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ปีการศึกษา 2555

ROLE OF *LACTOBACILLUS* IN THE ENHANCEMENT OF HUMAN
INTESTINAL EPITHELIAL BARRIER FUNCTIONS DESTROYED BY
CLOSTRIDIUM DIFFICILE

Miss Waraporn Siriterm

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	ROLE OF *LACTOBACILLUS* IN THE ENHANCEMENT OF HUMAN INTESTINAL EPITHELIAL BARRIER FUNCTIONS DESTROYED BY *CLOSTRIDIUM DIFFICILE*
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วราภรณ์ ศิริเติม : บทบาทของของแลคโตบาซิลลัสในการเพิ่มความสามารถกีดขวางของเยื่อบุผิวที่ถูกทำลายโดย คลอสทริเดียม ดิฟฟิไซล์ (ROLE OF *LACTOBACILLUS* IN THE ENHANCEMENT OF HUMAN INTESTINAL EPITHELIAL BARRIER FUNCTIONS DESTROYED BY *CLOSTRIDIUM DIFFICILE*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. สมหญิง ธัมวาสร, 78 หน้า.

แลคโตบาซิลลัส สายพันธุ์จำเพาะสามารถส่งเสริมความสมบูรณ์ของเยื่อบุลำไส้และป้องกันการทำลายหน้าที่กีดขวางของเยื่อบุผิวจากเชื้อก่อโรค วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อหาแลคโตบาซิลลัสสายพันธุ์ไทยที่สามารถเพิ่มความหนาแน่นของส่วนเชื่อมติดกันแน่นของเซลล์เยื่อบุลำไส้ โดยนำ แลคโตบาซิลลัสทั้งหมด 29 สายพันธุ์ ที่คัดแยกได้จาก อุจจาระเด็ก น้ำนมมารดา และชิ้นเนื้อกระเพาะอาหาร มาทดสอบความสามารถในการเพิ่มความหนาแน่นของส่วนเชื่อมติดกันแน่น โดยวิธี transepithelial electrical resistance (TEER) ในเซลล์เยื่อบุลำไส้ Caco-2 พบว่า มีแลคโตบาซิลลัส 8 สายพันธุ์ได้แก่ แลคโตบาซิลลัส เฟอร์เมนตัม L12 (LF-L12), แลคโตบาซิลลัส โอริส NL49 (LO-NL49), แลคโตบาซิลลัส มูรินัส B57 (LM-B57), แลคโตบาซิลลัส แพลนทารัม XB7 (LP-XB7), แลคโตบาซิลลัส ซาลิวาเรียส B37 (LS-B37), แลคโตบาซิลลัส ซาลิวาเรียส B60 (LS-B60), แลคโตบาซิลลัส แรมโนซัส L34 (LR-L34) และ แลคโตบาซิลลัส เคซิไอ L39 (LC-L39) สามารถป้องกันการทำลายส่วนเชื่อมติดกันแน่นจากคลอสทริเดียม ดิฟฟิไซล์

จากการคัดเลือก LR-L34 มาศึกษาต่อ พบว่า LR-L34 มีความสามารถในการป้องกันและแก้ไขการทำลายหน้าที่กีดขวางของเยื่อบุผิวจากคลอสทริเดียม ดิฟฟิไซล์ แม้ว่าระดับผลของการแก้ไขจะต่ำกว่าการป้องกันก็ตาม นอกจากนี้ยังพบว่า LR-L34 ที่มีชีวิตมีประสิทธิภาพมากกว่า LR-L34 ที่ถูกฆ่าด้วยรังสียูวี อีกทั้ง LR-L34 ที่มีชีวิตและที่ถูกฆ่าด้วยรังสียูวียังสามารถป้องกันการทำลายหน้าที่กีดขวางของเยื่อบุผิวจาก แคมไพโลแบคเตอร์ เจจูไนได้ แต่ไม่สามารถป้องกันการทำลายหน้าที่กีดขวางของเยื่อบุผิวจาก วิบริโอ คอเลอเร O1 Inaba และ ซัลโมเนลลา ไทฟีมิวเรียม ATCC 13311 การแสดงออกของโปรตีนที่เป็นองค์ประกอบของส่วนเชื่อมติดกันแน่นได้แก่ claudin-1 เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเซลล์เยื่อบุลำไส้ Caco-2 cells ถูกบ่มร่วมกับ LR-L34 เพียงอย่างเดียว นอกจากนี้การแสดงออกของ claudin-1 และ occludin เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเซลล์เยื่อบุลำไส้ Caco-2 cells ถูกบ่มร่วมกับ LR-L34 เป็นเวลา 3 ชั่วโมงก่อนบ่มกับคลอสทริเดียม ดิฟฟิไซล์ ดังนั้น LR-L34 จึงเป็นโพรไบโอติกส์สายพันธุ์ที่มีความสามารถเพิ่มความสมบูรณ์ของส่วนเชื่อมติดกันแน่น ป้องกัน และแก้ไขการทำลายส่วนเชื่อมติดกันแน่นจากคลอสทริเดียม ดิฟฟิไซล์

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต ....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก...

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KEYWORDS : PROBIOTICS/ INTESTINAL EPITHELIAL BARRIER FUNCTIONS/ TIGHT JUNCTIONS/ LACTOBACILLI

WARAPORN SIRITERM: ROLE OF *LACTOBACILLUS* IN THE ENHANCEMENT OF HUMAN INTESTINAL EPITHELIAL BARRIER FUNCTIONS DESTROYED BY *CLOSTRIDIUM DIFFICILE* ADVISOR: ASSOC. PROF. SOMYING TUMWASORN, Ph.D., 78 pp.

Specific strains of *Lactobacillus* spp. can promote intestinal integrity and prevent pathogen-induced damage of epithelial barrier function. This study aimed to find indigenous *Lactobacillus* Thai isolates with the ability to prevent and/or improve the integrity of epithelial tight junctions (TJs). Twenty-nine *Lactobacillus* isolates from infant feces, breast milk and gastric biopsies were tested for this ability by transepithelial electrical resistance (TEER) assay in Caco-2 cells. Eight *Lactobacillus* Thai isolates including *L. fermentum* L12 (LF-L12), *L. oris* NL49 (LO-NL49), *L. murinus* B57 (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 that of 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* but not by *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium ATCC 13311. The expression of claudin-1 significantly increased when differentiated Caco-2 cells were treated with LR-L34 alone. 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*. LR-L34 is thus a probiotic strain with the ability to enhance TJs integrity, protect and improve the destruction of TJs by *C. difficile.*

Field of Study : <u>Medical Microbiology</u>   Student's Signature ....

Academic Year : <u>2012</u>   Advisor's Signature ....

ACKNOWLEDGEMENTS

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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*
EAEC	enteroaggregative *Escherichia coli*
HIV-1	*Human immunodeficiency virus*-1
TEER	transepithelial electrical resistance
IECs	the intestinal epithelial cells
FAE	follicle-associated epithelium
kDa	kilodalton
C-terminal	carboxyl-terminal
N-terminal	amino-terminal
PMN	polymorphonuclear
MAGUK	the membrane-associated guanylate kinase
GK	guanylate kinase
SH3	Src homology 3
gp	glycoprotein
CDI	*Clostridium difficile* infection
IBD	inflammatory bowel disease
CDAD	*Clostridium difficile* antibiotic-associated diarrhea
CDT	binary toxin
AAD	antibiotic-associated diarrhea
Th	T helper cell
Ig	immunoglobulin
Treg	regulatory T cell

TGF	transforming growth factor
IFN	interferon
DC	dendritic cell
MΦ	macrophage
SCFA	short-chain fatty acid
NF-κB	nuclear factor-κB
ROS	reactive oxygen species
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 media
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	squared 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-(hydroxymethayl)-aminoethane
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	Dithiothretol
APS	Ammonium Persulfate
l	liter
M	molar
mM	millimolar
mg	milligram
rpm	round per minute
μl	microliter

CHAPTER I

INTRODUCTION

Clostridium difficile is an anaerobic, gram positive-spore forming bacillus which causes diarrhea and colitis [1]. The clinical symptom varies from asymptomatic to mild self-limited diarrhea and severe pseudomembranous colitis [2]. These symptoms result from the release of two protein exotoxins: toxin A (Tcd A), a 308-kD enterotoxin and toxin B (Tcd B), an approximately 270-kD cytotoxin [3, 4]. The pathogenic process starts with initial colonization in human intestinal epithelial and the production two toxins of *Clostridium 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 [5]. Toxin A and toxin B modify and inactivate Rho proteins via glucosylation. Glucosylation of Rho GTPases results in rearrangement of actin cytoskeleton, disruption of tight junctions, rounding up of cell, cell death and loss of intestinal epithelium barrier function [6, 7]. Previous studies showed that *Clostridium difficile* toxins cause tight junction disruption which was observed from the decrease in transepithelial electrical resistance and the increased paracellular permeability in epithelial cell lines incubated with toxins [8-10]. Disruptions of tight junctions facilitates TcdA and TcdB to cross the epithelium, reaching and activating monocytes, macrophages and mast cells to secrete several inflammatory cytokines. such as interleukin (IL)-8, tumor necrosis factor alpha (TNF- α), IL-1 and IL-6.These inflammatory cytokines, especially IL-8 cause neutrophil and lymphocyte influx resulting in pseudomembrane formation and diarrhea [11, 12].

