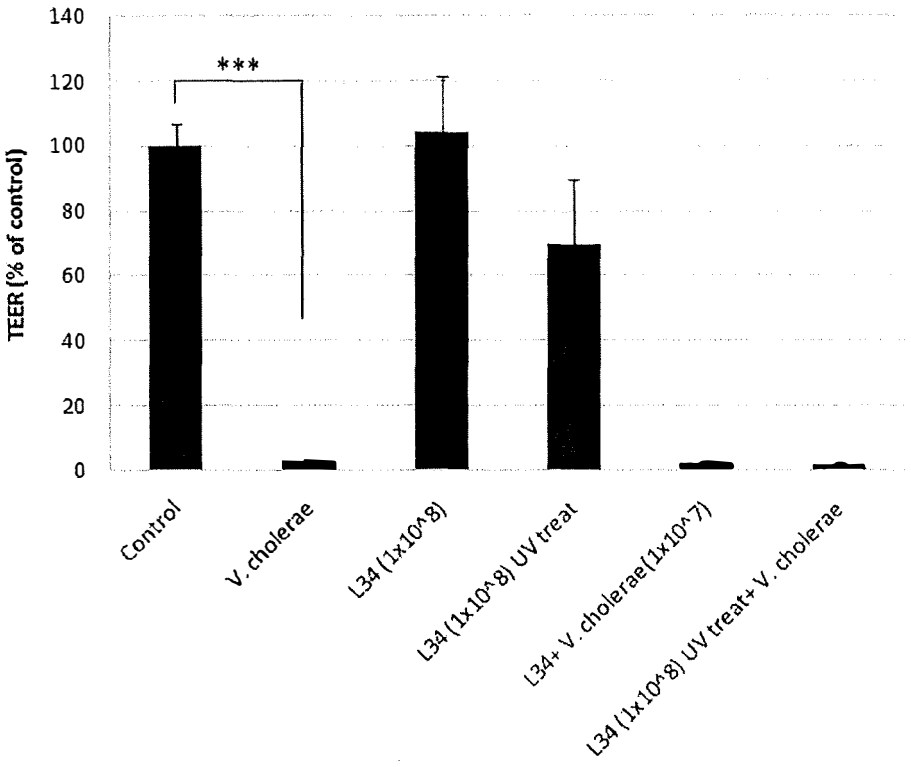


Figure 14. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Vibrio cholerae* O1 Inaba in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate. ***$p<0.001$

Table 14. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Salmonella* Typhimurium in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average (Ω.cm2)	**SD**		
Control	92.90	6.30	100	
S. Typhimurium	28.55	0.23	30.73	0.00238**
Live L34 ($1x10^8$)	87.29	0.70	93.96	0.16864
UV-treatedL34 ($1x10^8$)	89.43	7.93	96.27	0.33819
Live L34($1x10^8$) + *S.* Typhimurium($1x10^7$)	30.53	1.63	32.86	0.11589
UV-treated L34 ($1x10^8$) + *S.* Typhimurium($1x10^7$)	28.55	1.17	30.73	0.50000

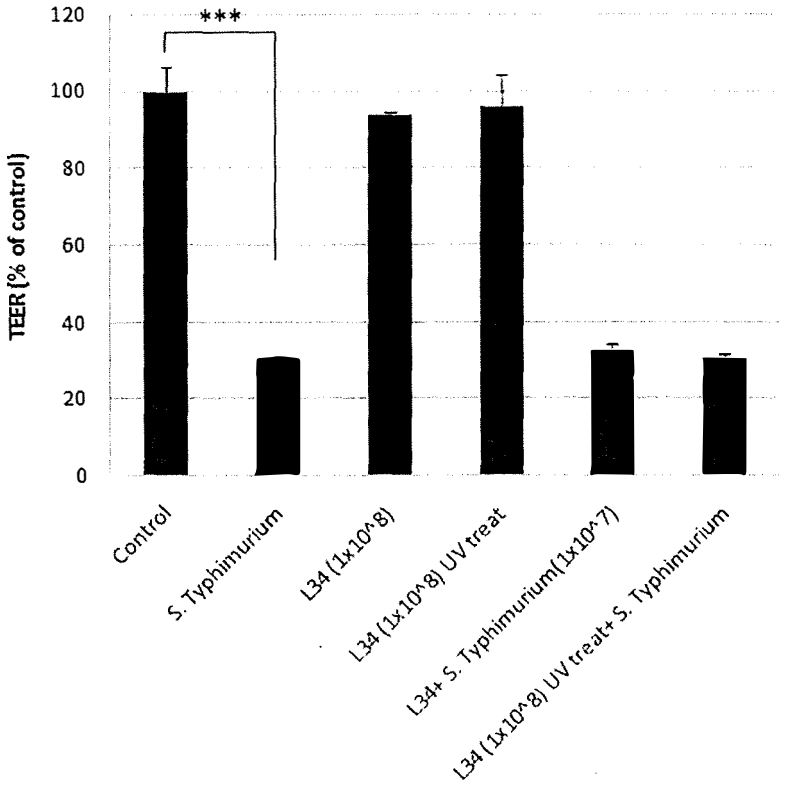


Figure 15. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Salmonella* Typhimurium in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate. ***p<0.001

Table 15. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Campylobacter jejuni* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed three times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours)		TEER (%)	*p*-value
	TEER average (Ω.cm2)	SD		
Control	101.97	11.20	100	
C. jejuni	51.15	2.33	50.16	0.01221*
Live L34 ($1x10^8$)	118.31	2.10	116.02	0.08993
UV-treated L34 ($1x10^8$)	94.88	3.03	93.04	0.23918
Live L34($1x10^8$) + *C. jejuni*($1x10^7$)	70.79	0.23	69.42	0.00353**
UV-treated L34 ($1x10^8$) + *C. jejuni*($1x10^7$)	96.36	3.73	94.50	0.00235**

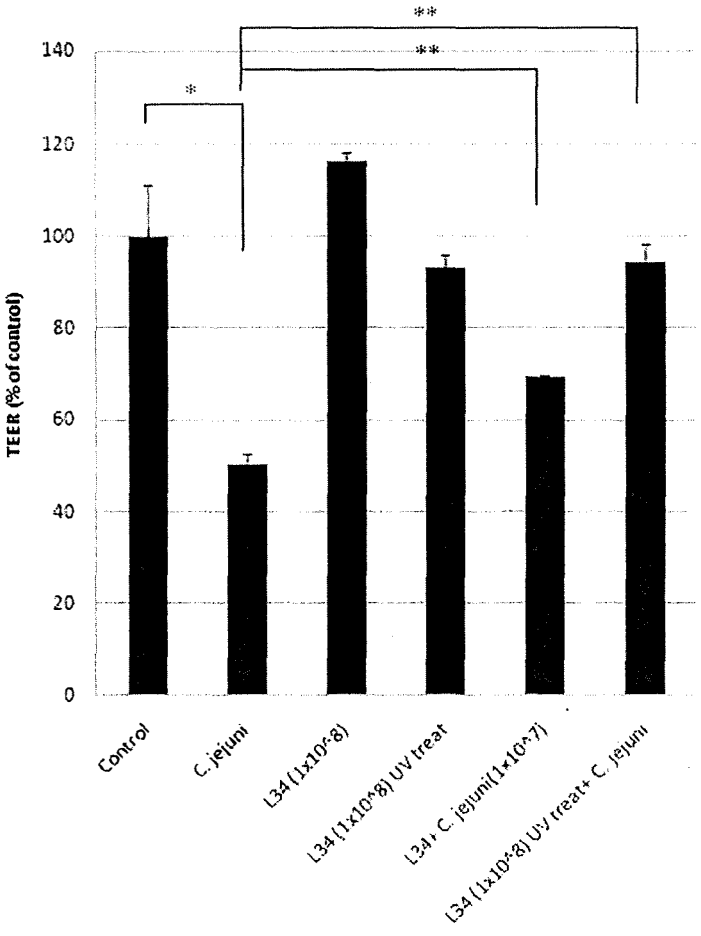


Figure 16. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *Campylobacter jejuni* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed three times in duplicate. *p<0.05, **p<0.01

Table 16. The enhancement effects of *Lactobacillus* spp. on transepithelial electrical resistance (TEER) when co-culture with *Helicobacter pylori* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed three times in duplicate.

Identity	Transepithelial electrical resistance (TEER) value at 24 hours)		TEER (%)	*p*-value
	TEER average (Ω.cm2)	SD		
Control	108.18	21.49	100	
XB7+*H. pylori*	37.39	7.47	34.57	0.152294
B37+*H. pylori*	30.05	8.20	27.78	0.047464
B57+*H. pylori*	38.24	13.68	35.35	0.183277
B60+*H. pylori*	33.41	18.71	30.89	0.400031
B78+*H. pylori*	38.94	8.04	36.00	0.154487
Helicobacter pylori	34.71	7.05	32.09	3.71E-14***

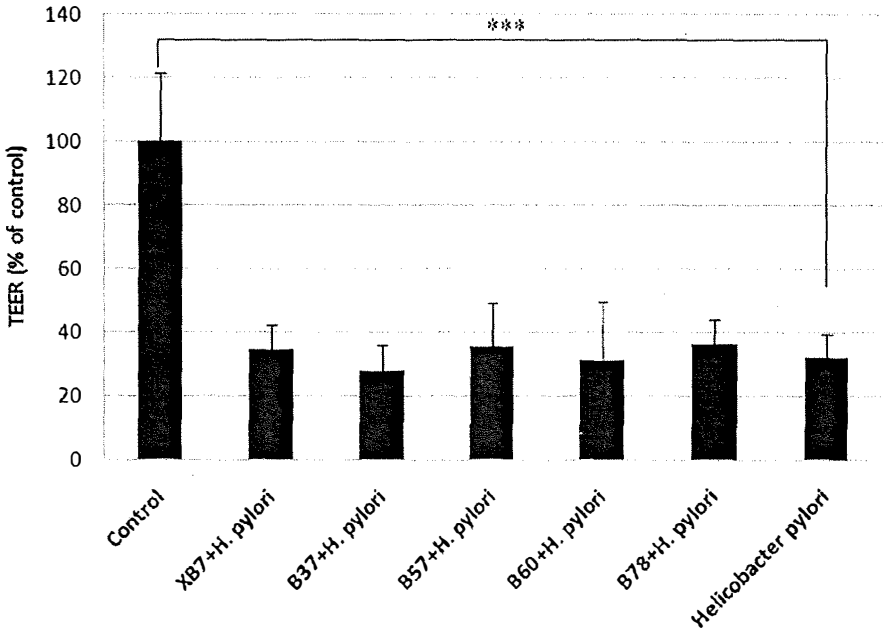
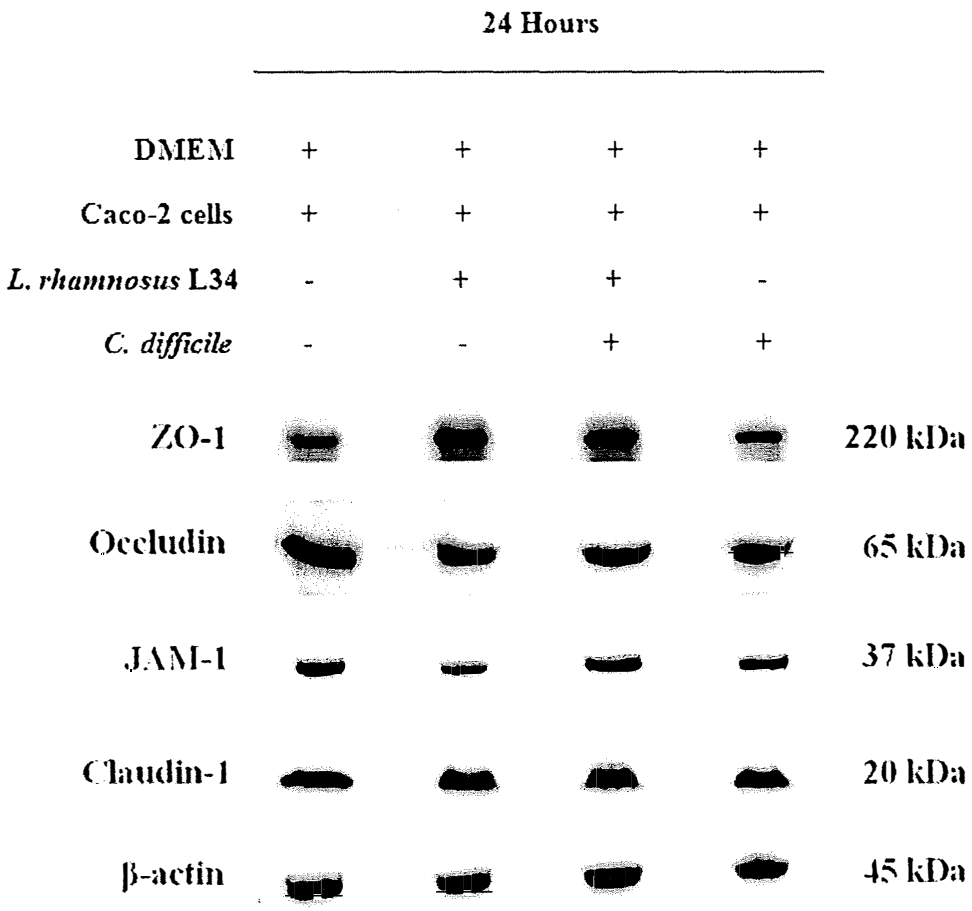