The intestinal epithelial barrier is a single layer of columnar epithelial cells on basement membrane. It separates the intestinal lumen from the underlying tissue. 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. Tight junctions are

complex structure proteins; transmembrane proteins (such as occludin, claudin and junctional adhesion molecules [JAM]) and plaque proteins (zonula occludens;ZOs). The plaque proteins are adaptor protein that is involved in the clustering and stabilization of transmembrane proteins which is located between transmembrane proteins and actin cytoskeleton. The cytoskeleton is important for maintaining the structure of eukaryotic cells because it is complex structure of protein filaments which extend throughout the cytosol of cell. Therefore, disruption of tight junction which is a part of the intestinal epithelial barrier is linked to the loss of intestinal barrier integrity and decrease paracellular permeability [13]. In addition, the intestinal epithelial barrier is regulated by Rho protein. The Rho proteins are important in maintaining tight junctions because the Rho proteins function is molecular switches of many eukaryotic signaling pathways which regulate the intestinal epithelial barrier function [7].

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 [14] and specific strain of *Lactobacillus plantarum* can protect enteroinvasive *Escherichia coli* (EIEC) and enteropathogenic *Escherichia coli* (EPEC)-induced change in intestinal epithelial barrier function[15, 16]. However, there is no report of *Lactobacillus* enhancement of TJs integrity disrupted by *Clostridium difficile*. Therefore, this study aims to search for specific strain(s) of *Lactobacillus* spp. which can prevent and/or enhance the integrity of tight junctions destroyed by *Clostridium difficile*. In addition, the ability of these lactobacilli to prevent TJs integrity destroyed by other intestinal bacterial pathogens including *Vibrio cholerae*, *Salmonella* Typhimurium and *Campylobacter jejuni* will be investigated.

Hypothesis

Specific strain(s) of *Lactobacillus* can prevent and/or improve the integrity of tight junctions destroyed by *Clostridium difficile* and other intestinal bacterial pathogens.

Objective

- Search for specific strain(s) of *Lactobacilllus* Thai isolates that can enhance intestinal epithelial resistance and prevent and/or improve *Clostridium difficile*-induced damage of the integrity of tight junctions
- Investigate their ability to prevent the damage of tight junction integrity caused by other intestinal bacterial pathogens including *Vibrio cholerae, Salmonella* Typhimurium and *Campylobacter jejuni*

Conceptual framework

Lactobacillus spp. isolated from infant feces, breast milk and gastric biopsies

Lactobacillus can prevent *Clostridium difficile*-induced damage of the integrity of tight junctions

Lactobacillus can improve *Clostridium difficile* -induced damage of the integrity of tight junctions

Lactobacillus can prevent the damage of tight junction integrity caused by other intestinal bacterial pathogens

Lactobacillus can improve the damage of tight junction integrity caused by other intestinal bacterial pathogens

Workflow

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┌─────────────────────────────────────────────────────────────┐
│   Lactobacillus spp. isolated from infant feces and breast milk │
└─────────────────────────────────────────────────────────────┘
                              │
                    ┌──────────────────┐
                    │    Caco-2 cells   │
                    └──────────────────┘
                              │
   ┌──────────┬──────────────┬──────────────┬──────────────┬──────────────┐
┌────────┐ ┌──────────┐ ┌──────────┐ ┌──────────┐ ┌──────────┐
│Untreated│ │Lactobacillus│ │Lactobacillus│ │Clostridium│ │Clostridium│
│         │ │  species  │ │  species  │ │ difficile │ │ difficile │
└────────┘ └──────────┘ └──────────┘ └──────────┘ └──────────┘
                           ┌──────────┐ ┌──────────┐
                           │Clostridium│ │Lactobacillus│
                           │ difficile │ │  species  │
                           └──────────┘ └──────────┘
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┌───┐
│ Measurement of transepithelial electrical resistance (TEER assay) │
└───┘

┌───┐
│ Western blotting for determining the distribution and
│ expression of tight junctions proteins │
└───┘

┌───┐
│ *Lactobacillus* with the ability to prevent and/or improve
│ the damage of the integrity of tight junctions by
│ *Clostridium difficile* │
└───┘

┌───┐
│ Test their ability to prevent and/ or improve the integrity
│ of tight junctions destroyed by other intestinal bacterial
│ pathogens │
└───┘

CHAPTER II

LITERATURE REVIEWS

Intestinal epithelial barrier functions

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 (Figure 1) [17]. The intestinal epithelial cells occur from stem cells in region of crypt that differentiate to special cell types including M cells, goblet cells, paneth cells, enteroendocrine cells and enterocyte (columnar epithelial cells). These special cell types are subset of the intestinal epithelial cells (IECs); they have different functions dependent on each cell types that show in Table 1 and Figure 2 [18, 19]. The most of special cell types in intestine are columnar epithelial cells (about 80%) that it is a single layer and reside underlying lamina propria [12, 18]. Furthermore, the intercellular space of intestinal epithelial cells is sealed by the junctional complexes which these are consisted tight junctions (TJs), adherens junctions, desmosomes and gap junctions [20, 21]. These are regulating permeability, polarity and integrity of intestinal epithelial barrier functions.
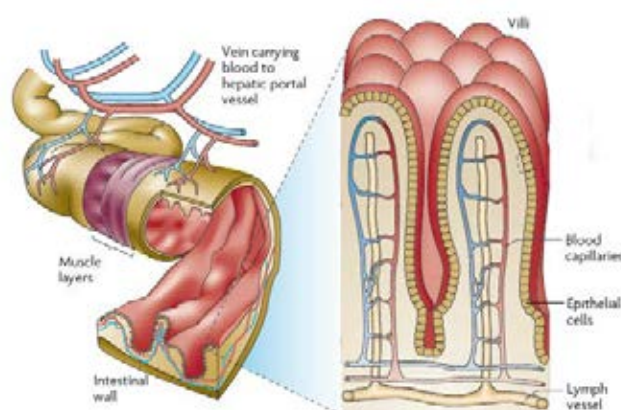
Figure 1. The human intestine (This figure was part of reference 12.). It has a length of 7 meters and composes of a single layer of columnar epithelial cells which has crucial role in not only the absorption of nutrients but also barrier functions that protect foreign agents and bacterial pathogens.

Table 1. The functions of the subset of intestinal epithelial cells (IECs) [18, 19]

The subset of intestinal epithelial cells (IECs)	Position	Functions
M cells	- In regions of follicle-associated epithelium (FAE)	- The mediator of the mucosal immune system
Globlet cells	- Upper the crypt	- The secretory cells produce constituent of mucus such as mucin, trefoil protein etc.
Paneth cells	- At the base of the crypt	- The secretory cells produce antimicrobial such as defensins, lysozyme etc.
Enteroendocrine cells	- Upper the crypt	- The secretory cells produce hormones and neuropeptides
Enterocytes (columnar epithelial cells)	-Upper the crypt	- Regulate permeability of nutrient, ion and foreign antigens
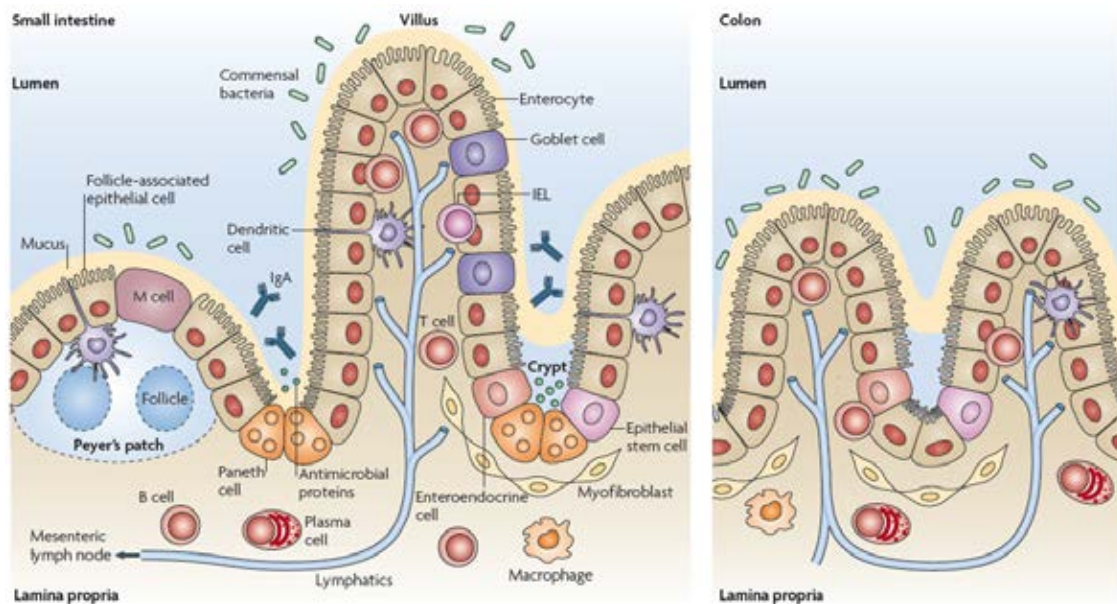
Figure 2. The intestinal epithelial cells (IECs) comprise several special cells type including M cells, goblet cells, paneth cells, enteroendocrine cells and enterocyte (columnar epithelial cells). These special cells type occurs from proliferation and differentiation of epithelial stem cells which special cells type are essential for maintenance of the balance between intracellular and extracellular environment in the intestine [22].

The tight junctions (TJs) is reside the apical intercellular junctions which it is a vital role of regulate diffusion of nutrient, ion, fluid and other molecule through paracellular space. However, the functions of TJs is classified two functions including fence and barrier functions that they are preserve polarity and regulate permeability of cells, respectively [23]. TJs are complexes structure of several proteins; transmembrane proteins that it is connected with actin cytoskeleton by adaptor proteins. This structure is join intercellular space and stabilizes integrity of the intestinal epithelial barrier [24, 25].

Transmembrane proteins are located intercellular and join paracellular space of epithelial cells. The members of transmembrane proteins are including occludin, claudin and junctional adhesion molecules (JAMs). Occludin is a ~65 kDa of molecular weights and composes four transmembrane domains, two extracellular loops, long cytoplasmic C-terminals and short cytoplasmic N-terminals [26]. It is

regulated by kinase and phosphatases that they are associated in assembly and disassembly process [27].

Claudin is a ~20 kDa of molecular weights and similarity with occludin that comprised 27 members, it is constitute four transmembrane domains, two extracellular loops and two cytoplasmic tails [28]. The classification of claudin is dependent on functions, for example as claudin-2 is increase permeability and the part of claudin-1,-3,-4,-5 and -8 are decrease permeability (tighten TJs) [27]. Moreover, claudin-1 and -2 were introduced into mouse L fibroblast lacking TJs that the expression of they are strong ability to form TJs strands. Furthermore, occludin was recruited along with claudin-1 strand when cotransfected with it [29].

Junctional adhesion molecules (JAMs) have a transmembrane domain, one N-terminal extracellular (constituent two extracellular Ig-like domain) and one C-terminal cytoplasmic tail. This protein is a member of immunoglobulin superfamily protein [30]. JAMs was first found by Ines Martin-Padura and coworker, they approve JAMs is a novel protein that it has a role in regulation monocyte transmigration [31]. Furthermore, JAMs has an important role in regulation the assembly of TJs and migration of polymorphonuclear leukocytes (PMN) through to TJs [32-35].

All transmembrane proteins including occluding, claudin and JAMs, these proteins are component of TJs. The tails of their proteins have a PDZ-binding motif that interaction with the actin cytoskeleton by binding zonular occludens (ZOs) that it is adaptor protein. ZOs proteins have three isoforms including ZO-1, ZO-2 and ZO-3. These proteins are cytoplasmic proteins involved with TJs and localized to the TJs of epithelial and endothelial cells. All ZO-1, ZO-2 and ZO-3 are member of the membrane-associated guanylate kinase (MAGUK) family of proteins. MAGUK is a core structure including of several PDZ domains, SH3 (Src homology 3) domain and guanylate kinase (GK) domain. The PDZ domains are mediated of interaction between ZOs proteins with transmembrane proteins [26, 30, 36, 37]. This structure complexes of TJs is beside a role of regulation paracellular permeability, these structure are associated in the regulation of gene expression, polarity, proliferation, differentiation and integrity of epithelial cells. Furthermore, these structure complexes of TJs are the first line of defense against pathogens. However, TJs are destroyed by

pathogens and other factors such as cytokines that they use different mechanism in destruction TJs which lead to various diseases (Table 2) [38].
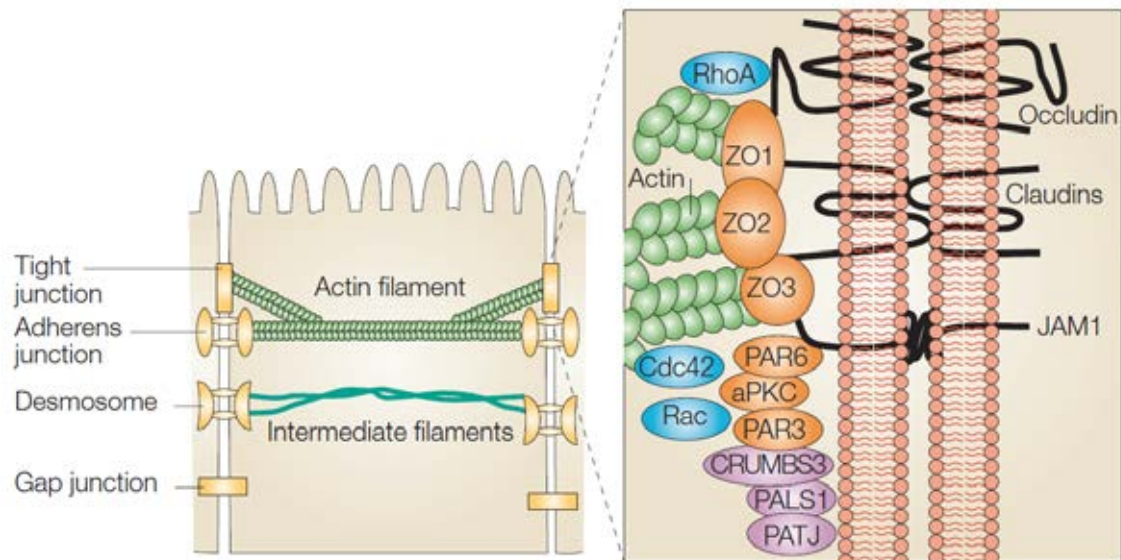
Figure 3. Structure of tight junctions (TJs). It is composed of transmembrane proteins, such as occludin, claudin, junctional adhesion molecules (JAMs) and plaque proteins (zonula occludens; ZOs). The plaque protein is adaptor protein which links transmembrane proteins with actin cytoskeleton. This structure is important in the regulation of permeability and integrity of TJs [7].

Table 2. Summary of researches on tight junctions (TJs) destroyed by various pathogens.

Pathogens	Study summary	Reference
Enterohemorrhagic *Escherichia coli* (EHEC)	EHEC increases expression of claudin-2 and redistribution of claudin-3 and occludin that increase intestinal permeability.	[39]
Enteroaggregative *Escherichia coli* (EAEC)	EAEC causes dissociation of claudin-1 which leads to disruption of TJs.	[40]
Enteropathogenic *Escherichia coli* (EPEC)	EPEC causes disruption of TJs and redistribution of occludin.	[41]
Enteropathogenic *Escherichia coli* (EPEC)	EPEC induces alterations in occludin and increases TJs permeability.	[42]
Salmonella enterica serovar Typhimurium	SopB, SopE, SopE2 and Sip A are effectors of *Salmonella enterica* serovar Typhimurium that cause disruption of TJs.	[43]
Human immunodeficiency virus-1 (HIV-1)	Viral envelope glycoprotein (gp) 120 can increase the permeability of the epithelial barrier.	[44]

Clostridium difficile

Epidemiology

Clostridium difficile is an anaerobic, gram positive-spore forming bacillus that is the most common infectious cause of antibiotic-associated diarrhea and colitis [1]. 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 and distribute worldwide. The incidence rate of CDI has increased from 82,000 in 1996 to 178,000 in 2003 in the United States.[45] Furthermore, in the United