Figure 17. The enhancement effects of *Lactobacillus* spp. on transepithelial electrical resistance (TEER) when co-culture with *Helicobacter pylori* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, ***$p<0.001$

A


24 Hours
DMEM + + + +
Caco-2 cells + + + +
L. rhamnosus L34 - + + -
C. difficile - - + +
ZO-1
220 kDa
Occludin
65 kDa
JAM-1
37 kDa
Claudin-1
20 kDa
β-actin
45 kDa

B

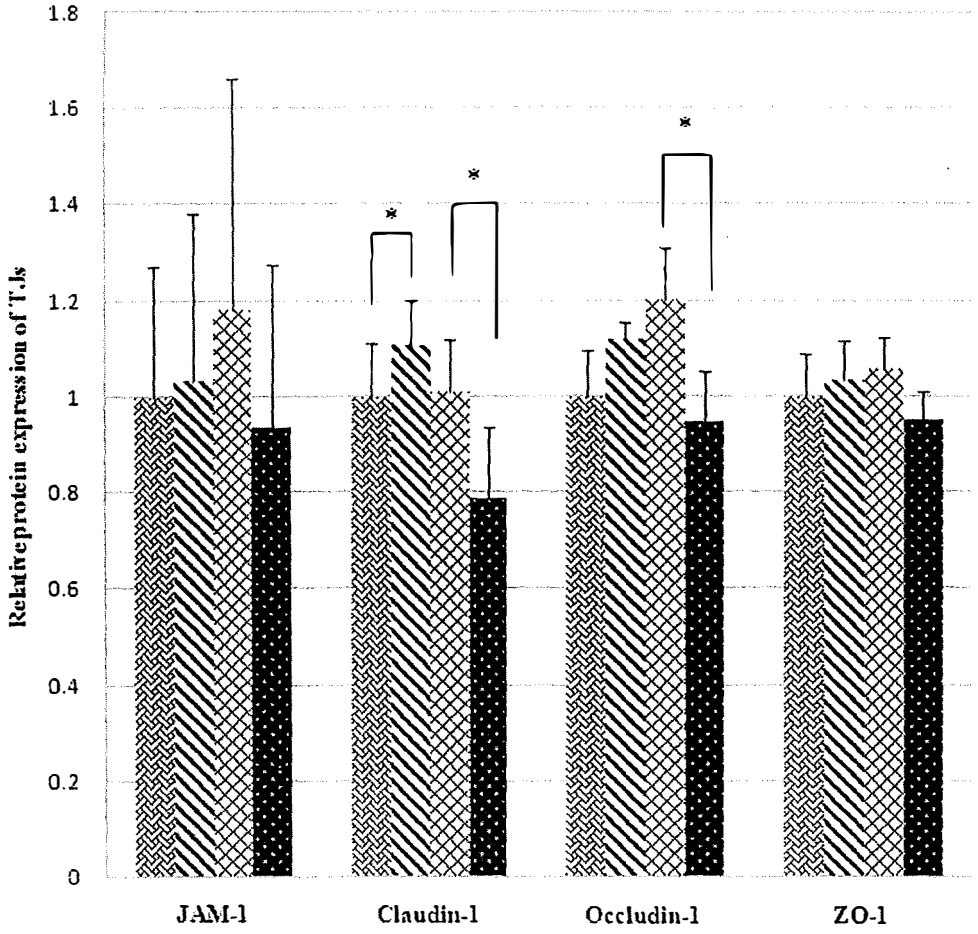


Figure 18. The expression of TJs proteins. A) Representative result of western blot analysis of JAM-1, Claudin-1, Occludin and ZO-1. B) Semi-quantitative analysis of western blot showed protein expression at different conditions. Values were calculated by Student's *t*-test.

Table 17. The effects of *Bifidobacterium* spp. isolated from breast milk and infant feces on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.
TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	131.92	8.00	100	
NB2 *(B. pseudocatenulatum)*	98.18	14.35	74.42	0.0197
NB45 *(B.pseudocatenulatum)*	99.66	10.89	75.55	0.0142
NB14 *(B. dentium)*	91.91	7.09	69.67	0.0040
B36 *(B. longum)*	102.47	4.79	77.67	0.0087
B57 *(B. pseudocatenulatum)*	102.96	0.99	78.05	0.0071

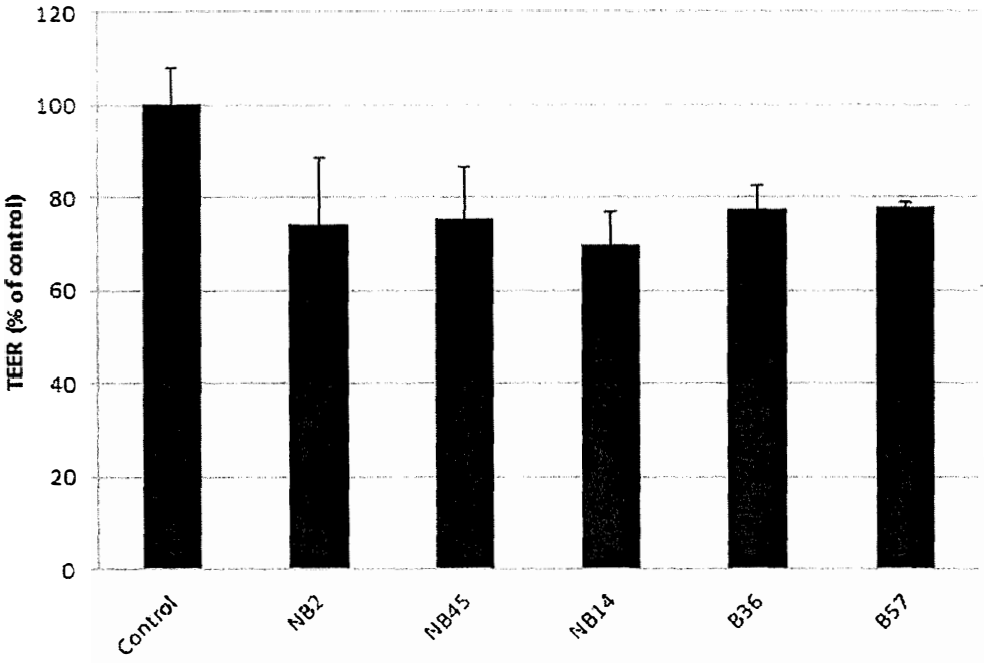


Figure 19. Change in the TEER by *Bifidobacterium* spp. isolated from breast milk and infant feces across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 18. The effects of *Bifidobacterium* spp. isolated from breast milk and infant feces on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.
TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	160.71	2.80	100	
Bif29 *(B. breve)*	145.70	1.63	90.66	0.0113
NB11 *(B. dentium)*	122.43	30.80	76.18	0.1111
NB13 *(B. bifidum)*	123.26	1.17	76.69	0.0016
B11 *(B. pseudocatenulatum)*	83.82	13.07	52.16	0.0074
B9 *(B. longum)*	129.20	1.17	80.39	0.0023
B14 *(B. adolescentis)*	124.58	2.10	77.52	0.0023
B38 *(B. catenulatum)*	115.67	2.57	71.97	0.0018

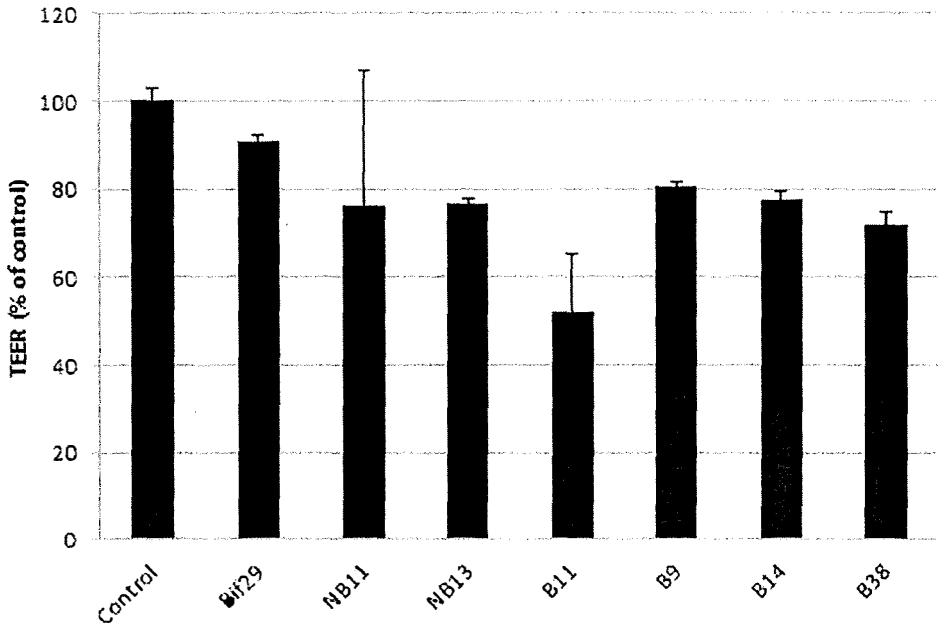


Figure 20. Change in the TEER by *Bifidobacterium* spp. isolated from breast milk and infant feces across Caco-2 human colorectal adenocarcinoma cells. (*p<0.05)

Table 19. The effects of *Bifidobacterium* spp. isolated from breast milk and infant feces on transepithelial electrical resistance (TEER) in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.
TEER ($\Omega.cm^2$monolayer) = (Total resistance – Blank resistance) (Ω) x Area (cm^2)