States a significant mortality has been reported that 15,000 to 20,000 patients die from CDI [46]. The estimated 348,950 hospitalizations in the United States revealed that CDI are increase in 1998 to 2008 as shown in Figure 4 [47]. There was 4-fold increase rate of CDI in Quebec, Canada [48]. The major risk factors of CDI are antibiotic treatment (Table 3 and Figure 5) and hospitalizations which lead to various clinical symptoms from asymptomatic to mild self-limited diarrhea and severe pseudomembranous colitis [2]. 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 [49, 50]. In addition, CDI are reported in non-risk group such as children, pregnant women, community-acquired infection and no previous exposure to antibiotic [47].
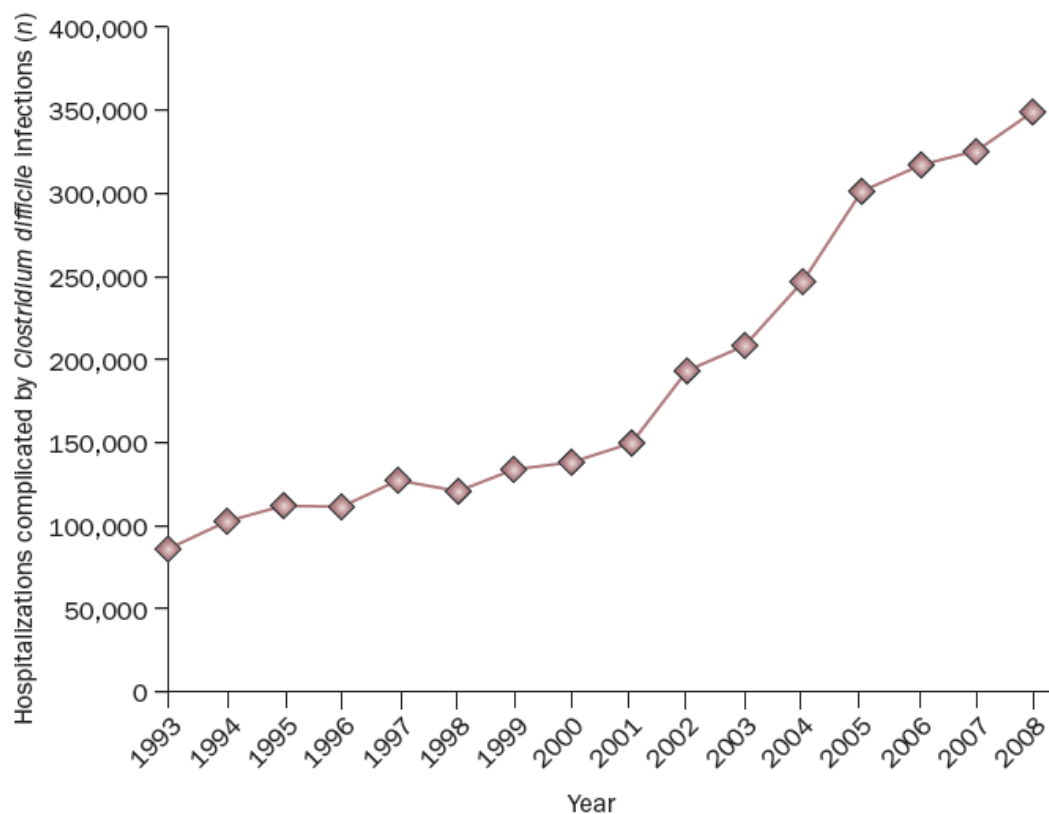
Figure 4. The increasing number of hospitalizations in the United State complicated by *Clostridium difficile* infection (CDI) [47]
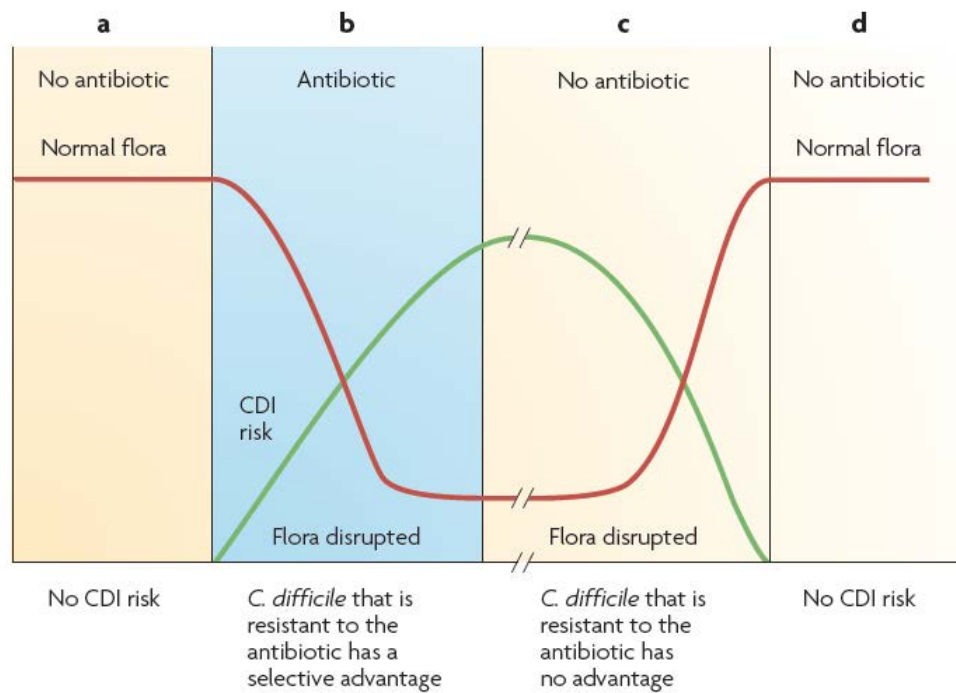
Figure 5. The effect of antibiotic to normal flora in the intestine. (a) Antibiotics are not affect to normal flora of Patients which resistant to CDI. (b) When antibiotic treatment starts, infection with *C. difficile* strain that it is resistant to antibiotic and the antibiotics are remain in the intestine. (c) When antibiotic treatment stops, the antibiotic in the intestine has low level, but normal floras are disturbed from antibiotic for a variable period of time (indicated by the break in the graph) which depending on the antibiotic given. (d) During this time, patients can be infected with either resistant or susceptible *C. difficile*. However, when the normal flora recovers, homeostasis of the intestine is restored [46].

Table 3. Antimicrobial agents that predispose to *C. difficile* diarrhea and colitis [51].

Frequently	Infrequently	Rarely or never
Ampicillin and amoxicillin	Tetracyclines	Parenteral aminoglycosides
Cephalosporins	Sulfonamides	Bacitracin
Clindamycin	Erythromycin	Metronidazole
	Chloramphenicol	Vancomycin
	Trimethoprim	
	Quinolones	

Virulence factors and pathogenesis

Clostridium difficile antibiotic-associated diarrhea (CDAD) is as part of CDI occurs 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 that they are the important of virulence factors of *C. difficile*. An additional binary toxin (CDT) is also expressed in some virulent strain groups [3, 4, 52]. The pathogenic process starts with initial colonization in human intestinal epithelial and the production two toxins of *Clostridium 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[5]. Toxin A and toxin B modify and inactivate Rho proteins via glucosylation. Glucosylation of Rho GTPases results in rearrangement of actin cytoskeleton, disruption of tight junctions, rounding up of cell, cell death and loss of intestinal epithelium barrier function [6, 7, 53]. Previous studies showed that *Clostridium difficile* toxins disrupt tight junctions which observed from the decrease in transepithelial electrical resistance and the increase paracellular permeability in epithelial cell lines incubated with toxin [8-10]. Disruptions of tight junctions grant the toxin move through the cell membrane. The toxins 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 (Figure 6) [11, 12].
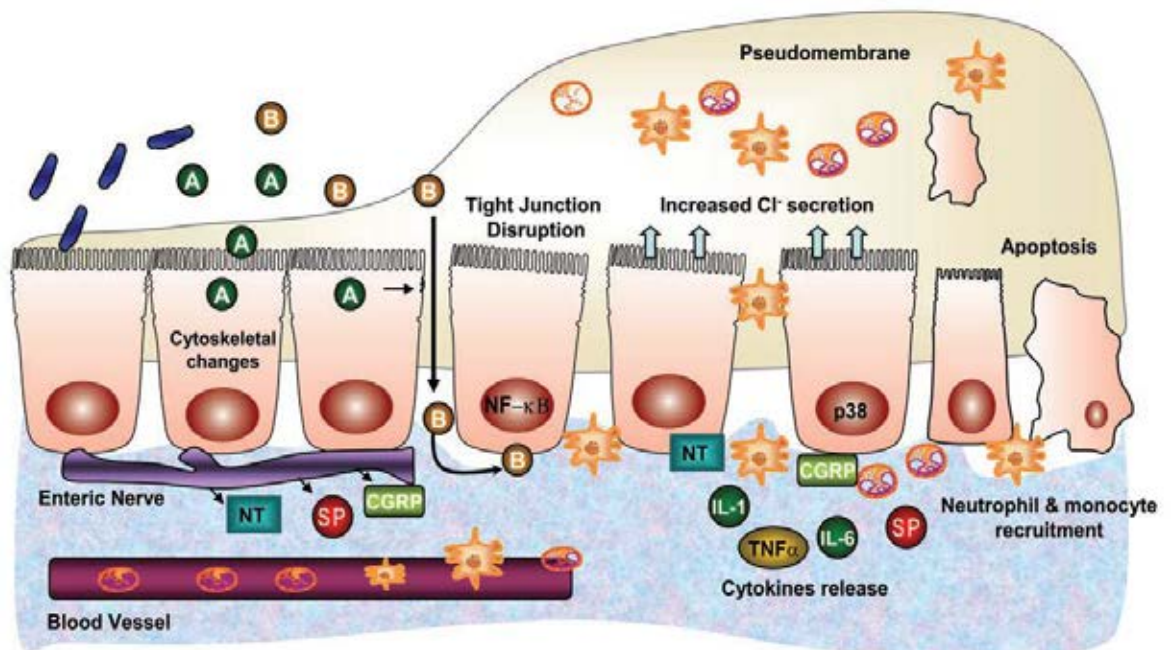
Figure 6. Pathogenesis of *Clostridium difficile*. *C. difficile* produce toxin A (Tcd A) and toxin B (Tcd B), toxin A binds to specific receptor on the surface of the intestinal epithelium that it is affect to disruption of tight junctions, then toxin B can move through the basolateral side of cell membrane. The toxins induce disruption of tight junctions and releasing of proinflammatory cytokines which lead to recruitment of neutrophils and monocytes, cell apoptosis and connective tissue degradation, resulting in pseudomembrane formation and diarrhea [5].

Treatment

The common agents for CDI treatment are metronidazole and vancomycin [54]. The metronidazole has failure to treatment patients with severe disease when compare with vancomycin (Figure 7). Thus patients with mild-moderate disease were treated metronidazole and the patients with severe disease were treated vancomycin. However, the present these agent has efficacy to treatment patients with CDI but 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 [55]. The recurrence of disease occurs two form including relapse and reinfection. The relapse is infection of the same strain (original

strain) with initial episode. The reinfection is infection of different strain which it is occur more than relapse [3, 49, 56]. The present, probiotics can reduce the recurrence of disease. The study the effect of *Saccharomyces boulardii*, *Lactobacillus* GG and *Lactobacillus plantarum* 299V in clinical trial that the results show reduction of recurrent of disease [57-59].
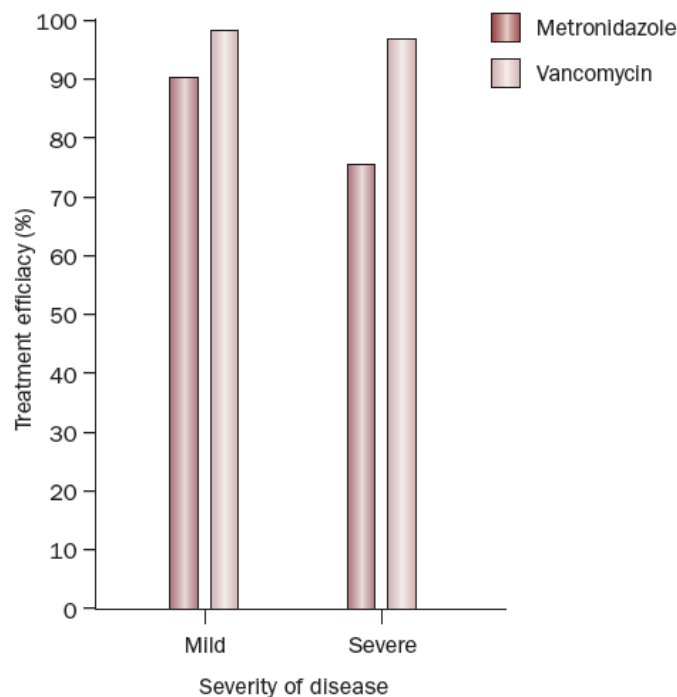
Figure 7. Comparison of the effect of metronidazole and vancomycin for patients with *Clostridium difficile* infection (CDI). Metronidazole has failure to treatment patient with severe disease when compared with vancomycin [47, 60].

Probiotics

The FAO/WHO provide the definition of probiotics that are 'live organisms which when administered in adequate amounts confer a health benefit on the host' [61]. The most common probiotics include *Lactobacillus species*, *Bifidobacterium species*, *Escherichia coli* (such as *E. coli* Nissle 1917) and yeast (such as *Saccharomyces boulardii*) [61]. These probiotics were used to prevent and treat in patient with gastrointestinal disorder, especially antibiotic-associated diarrhea (AAD) and *Clostridium difficile* infection [62, 63]. The treatments with antibiotics disturb and

inhibit growth of normal flora in intestine. In contrast, probiotics can restore and preserve homeostasis of normal flora in intestine as shown in Figure 8 [61]. Furthermore, probiotics have other beneficial effects to host including increase integrity of tight junction, immunomodulate and compete with pathogen in adherence to surface of intestinal epithelium (Figure 9) [64, 65].
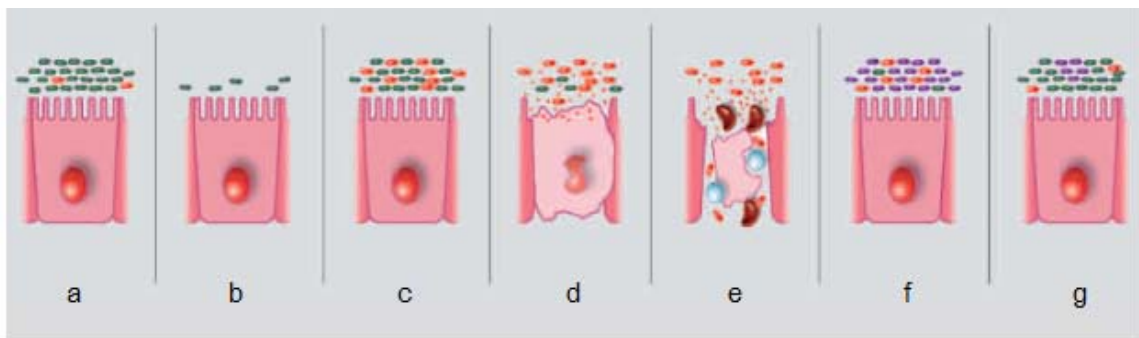
Figure 8. Homeostasis of normal flora in intestine (adapted from [66]). (a) Homeostasis of normal flora (green) and pathogen (red) that they are distributed on surface of intestinal epithelium. (b) Normal flora is destroyed by antibiotics or other factor (such as pathogen, stress etc.). (c) Pathogen is overgrowth. (d) The intestinal barrier and cells are destroyed. (e) The intestinal barriers are disrupted and leak. (f) Probiotic (purple) growth is numerous. (g) Probiotics can restore normal flora and homeostasis in intestine [61].
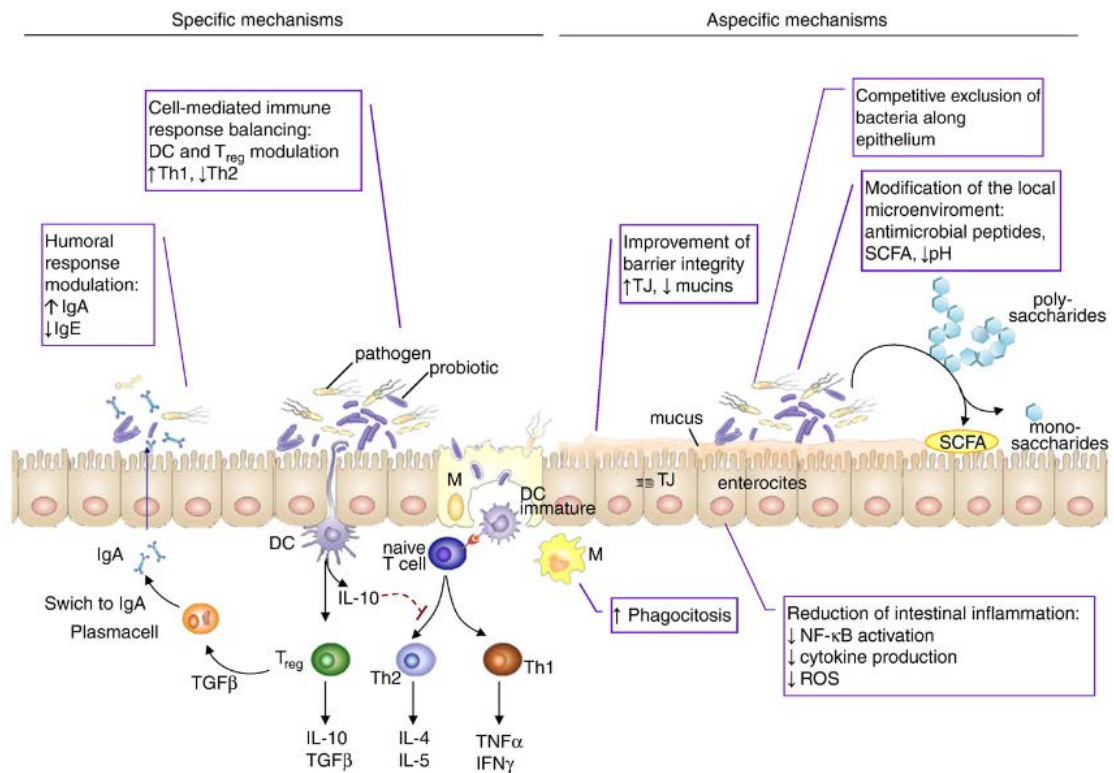
Figure 9. Mechanisms of probiosis which include specific and aspecific mechanisms. Th, T helper cell; Ig, immunoglobulin; Treg, regulatory T cell; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IFN, interferon; M, M cell; DC, dendritic cell; TJ, tight junction; MΦ, macrophage; SCFA, short-chain fatty acid; NF-κB, nuclear factor-κB; ROS, reactive oxygen species [67].

Lactobacillus

Lactobacilli are lactic acid bacteria (LAB) which are gram-positive, nonspore-forming rods or coccobacilli with low G+C content. They are fermentative which classified into three groups including obligately homofermentative, facultatively heterofermentative and obligately heterofermentative [68, 69]. Lactobacilli were isolated from several sources of human and animal including GI tract, vaginal tract, and oral which they have benefit to human body. Some strains of lactobacilli were used in food industry while some strains of lactobacilli are probiotics [68]. At present, *Lactobacillus* was used as probiotics in order to prevent disease in humans. Current researches reported that *Lactobacillus* can enhance the integrity of intestinal barrier

function and prevent pathogen-induced damage in epithelial barrier function (Table 4).

Table 4. Summary of the studies ofthe effect of lactobacilli on the enhancement of intestinal barrier   function.