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	84.65	10.97	100.00	
NB48 *(B. pseudocatenulatum)*	153.12	11.67	180.90	0.0131*
NB42 *(B. bifidum)*	150.98	3.50	178.36	0.0074**
NB38*(B. catenulatum)*	130.85	24.50	154.58	0.0677
B103*(B. longum)*	112.70	19.83	133.14	0.1111
B24 *(B. adolescentis)*	94.05	3.27	111.11	0.1825

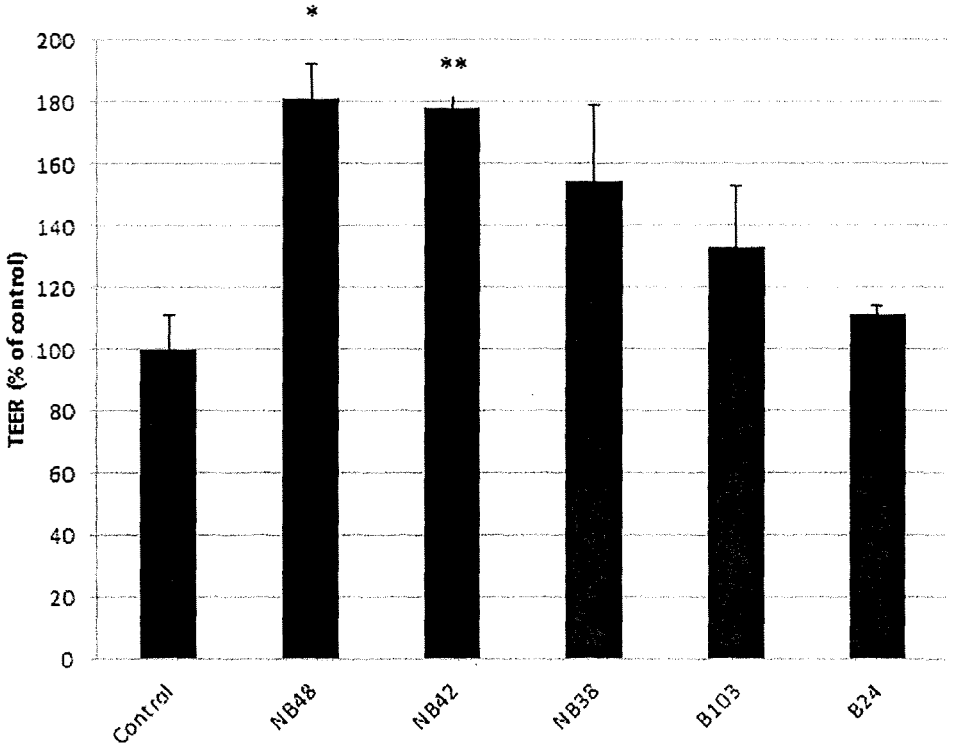


Figure 21. Change in the TEER by *Bifidobacterium* spp. isolated from breast milk and infant
feces across Caco-2 human colorectal adenocarcinoma cells.(*p<0.05, **p<0.01)

Table 20. *Bifidobacterium* spp. which increased intestinal epithelial resistance of Caco-2 cells and were chosen for further investigation

Bifidobacterium spp. which increased TEER significantly	*Bifidobacterium* spp. which increased TEER non- significantly
B. bifidum NB42 (BB-NB42) *B. pseudocatenulatum* NB48 (BP-NB48)	*B. adolescentis* B24 (BA-B24) *B. catenulatum* NB38 (BC-NB38) *B. longum* B103 (BL-B103)

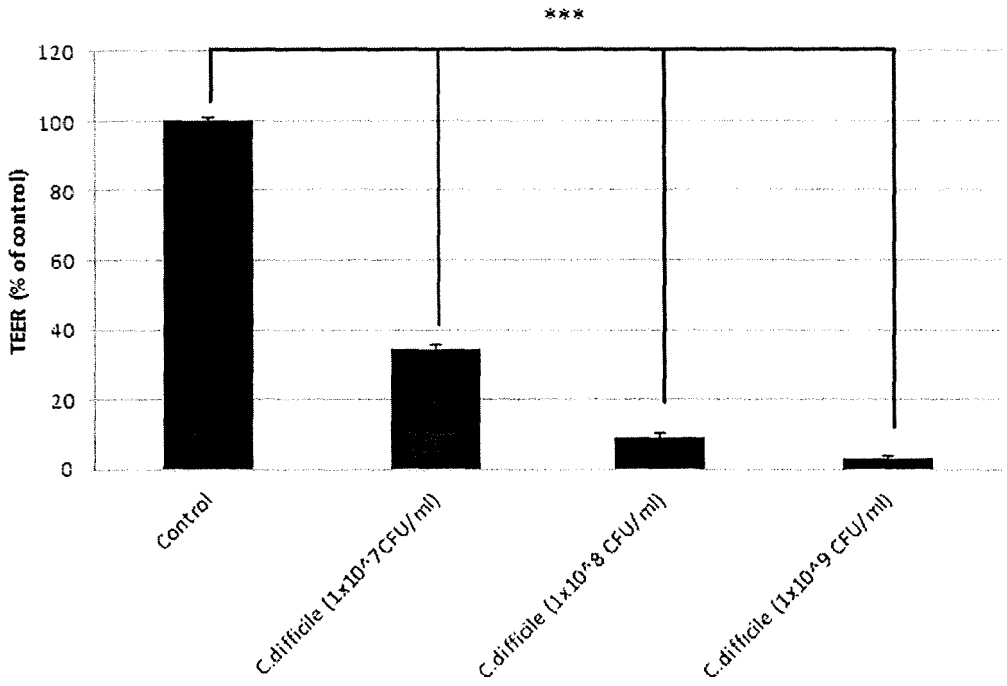


Figure 22. Effect of *Clostridium difficile* on resistance in Caco-2 human colorectal adenocarcinoma cells. SD, standard deviation; significantly lower than the control (DMEM, Caco-2 media control), *** $p<0.001$.The experiments were performed once in duplicate.

Table 21. The enhancement effects of *Bifidobacterium* spp. on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	114.76	23.62	100	
C. difficile	13.37	2.33	11.65	0.00007***
NB42 + *C. difficile*	44.47	17.69	38.75	0.0065**
B24 + *C. difficile*	24.42	7.00	21.28	0.0121*
NB48 + *C. difficile*	23.60	8.42	20.56	0.0288*
NB38 + *C. difficile*	19.47	5.99	16.97	0.0531
B103 +*C. difficile*	18.65	6.15	16.25	0.0796

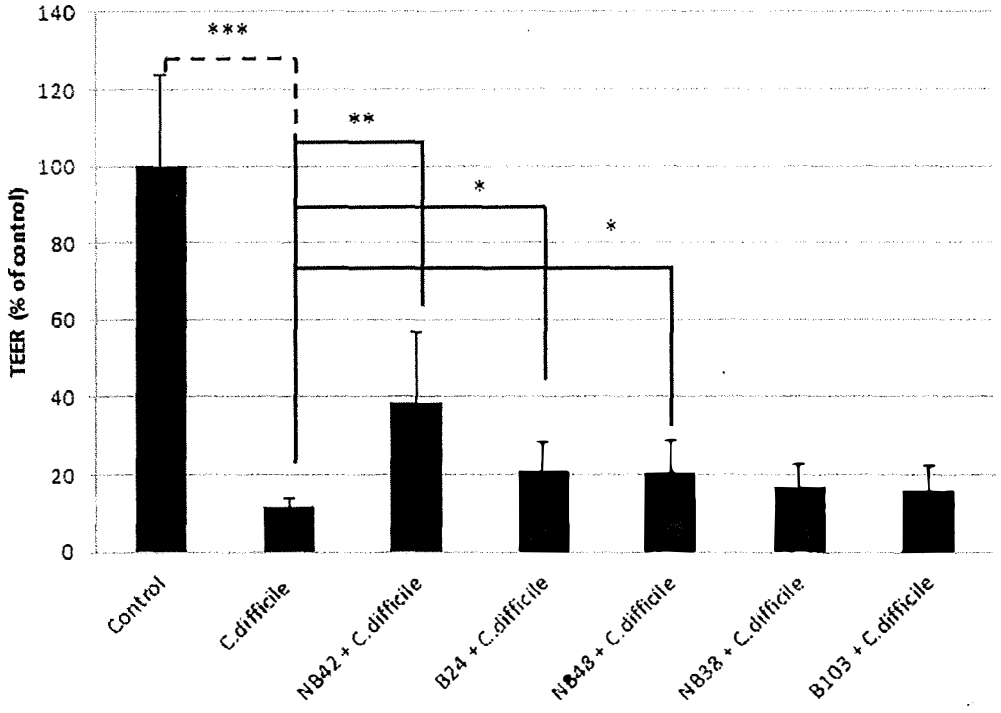


Figure 23. The enhancement effects of *Bifidobacterium* spp. on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *p<0.05, **p<0.01, ***p<0.001

Table 22. The effects of BB-NB42 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	98.59	4.9049	100	
C. difficile	19.31	6.8031	19.58	0.00000071***
NB42 ($1x10^9$)	129.69	35.4654	131.55	0.0665
C.difficile ($1x10^8$) + NB42 ($1x10^9$)	20.79	2.1044	21.09	0.3456

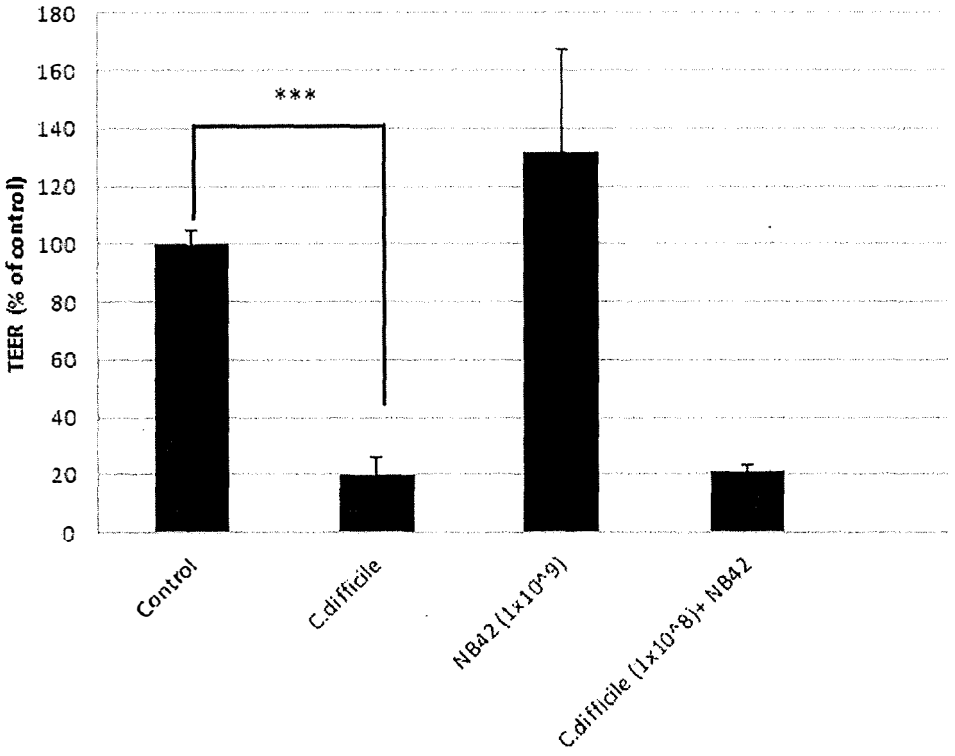


Figure 24. The enhancement effects of BB-NB42 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, $^{**}p<0.01$, $^{***}p<0.001$

Table 23. The effects of BA-B24 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	98.59	4.9049	100	
C. difficile	19.31	6.8031	19.58	0.00000071***
B24 ($1x10^9$)	115.25	26.4225	116.90	0.1306
C. difficile ($1x10^8$) + B24 ($1x10^9$)	15.43	2.5042	15.65	0.1629

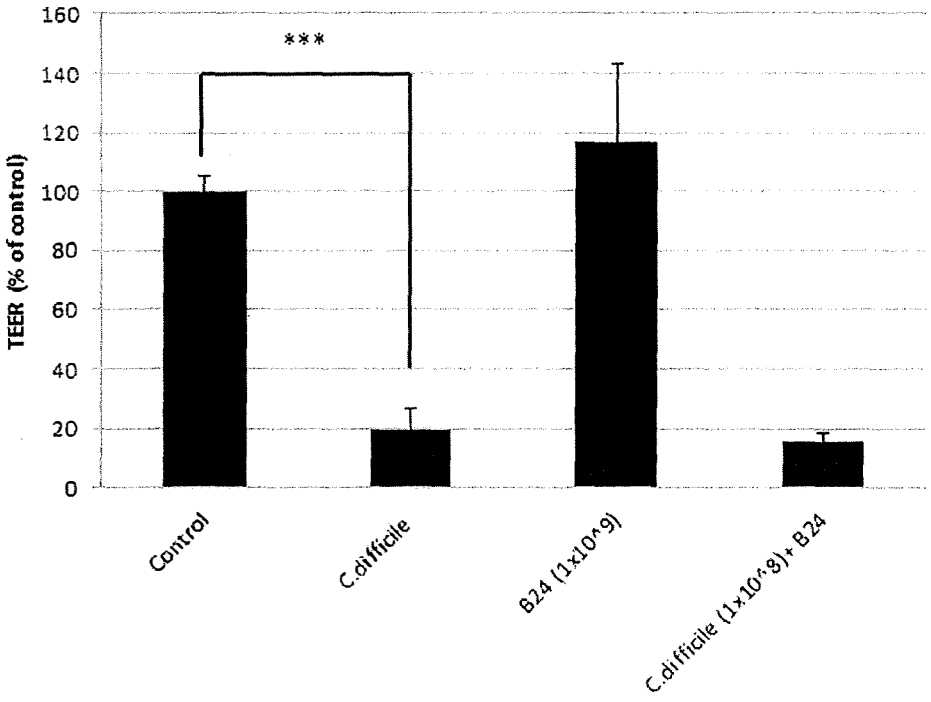


Figure 25. The enhancement effects of BA-B24 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, ***$p<0.001$

Table 24. The effects of BP-NB48 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	98.59	4.9049	100	
C. difficile	19.31	6.8031	19.58	0.00000071***
NB48 ($1x10^9$)	81.59	20.3272	82.76	0.0776
C. difficile ($1x10^8$) + NB48 ($1x10^9$)	16.67	3.1249	16.90	0.2535

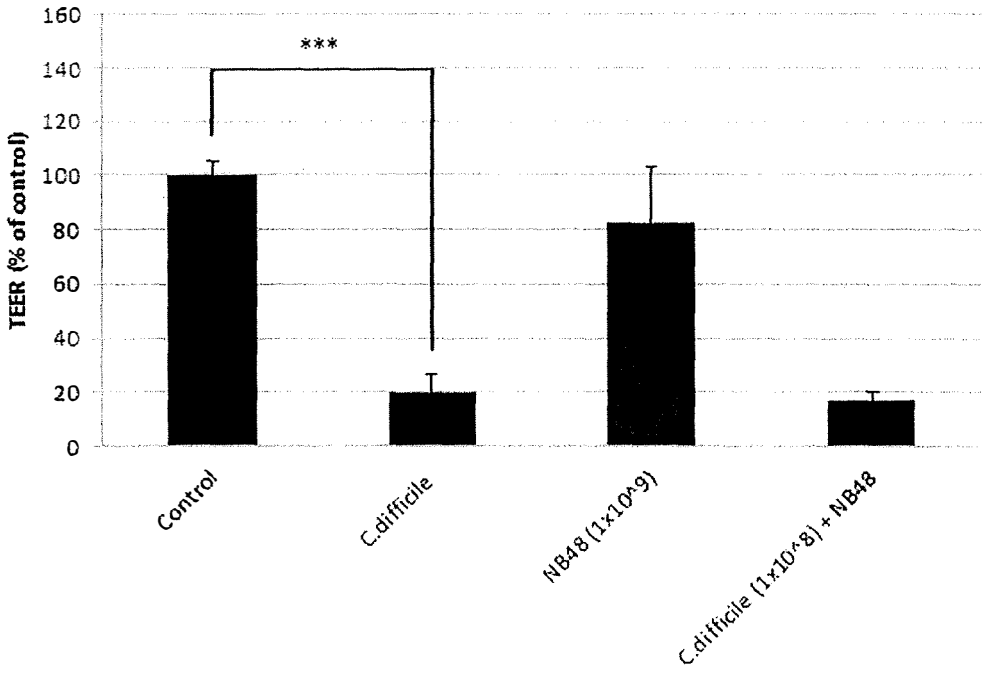


Figure 26. The enhancement effects of BP-NB48 on transepithelial electrical resistance (TEER) when added after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, ***$p<0.001$

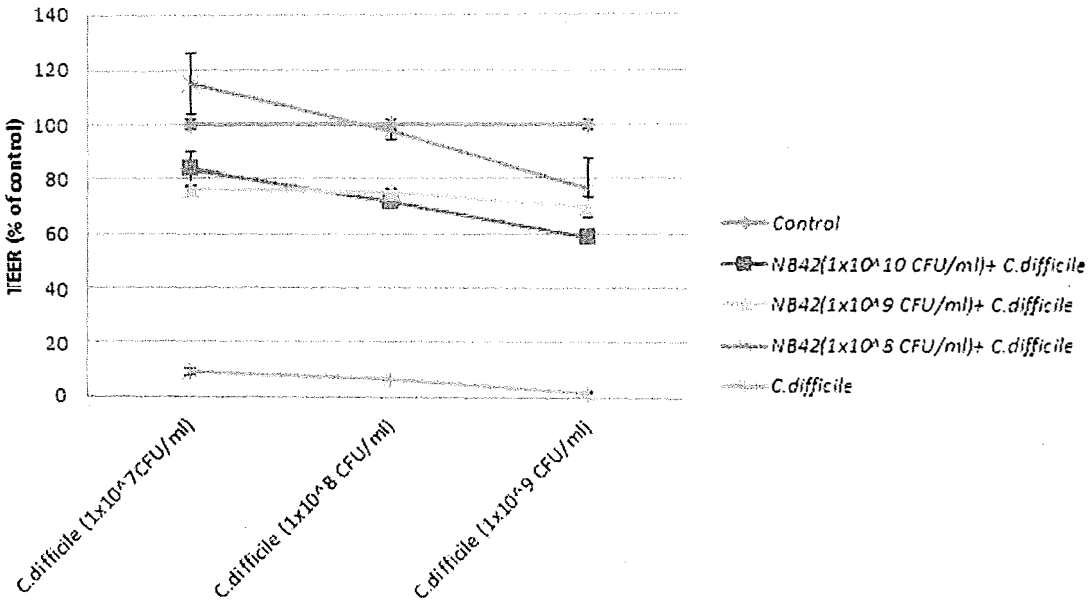


Figure 27. TEER from different proportion of BB-NB42 and *Clostridium difficile*. The experiments were performed once in duplicate.

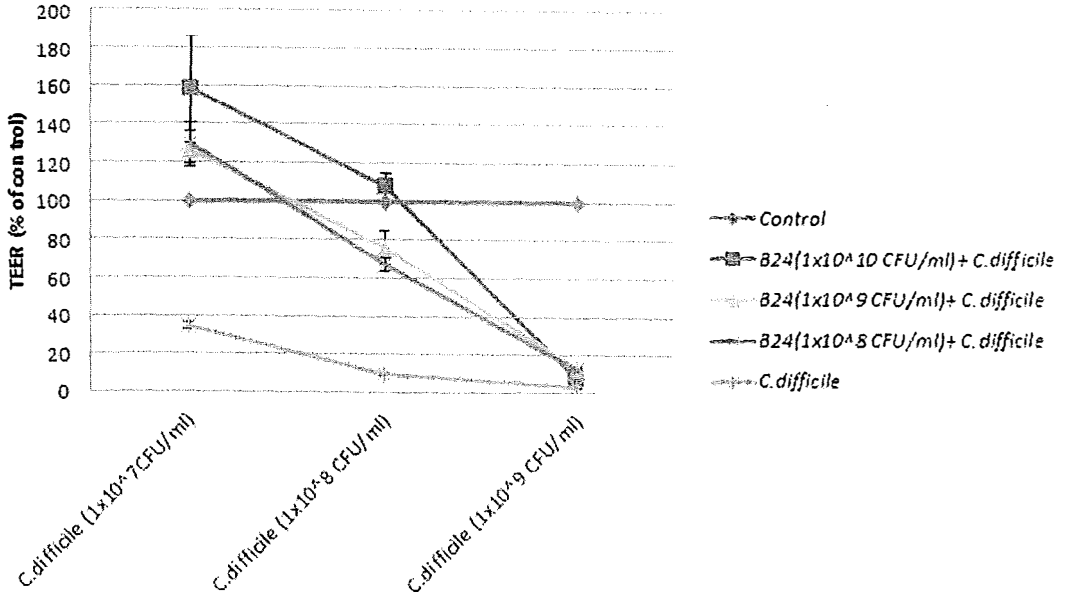


Figure 28. TEER from different proportion of BA-B24 and *Clostridium difficile*. The experiments were performed once in duplicate.

Table 25. The enhancement effects of live or UV-treated BB-NB42 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	81.39	7.5188	100	
C. difficile	45.42	7.0710	55.80	0.000000056***
Live NB42 ($1x10^8$)	99.50	17.5703	122.25	0.008972294**
UV-treated NB42 ($1x10^8$)	94.50	21.8833	116.12	0.065576293
NB42 ($1x10^8$) + *C. difficile* ($1x10^7$)	97.85	22.4129	120.22	0.000009618***
UV-treated NB42 ($1x10^8$) + *C. difficile*	91.66	16.5900	112.62	0.000002104***