Lactobacillus species	Study summary	References
Lactobacillus plantarum MB452	*Lactobacillus plantarum* MB452 increases the integrity of TJs as determined by the increased transepithelial electrical resistance (TEER) in a dose-dependent manner.	[70]
Lactobacillus plantarum DSM 2648	*L. plantarum* DSM 2648 enhances the integrity of TJs destroyed by enteropathogenic *Escherichia coli* (EPEC) O127:H6 (E2348/69)	[71]
Lactobacillus plantarum	*L. plantarum* surface layer adhesive protein (SLAP) increases both mRNA and protein level of TJs proteins, decreases level of adhesion of enteropathogenic *Escherichia coli* (EPEC) on Caco-2 cells.	[72]
Lactobacillus rhamnosus strain GG	*L. rhamnosus* strain GG prevents the redistribution of TJs induced by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7	[14]
Lactobacillus plantarum	*L. plantarum* prevents the changing of TJs induced by enteroinvasive *Escherichia coli* (EIEC) and enteropathogenic *Escherichia coli* (EPEC)	[15, 16]

CHAPTER III

MATERIAL AND METHODS

1. Bacterial strains and culture conditions

A total of 29 *Lactobacillus* spp. which were previously isolated from infant feces, breast milk and gastric biopsies were used in this study (Table 5). They were inoculated on de Man, Rogosa and Sharpe agar (MRS, Oxoid, Oxoid Ltd., Basingstoke, Hampshire, England) and 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.

Clostridium 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. 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.

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

Caco-2 human colorectal adenocarcinoma cells (ATCC HTB-37) were grown as previously described by Anderson *et al*.[71] with modification. 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 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 ^{0}C 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. After incubation for 24 h, TEER 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:

$$\text{TEER } (\Omega.cm^2) = (\text{Total resistance} - \text{Blank resistance}) \ (\Omega) \ x \ \text{Area } (cm^2).$$

3. *Lactobacillus* spp. and pathogens coculture

Lactobacillus spp. and pathogens were prepared as described above. *Lactobacillus* spp. 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* spp. Each pathogen was also tested for its effect on TEER. One hundred microlitres of $1.0x10^8$ -$1.0x10^9$ CFU/mL *Lactobacillus* spp. and 100 µL of $1.0x10^7$ -$1.0x10^8$ CFU/mL pathogens were added at the apical side. After incubation for 24 h, TEER was measured as described above.

4. The effect of cells viability on TEER

Selected *Lactobacillus* species was killed by 254-nm ultraviolet irradiation for 45 minute 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 agar and 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.

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$^®\Delta$, 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. 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.

CHAPTER IV

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 6-9 and Figures 10-13. 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[73]. 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[73] were selected for further study although they decreased TEER. Summary of selected *Lactobacillus* spp. for further investigation was shown in Table 10.

Table 5. *Lactobacillus* used in this study

Lactobacillus isolated from infant feces	*Lactobacillus* 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)		*L. casei* B106(LC-B106)
L. rhamnosus L34(LR-L34)		
L. rhamnosus L35(LR-L35)		
L. casei L39 (LC-L39)		

Table 6. 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
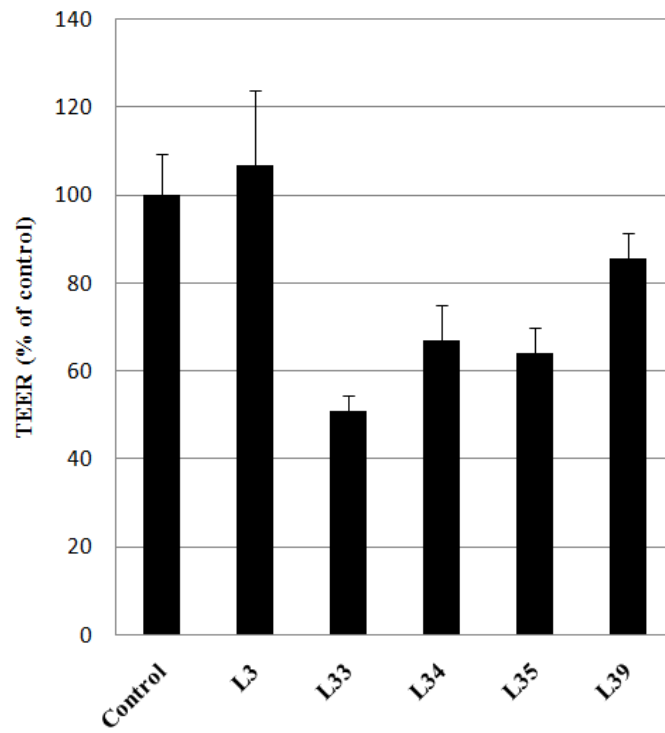
Figure 10. Change in the TEER by *Lactobacillus* spp. isolated from infant feces across Caco-2 human colorectal adenocarcinoma cells. (*$p<0.05$)

Table 7. 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
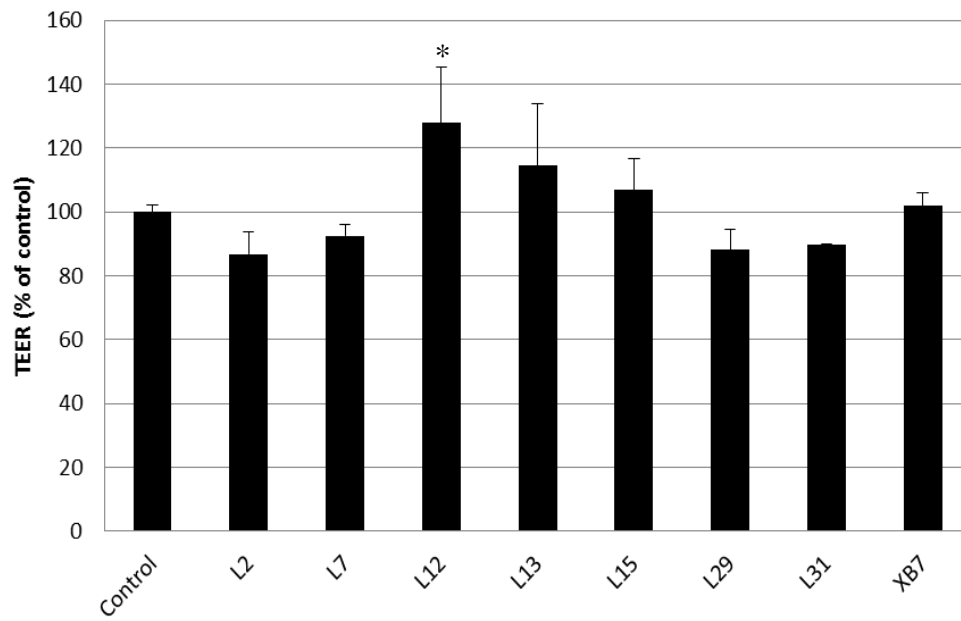
Figure 11. Change in the TEER by *Lactobacillus* spp. isolated from infant feces and gastric biopsies across Caco-2 human colorectal adenocarcinoma   cells. (*$p<0.05$)

Table 8. 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
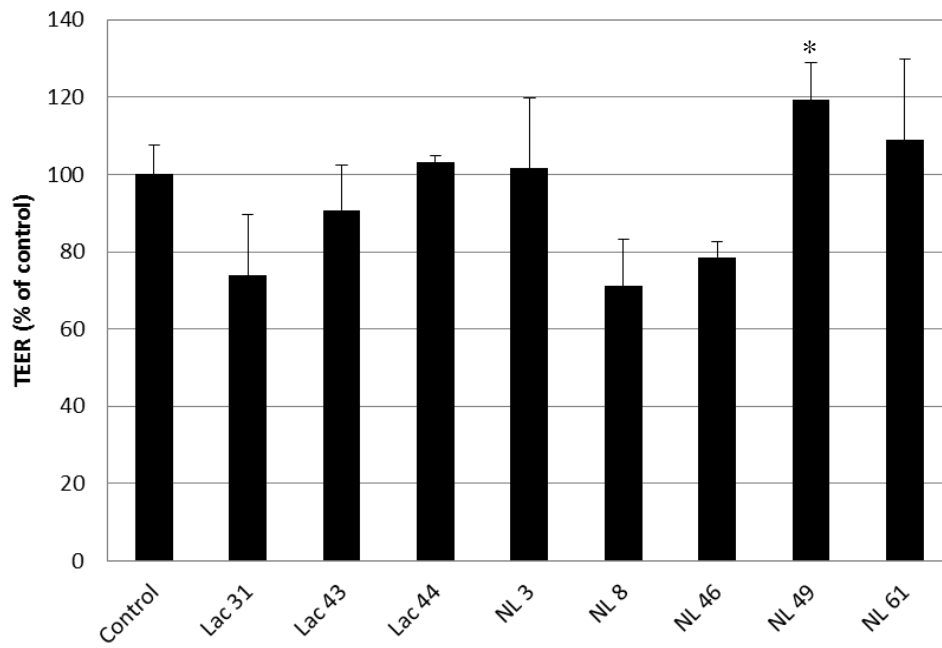
Figure 12. Change in the TEER by *Lactobacillus* spp. isolated from breast milk across Caco-2 human colorectal adenocarcinoma cells. (*$p<0.05$)