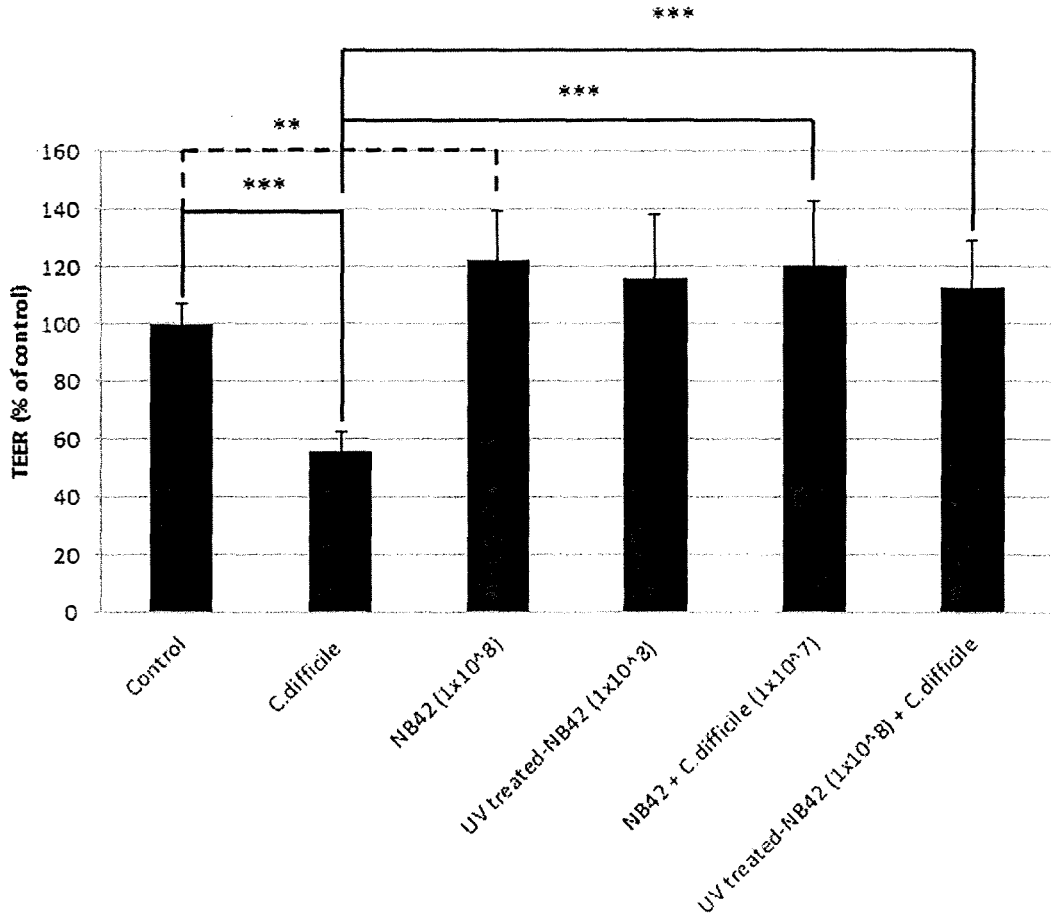


Figure 29. The enhancement effects of live or UV-treated BB-NB42 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate. $^{**}p<0.01$, $^{***}p<0.001$

Table 26. The enhancement effects of live or UV-treated BA-B24 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.

Subject	Transepithelial electrical resistance (TEER) value at 24 hours		TEER (%)	*p*-value
	TEER average ($\Omega.cm^2$)	SD		
Control	81.39	7.5188	100	
C. difficile	45.42	7.0710	55.80	0.000000056***
Live B24 ($1x10^8$)	102.14	21.7514	125.49	0.011558751*
UV-treated B24 ($1x10^8$)	89.38875	15.2978	109.83	0.102732924
B24 ($1x10^8$) + *C. difficile* ($1x10^7$)	96.23625	9.5772	118.25	0.000000004***
UV-treated B24 ($1x10^8$) + *C. difficile*	80.9325	8.6392	99.44	0.000000170***

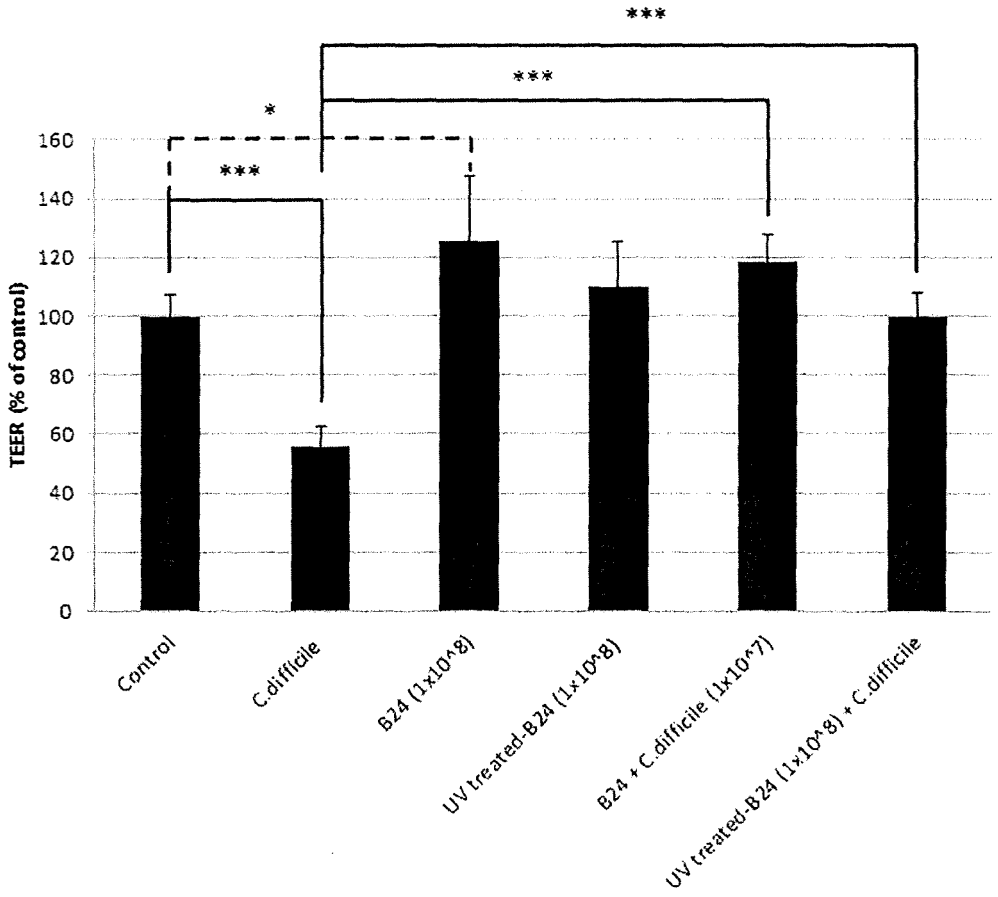


Figure 30. The enhancement effects of live or UV-treated BA-B24 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.*p<0.05, ***p<0.001

Table 27. The enhancement effects of live or UV-treated BP-NB48 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	81.39	7.5188	100	
C. difficile	45.42	7.0710	55.80	0.000000056***
Live NB48 ($1x10^8$)	90.92	22.6072	111.71	0.138480858
UV-treated NB48 ($1x10^8$)	78.13	10.1326	96.00	0.238565374
NB48 ($1x10^8$) + *C. difficile* ($1x10^7$)	97.39	19.7090	119.67	0.000003023***
UV-treated NB48 ($1x10^8$) + *C. difficile*	76.89	14.2551	94.48	0.000033057***

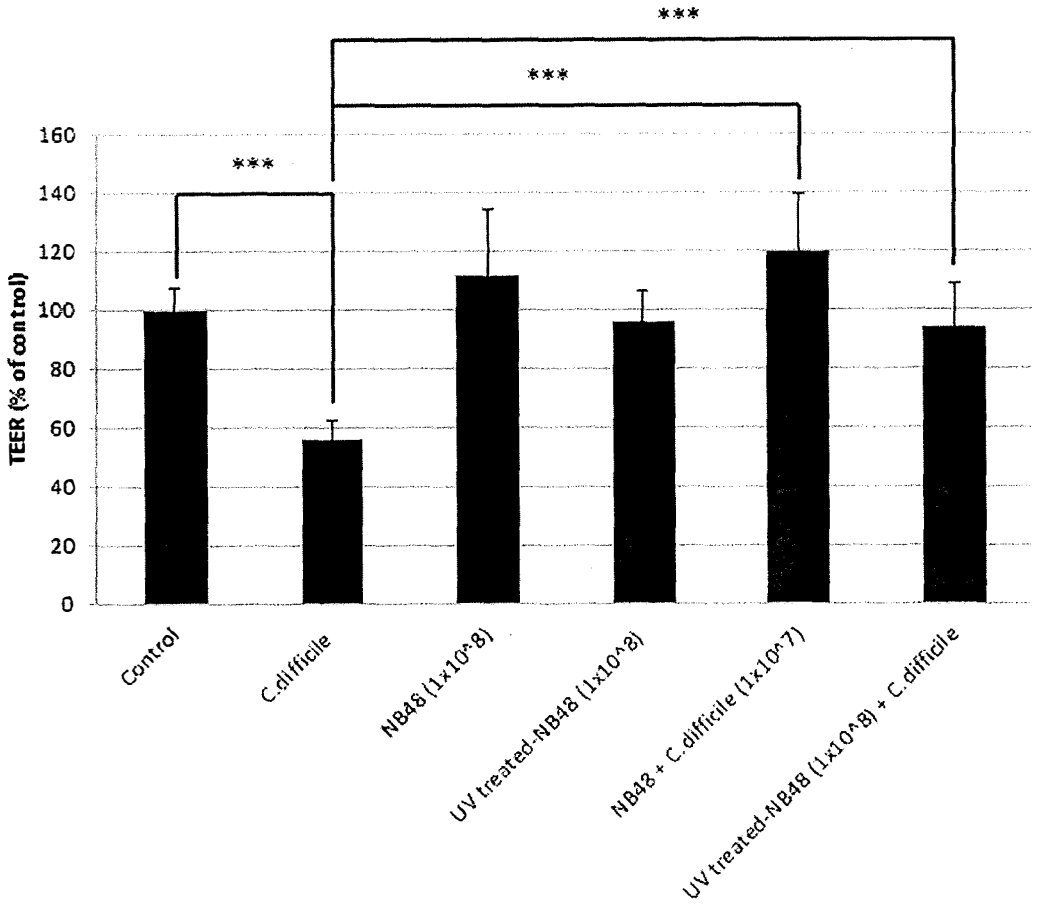


Figure 31. The enhancement effects of live or UV-treated BP-NB48 on TEER when coculture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate. ***$p<0.001$

Table 28. The effects of BB-NB42 on TEER when coculture with *Vibrio cholerae* O1 Inaba in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	82.01	3.58	100.00	
V. cholerae	1.82	0.23	2.21	0.0001***
NB42 ($1x10^8$)	93.89	14.23	114.49	0.2130
NB42 ($1x10^8$) + *V. cholerae* ($1x10^7$)	2.97	0.93	3.62	0.1158

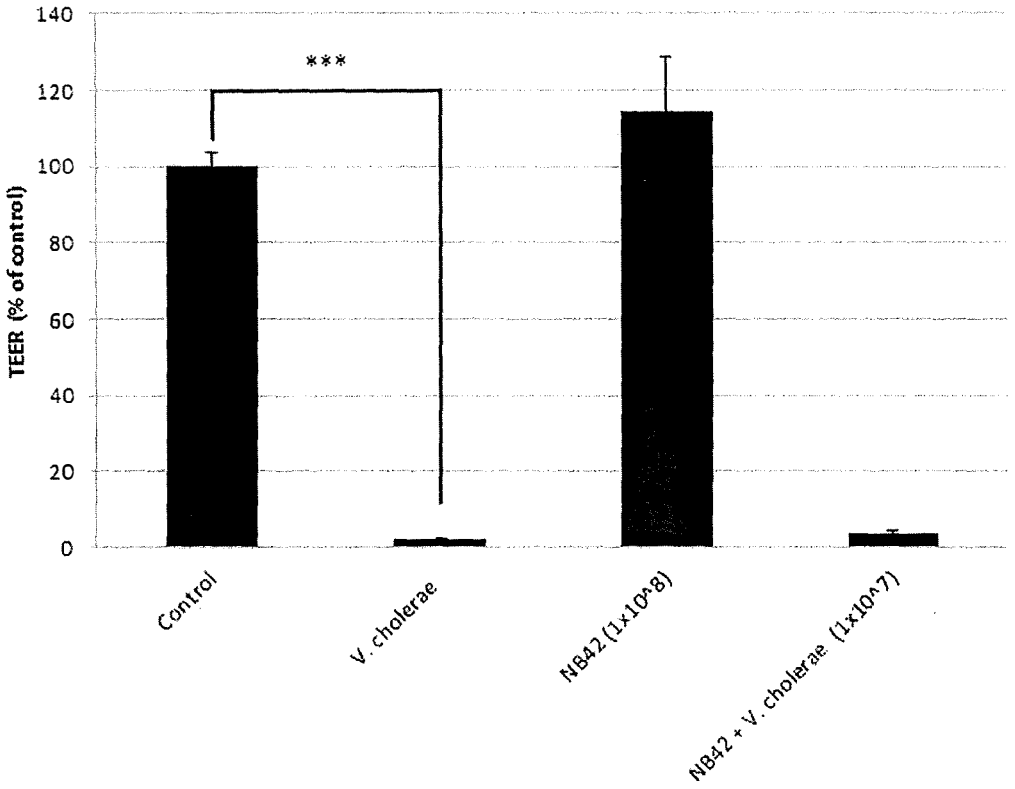


Figure 32. The effects of BB-NB42 on TEER when coculture with *Vibrio cholera* O1 Inaba in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate. ***p<0.001

Table 29. The effects of BB-NB42 on TEER when coculture with *Salmonella* Typhimurium in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	82.01	1.87	100.00	
S. Typhimurium	19.47	6.53	23.74	0.0028**
NB42 ($1x10^8$)	93.89	14.23	114.49	0.2130
NB42 ($1x10^8$) + *S.* Typhimurium ($1x10^7$)	50.49	3.27	61.57	0.0133*

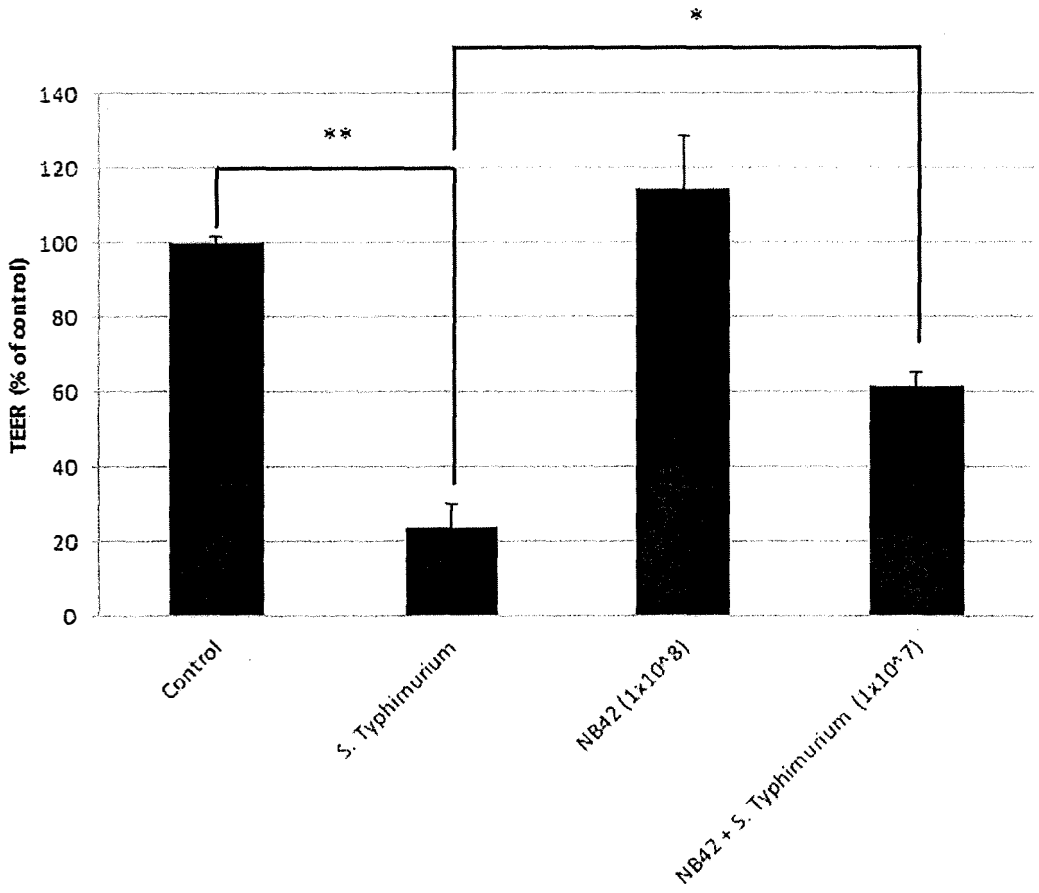


Figure 33. The effects of BB-NB42 on TEER when coculture with *Salmonella* Typhimurium in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate. *p<0.05, **p<0.01

Table 30. The effects of BB-NB42 on TEER when coculture with *Campylobacter jejuni* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate.

Subject	**Transepithelial electrical resistance (TEER) value at 24 hours**		**TEER (%)**	***p*-value**
	TEER average ($\Omega.cm^2$)	**SD**		
Control	84.48	6.78	100.00	
C. jejuni	28.55	2.57	33.79	0.0034**
NB42 ($1x10^8$)	93.89	14.23	111.13	0.3448
NB42 ($1x10^8$) + *C. jejuni* ($1x10^7$)	50.66	6.30	59.96	0.0221*

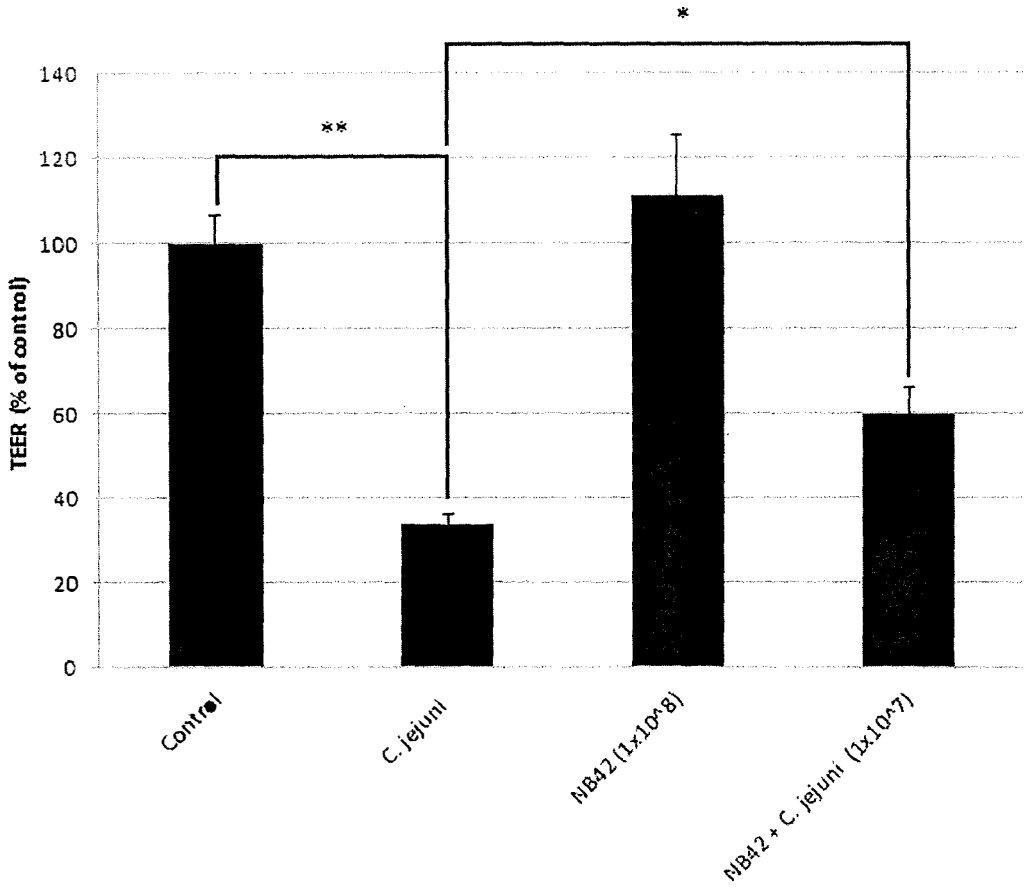
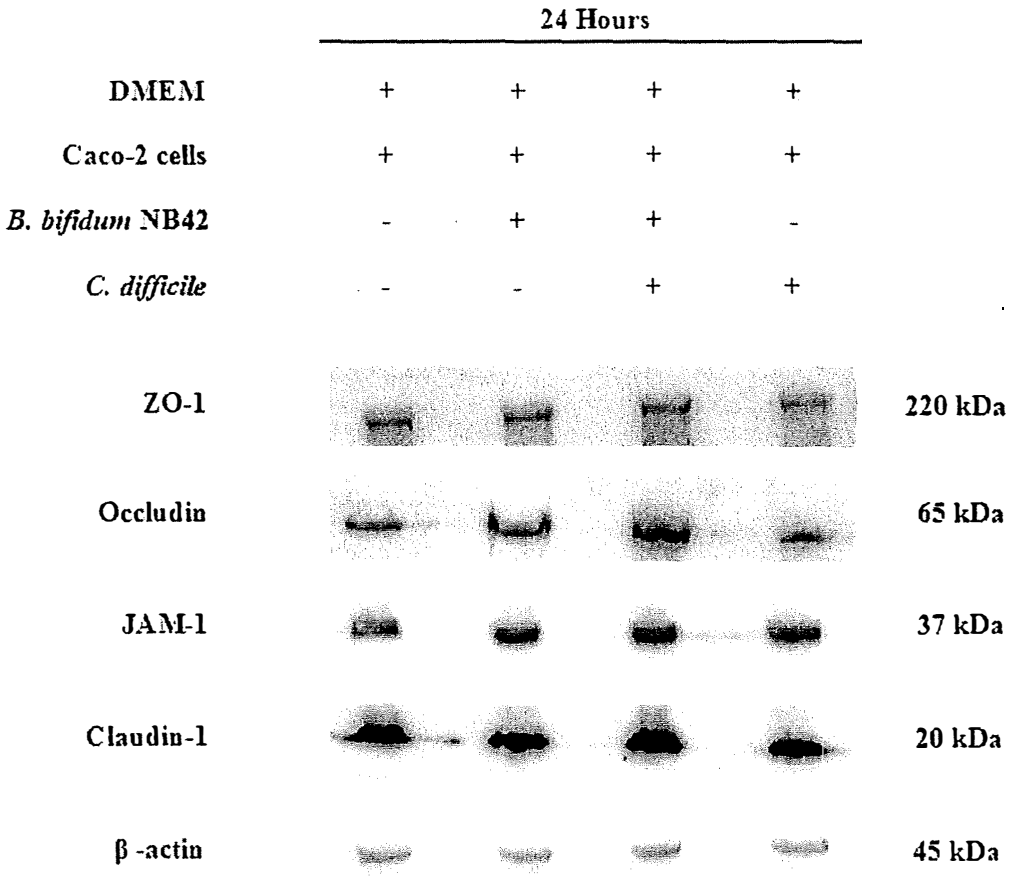


Figure 34. The effects of BB-NB42 on TEER when coculture with *Campylobacter jejuni* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed once in duplicate. *p<0.05, **p<0.01