Table 9. 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
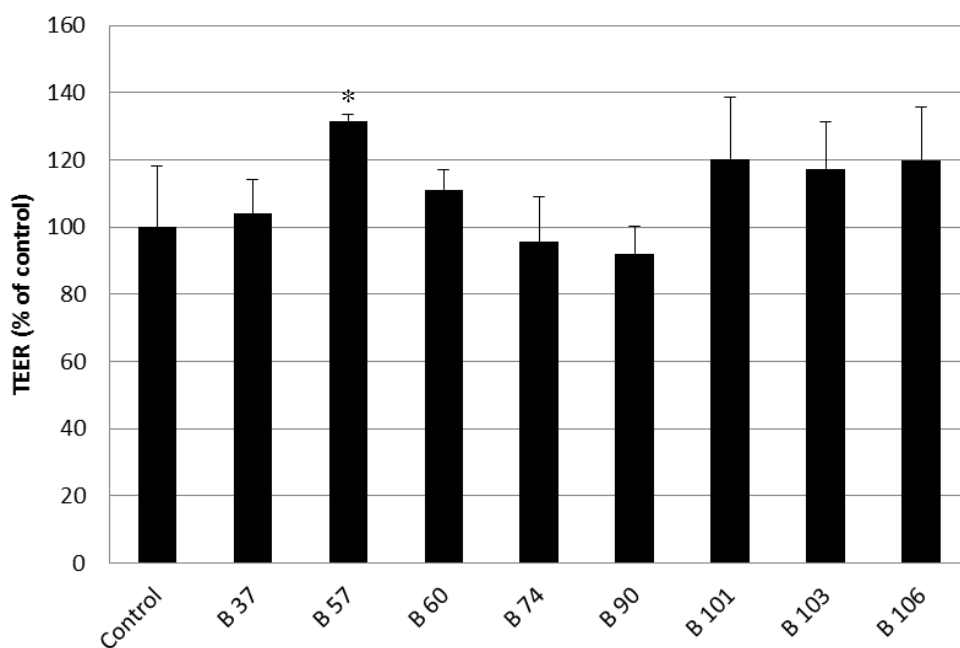
Figure 13. Change in the TEER by *Lactobacillus* spp. isolated from gastric biopsies across Caco-2 human colorectal adenocarcinoma cells. (*$p<0.05$)

Table 10. 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. oris* NL49 (LO-NL49) *L. murinus* B57 (LM-B57)	*L. gasseri* L3 (LG-L3) *L. plantarum* XB7 (LP-XB7) *L. salivarius* B37 (LS-B37) *L. salivarius* B60 (LS-B60)	*L. rhamnosus* L34 (LR-L34) *L. casei* L39 (LC-L39) *L. plantarum* B90 (LP-B90)

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 cholerae* 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 (1×10^7 - 1×10^9 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 14 and 15, 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.
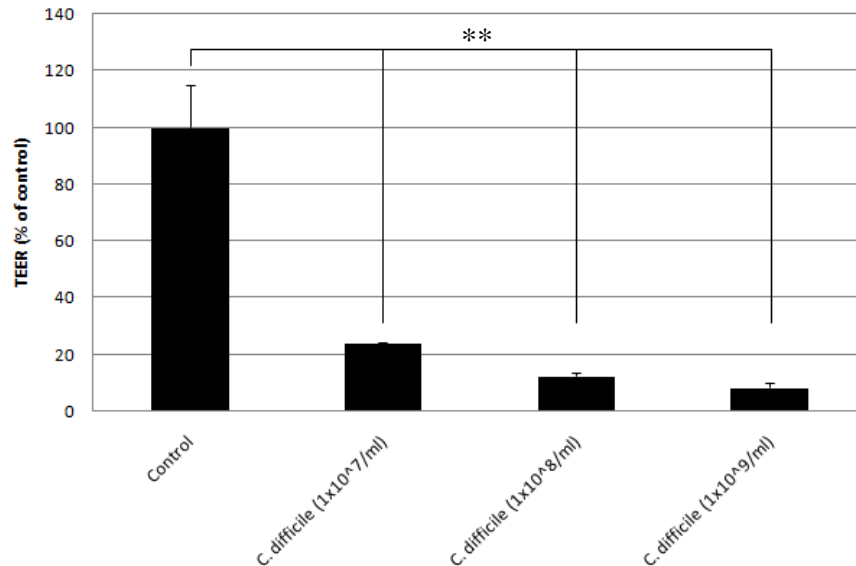
Figure 14. The effects of *C. difficile* on tight junctions in Caco-2 human colorectal adenocarcinoma cells. SD, standard deviation; significantly lower than the control (DMEM, Caco-2 media control), ** *p*<0.01. The experiments were performed once in duplicate.
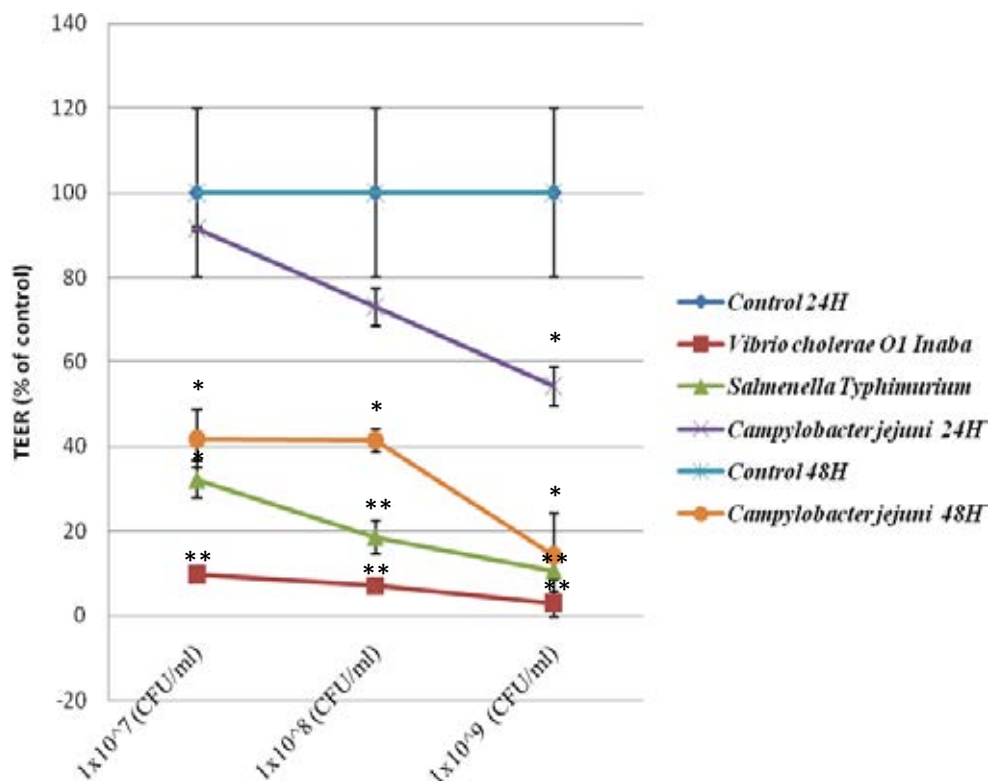
Figure 15. The effects of *V. cholerae* 01 Inaba*, S.* Typhimurium ATCC 13311 and *C. jejuni* on tight junctions in Caco-2 human colorectal adenocarcinoma cells. SD, standard deviation; significantly lower than the control (DMEM, Caco-2 media control), * $p<0.05$, ** $p<0.01$. The experiments were performed once in duplicate.

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. (1×10^9 CFU/ml) for 3 hours and infected with *C. difficile* (1×10^8 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 11 and Figure 16.

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.

Table 11. 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
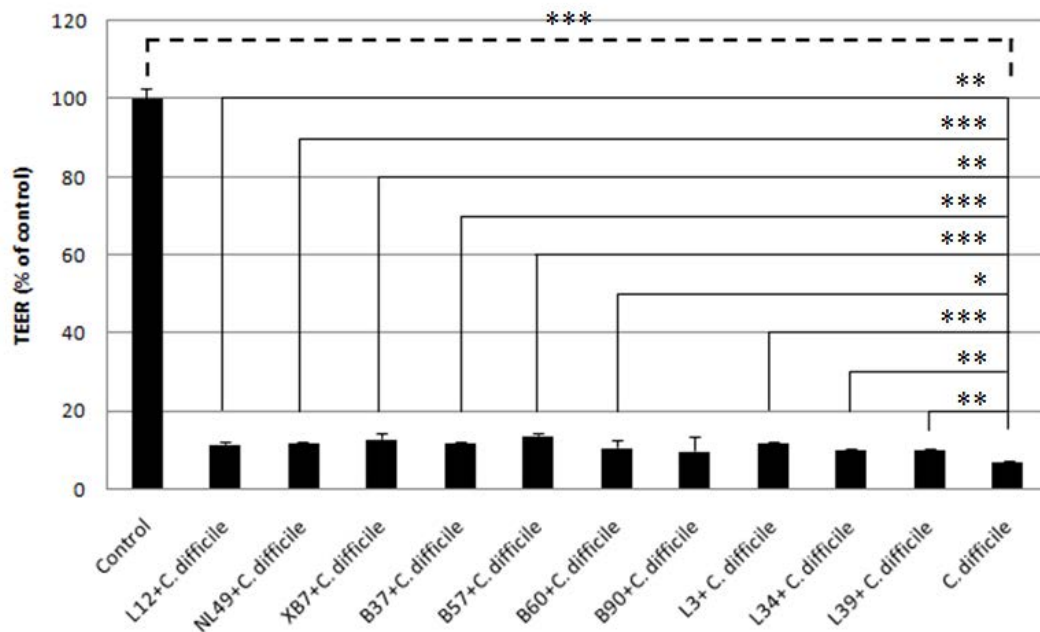
Figure 16. The enhancement effects of *Lactobacillus* spp. on transepithelial electrical resistance (TEER) when co-culture with *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, **$p<0.01$, ***$p<0.001$