A


24 Hours
DMEM + + + +
Caco-2 cells + + + +
B. bifidum NB42 - + + -
C. difficile - - + +
ZO-1
220 kDa
Occludin
65 kDa
JAM-1
37 kDa
Claudin-1
20 kDa
β -actin
45 kDa

B

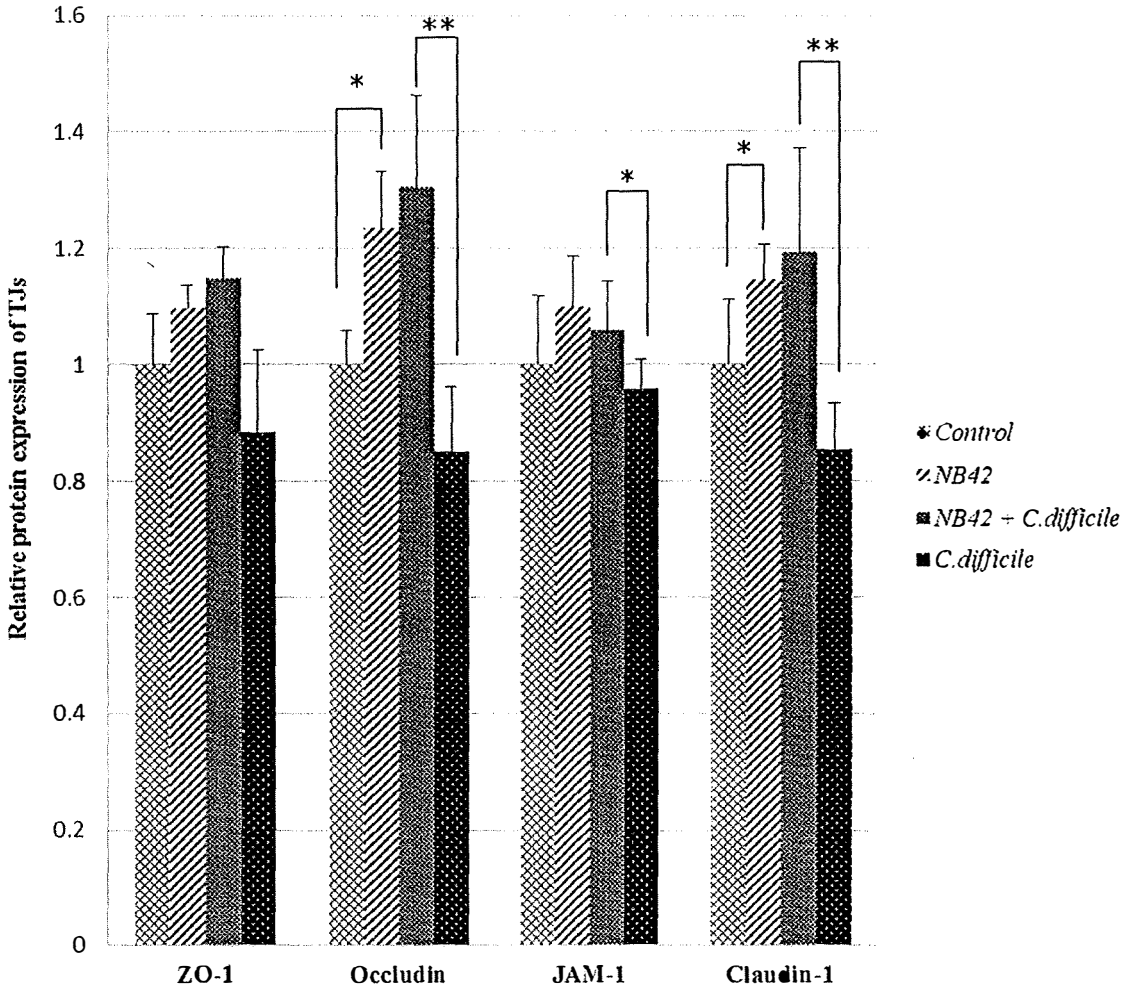


Figure 35. BB-NB42 prevents tight junction proteins expression disrupted by *Clostridium difficile* (A) Representative western blotting analysis for ZO-1, Occludin, JAM-1 and Claudin-1 proteins in Caco-2 cells. (B) Semi-quantitative analysis of western blot showed tight junction proteins expression at different conditions. *p<0.05, **p<0.01. Values were calculated by Student's t-test.

Table 31. Immunomodulatory effects of LR-L34 on IL-8 productions in *Clostridium difficile*-stimulated HT-29 human colon adenocarcinoma cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation. The experiment of each LCM was performed in three technical replicates.

Sample	TNF suppression (%)	48h LCM		48h LCM Plus *C. difficile*		IL-8 suppression (%)[c]	p-value
		IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
MRS media control	-	189.74	14.83	1078.15	371.52	-	-
LR-L34 (I)	38.79*	156.92	23.44	516.43	181.41	52.10*	0.0391
MRS media control	-	195.66	24.14	975.31	121.23	-	-
LR-L34 (II)	38.79*	141.54	141.54	430.58	179.13	55.85**	0.0060
MRS media control	-	359.11	7.26	1407.01	155.20	-	-
LR-L34 (III)	38.79*	153.31	88.04	652.60	377.48	53.62*	0.0164

[c] IL-8 suppression was calculated from the difference of IL-8 value of HT-29 cells co-cultured with MRS media control + *C. difficile* and LCM + *C. difficile*. Significantly different from control: ***p-value < 0.001, **p-value < 0.01 and * p-value < 0.05

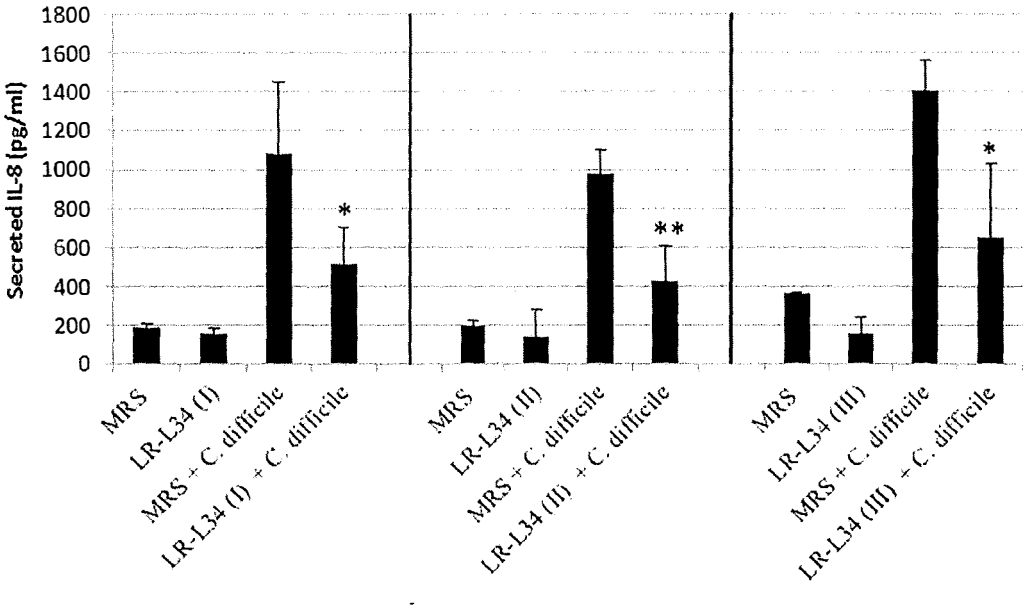


Figure 36. Inhibitory effects of LR-L34 on IL-8 production in *Clostridium difficile*-stimulated HT-29 human colon adenocarcinoma cells. MRS, bacterial media control. Significantly different from MRS media control, ** p-value <0.01and, * p-value <0.05. The experiment of each LCM was performed in three technical replicates.

Table 32. Immunomodulatory effects of BB-NB42, BA-B24 and BP-NB48 on IL-8 productions in *Clostridium difficile*-stimulated HT-29 human colon adenocarcinoma cells. BCM, *Bifidobacterium* conditioned media; BHI, bacterial media control; SD, standard deviation. Results were presented as mean values from three biological and three technical replicates (n=9).

Sample	48h BCM		48h BCM Plus *C. difficile*		IL-8 suppression (%)	p-value
	IL-8 conc. (pg/ml)	SD	IL-8 conc. (pg/ml)	SD		
BHI media control	153.27	5.09	745.73	138.92	-	-
BB-NB42	136.54	12.25	777.15	166.58	0.0421	0.3882
BHI media control	153.27	5.09	745.73	138.92	-	-
BA-B24	185.79	22.89	943.52	210.95	0.2652	0.0831
BHI media control	153.27	5.09	745.73	138.92	-	-
BP-NB48	146.71	7.52	732.96	108.34	-0.0171	0.4356

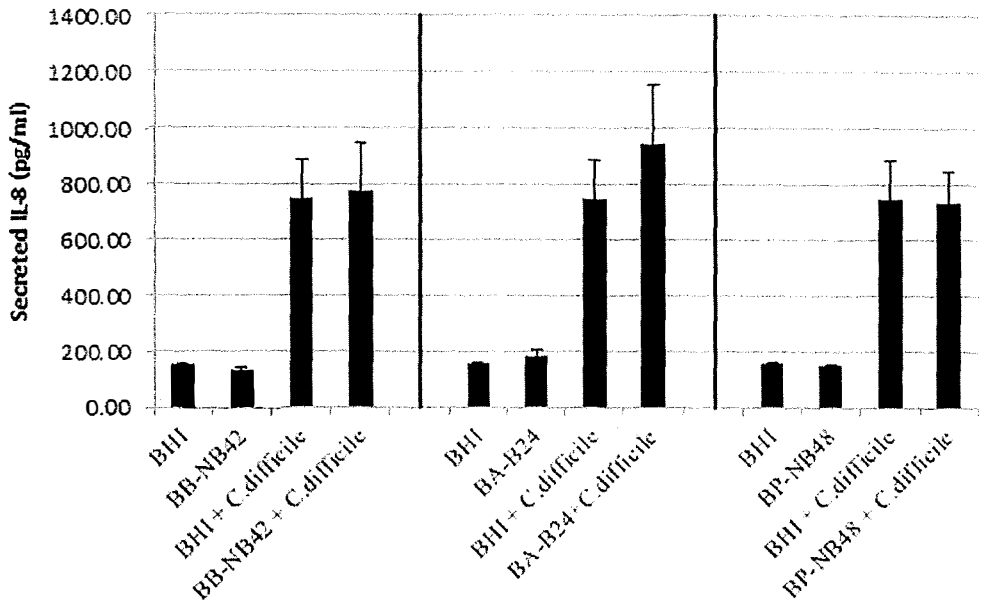


Figure 37. Inhibitory effects of BB-NB42, BA-B24 and BP-NB48 on IL-8 production in *Clostridium difficile*-stimulated HT-29 human colon adenocarcinoma cells. BHI, bacterial media control. Results were presented as mean value from three biological and three technical replicates (n=9), bars indicate standard deviation.

DISCUSSION

The intestine contains normal flora about 10 trillion microbes with many different species, amounting to 1-2 kg in weight [30, 53, 54]. Some normal flora are probiotic bacteria which confer health benefit to host in preservation of homeostasis in the intestine, protection of the harmful effect of pathogens by various mechanisms such as immunomodulation, competitive adherence to epithelial cells, increasing the integrity of tight junctions (TJs) and restore normal flora when host was encroached from pathogens [33, 54]. Furthermore, the efficacy of probiotics is thought to be strain-specific to each group of population [55]. This study showed that *Lactobacillus* Thai isolates could increase the integrity the tight junctions as determined by the increased transepithelial electrical resistance (TEER). Only three isolates of *Lactobacillus* spp. including *L. fermentum* L12, *L. oris* NL49 and *L. murinus* B57 increased TEER significantly ($p<0.05$).The enhancement effect is strain-specific since only *L. fermentum* L12 increased TEER whereas L7 and Lac31 did not.

TJs can be destroyed by pathogens such as *Clostridium difficile*, enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), *Bacteroides fragilis*, *Clostridium perfringens*, *Vibrio cholerae* and *Salmonella* Typhimurium with different destruction mechanism [56, 57]. In addition, TJs can be destroyed by other factors such as pro-inflammatory cytokines and oxidative stress [58, 59]. Current researches reported that *C. difficile, V. cholerae*, *S.* Typhimurium and *Campylobacter jejuni* can disrupt TJs proteins resulting in the decrease of TEER [21, 60-64]. Our results showed that *C. difficile, V. cholerae* O1 Inaba, *S.* Typhimurium ATCC 13311 and *C. jejuni* decreased TEER on Caco-2 cells and the decrease was dose-dependent.

At present, probiotics are used for prevention and treatment of patients with gastrointestinal disorder [30]. Most used probiotics include *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli* (such as *E. coli* Nissle 1917), *Streptococus thermophilus* and yeast (such as *Saccharomyces boulardii*). In general, probiotics have the activities in one or more of the followings: the enhancement of TJs by increasing expression of TJs protein, stimulating mucus and antimicrobial agents, promotion of secretory IgA secretion, prevention of cell apoptosis and entry of pathogens [65-67]. The ability of probiotics to protect the disruption of TJs by pathogens was previously reported. For examples, *L. rhamnosus* strain GG prevents the redistribution of TJs induced by

enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 [37]. *L. plantarum* prevents the changing of TJs induced by enteroinvasive *Escherichia coli* (EIEC) and enteropathogenic *Escherichia coli* (EPEC) [38, 39]. Our study showed that *L. rhamnosus* L34 (LR-L34) at $1x10^7$ CFU per well increased TEER when cells were treated with LR-L34 alone. LR-L34 increased TEER significantly ($p< 0.001$) when cells were pretreated with LR-L34 for 3 hours before the addition of *C. difficile*. UV-treated LR-L-34 also increased TEER significantly ($p< 0.05$) which suggested that LR-L34 cells hinder the binding of *C. difficile* toxins to initiate the activities. However, live LR-L-34 increased TEER more than UV-treated LR-L-34 which suggested that LR-L34 secretes bioactive product capable of modulation of TJs production and redistribution.

This study demonstrated that the expression of claudin-1 increased significantly when cells were treated with LR-L34 alone as compared with control while the expression of JAM-1 and occludin non-significantly increased. Furthermore, the expression of claudin-1 and occludin increased significantly when cells were pretreated with LR-L34 for 3 hours followed by the infection with *C. difficile*, whereas the expression of JAM-1 non-significanly increased as compared with cells infected *C. difficile* alone. Moreover, the expression of ZO-1 could not be detected in this study. The experiment will be repeated for the detection of ZO-1.

For the effect of LR-L34 on the enhancement of TJs integrity disrupted by other pathogens, LR-L34 could not prevent the intestinal epithelial barrier functions destroyed by other intestinal bacterial pathogens such as *V. cholerae* O1 Inaba, and *S.* Typhimurium ATCC 13311. However, live LR-L34 and UV- treated LR-L34 could prevent the intestinal epithelial barrier functions destroyed by *C. jejuni* as demonstrated by the significantly increased TEER. Surprisingly, UV- treated LR-L34 increased TEER more than live LR-L34 although UV has effect only on DNA resulting in the viability of bacteria.

The most frequent genera used as probiotics are not only *Lactobacillus* but also *Bifidobacterium* [41, 68]. It has been reported that *B. infantis* conditioned medium had the greater effect on TER in T84 cells than probiotics VSL#3 [43]. Furthermore, another study have shown that cell-free supernatant of *B. lactis* 420 was able to increase epithelial resistance on Caco-2 cells [44]. In this study, similar results showed that *B. psedocatenulatum* NB48 (BP-NB48) and *B.* bifidum NB42 (BB-NB42) increased TER significantly in Caco-2 cells. Only strain NB48 from five *B. psedocatenulatum* isolates and strain NB42 from two *B. bifidum* isolates increased TER. This implied that the

enhancement effect of *Bifidobacterium* spp. on TJs integrity is strain-specific. The increase of TER in Caco-2 cells by these bifidobacteria suggests that BP- NB48 and BB-NB42 are able to enhance intestinal barrier function.

For the prevention of TJ damage, pretreatment of Caco-2 cells with live BB-NB42 for 3 h before the addition of *C. difficile* resulted in a significant increase in TEER when compared with *C. difficile*-infected control ($p< 0.001$). These result suggested that BB- NB42 can prevent *C. difficile*-induced damage of tight junctions. Furthermore, UV-treated BB-NB42 also increased TEER significantly ($p< 0.001$) which suggested that BB-NB42 cells may hinder *C. difficile* toxins binding to initiate the activities. However, live BB-NB42 increased TEER more than UV-treated BB-NB42 which suggested that secreted bioactive factors from BB- NB42 was able to modulate TJs production and redistribution. Although both live BA-B24 and BP-NB48 also increased TEER significantly ($p< 0.001$), pretreatment of Caco-2 with live BA-B24 and BP-NB48 prior to *C. difficile* infection had less positive effects than that of BB-NB42. Thus, BB-NB42 had the greatest effect on TEER suggesting its most potential to prevent *C. difficile*-induced damage of tight junctions in this study.

V. cholerae, S. Typhimurium and *C. jejuni* can damage intestinal epithelial barrier function by different mechanisms. The causes of the alteration of intestinal epithelial barrier function by *V. cholerae* are toxins, including hemagglutinin/protease (HA/P), RTX and zonula occluden toxin (Zot). HA/P causes the TJ disruption by the cleavage of occludin while RTX interferes with the contractile actin ring and Zot causes the dissociation of ZO-1 from junctional complex [15]. *S.* Typhimurium disrupts TJ integrity through the use of 4 effectors, including SipA, SopB, SopE and SopE2 [69]. Although the mechanism of *C. jejuni* infection is unclear, Chen *et al.* reported that *C. jejuni* infection caused redistribution of TJ proteins and increasing of IL-8 secretion which support the pathogenesis of *C. jejuni*-induced enterocolitis [70]. At present, there is no report of the effect of *Bifidobacterium* on the tight junction loss caused by *V. cholerae*, *S.* Typhimurium and *C. jejuni*. This study showed that *B. bifidum* NB42 might be beneficial in preventing the damage of tight junction integrity caused by *S.* Typhimurium ATCC 13311 and *C. jejuni* but not *V. cholerae* O1 Inaba. Since the experiments were performed in only once in duplicate, further experiments are needed to confirm the results.

Prevention of tight junction damage by probiotic bacteria has been evidenced by the increase in expression and rearrangement of tight junction proteins. Qin *et al.* reported

that *L. plantarum* prevented EIEC-induced decrease in expression of Claudin-1, Occludin, JAM-1 and ZO-1 proteins [71]. In addition, *B. infantis* conditioned medium increased TER by altering the expression of tight junction proteins by increasing ZO-1 and occludin expression but decreasing claudin-2 expression [43]. In this study, BB-NB42 was the only strain chosen for the investigation of its effect on the expression of tight junction proteins. BB-NB42 alone increased the expression of occludin and claudin-1 significantly and ZO-1 and JAM-1 non-significantly in Caco-2 cells. While in *C. difficile*-infected Caco-2 cells, BB-NB42 increased the expression of occludin, JAM-1 and claudin-1 significantly and ZO-1 non-significantly. Regulation of the synthesis of tight junction proteins is known to be mediated in part by signaling pathways including protein kinase C (PKC), myosin light chain kinases (MLCK), Rho kinase (ROCK) and mitogen-activated protein kinase (MAPK) [68]. Ewaschuk *et al.* reported the protective effect of *B. infantis* conditioned medium on intestinal barrier disruption through MAPK pathway with increased levels of phospho-ERK [43]. It is thus interesting to investigate the effect of BB-NB42 on the modulation signaling pathway associated with the expression of these tight junction proteins.

CONCLUSION

Three isolates of *Lactobacillus* spp. including *L. fermentum* L12 (LF-L12), *L. oris* NL49 (LO-NL49) and *L. murinus* (LM-B57) increased TER significantly. Eight isolates including LF-L12, LO-NL49, LM-B57, *L. plantarum* XB7 (LP-XB7), *L. salivarius* B37 (LS-B37), *L. salivarius* B60 (LS-B60), *L. rhamnosus* L34 (LR-L34) and *L. casei* L39 (LC-L39) prevent the destruction of TJs by *C. difficile*. LR-L34 which was selected for further investigation had the ability to protect and improve the intestinal epithelial barrier destroyed by *C. difficile* although the magnitude of improvement is lower than protection. Live LR-L34 had more effect than UV-treated LR-L34. Live and UV-treated LR-L34 had protection effect on the destruction of intestinal epithelial barrier by *C. jejuni* not *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium ATCC 13311. . LR-L34 was able to increase the expression of claudin-1 significantly and its pretreatment prevented *C. difficile*-induced decrease in the expression of claudin-1 and occludin significantly. Furthermore, LR-L34 has the ability to suppress *C. difficile*-induced interleukin-8 (IL-8) production. LR-L34 is thus a potential probiotic strain with the ability to enhance TJs integrity, protect and improve the destruction of TJs by *C. difficile* together with anti-inflammation by IL-8 suppression. In addition, it can probably prevent the damage of tight junctions by *C. jejuni* and confirmation of this ability is needed in further investigation.

Five *Bifidobacterium* Thai isolates including *B. adolescentis* B24 (BA-B24), *B. bifidum* NB42 (BB-NB420, *B. catenulatum* NB38(BC-NB38), *B. longum* B103(BL-B103) *and B. psedocatenulatum* NB48 (BP-NB48) can enhance intestinal epithelial resistance of human Caco-2 colorectal adenocarcinoma cells. From five *Bifidobacterium* isolates, three *Bifidobacterium* spp. including BA-B24, BB-NB42 and BP-NB48 can prevent *C. difficile*-induced damage of the integrity of tight junctions. In contrast, these bifidobacteria cannot restore the integrity of tight junctions destroyed by *C. difficile*. Although, live and UV-treated BA-B24, BB-NB42 and BP-NB48 can prevent *C. difficile*-induced damage of the integrity of tight junctions, live bifidobacteria had more effect than UV-treated bacteria. BB-NB42 had greatest effect in the prevention of *C. difficile*-induced damage of tight junctions. BB-NB42 can prevent the damage of tight junction integrity caused by *S.* Typhimurium ATCC 13311 and *C. jejuni* but not *V. cholerae* O1

Inaba. BB-NB42 increased the expression levels of occludin and claudin-1 significantly while non-significantly increased those of ZO-1 and JAM-1. The pretreatment of Caco-2 cells with BB-NB42 before *C. difficile* infection can prevent *C. difficile*-induced decrease in expression of tight junction proteins as shown by the significantly increased expression of occludin, JAM-1 and claudin-1. *B. bifidum* NB42 is thus a potential probiotic strain in enhancing intestinal epithelial resistance and prevention of *C. difficile*-induced damage of tight junction integrity. In addition, it can probably prevent the damage of tight junctions by *Salmonella* Typhimurium and *Campylobacter jejuni* and confirmation of this ability is needed in further investigation.

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