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 1×10^{8}-1×10^{10} CFU/mL LC-L39 were co-cultured with 1×10^{7}-1×10^{9} CFU/mL *C. difficile* and TEER was determined in each combination. The result in Figure 17 indicated that the proportion of 1.0×10^{8} CFU/mL LC-L39 and 1.0×10^{7} CFU/mL *C. difficile* was appropriate and used in further experiment.
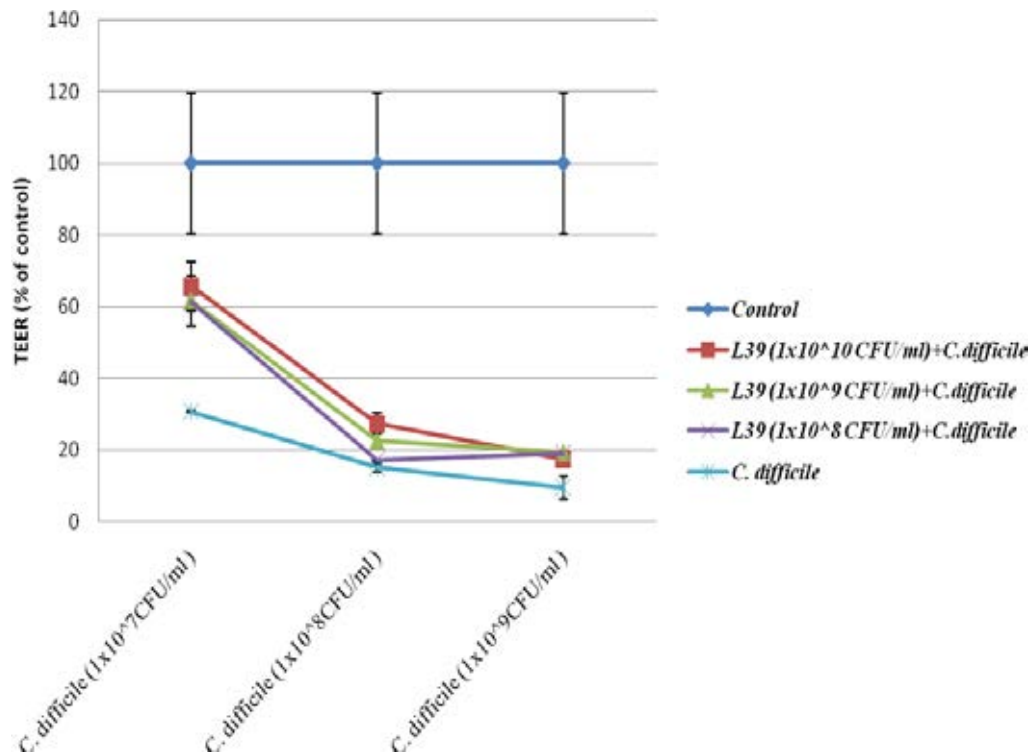
Figure 17. TEER from different proportion of *Lactobacillus casei*-L39 and *C. difficile*. The experiments were performed once in duplicate**.**

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 12-15), whereas LF-XB7 alone decreased TEER (Table 15). 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 12-15 and Figures 18-21). 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.

Table 12. 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 (1×10^8)	115.95	21.24	110.67	0.1009489
L34(1×10^8)+ *C. difficile*(1×10^7)	67.77	25.45	64.69	0.0002926***
C. difficile(1×10^7)+ L34(1×10^8)	38.53	18.83	36.77	0.0413885*
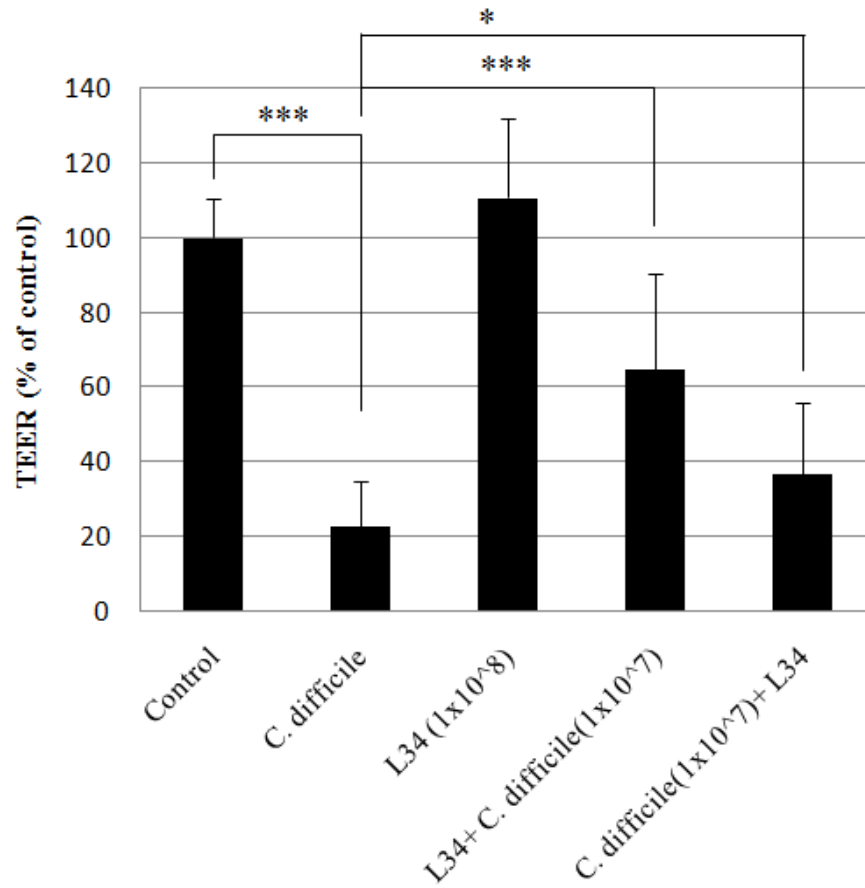
Figure 18. The enhancement effects of LR-L34 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *p*<0.05, **p*<0.01, ***p*<0.001

Table 13. 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*
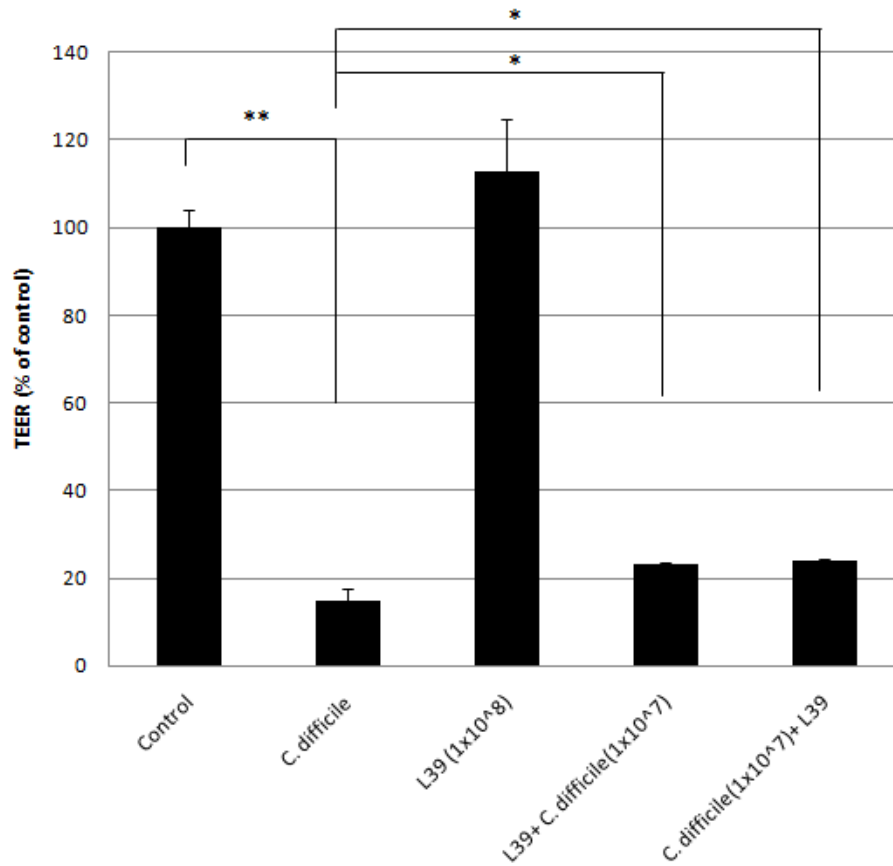
Figure 19. The enhancement effects of LC-L39 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, **$p<0.01$

Table 14. 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
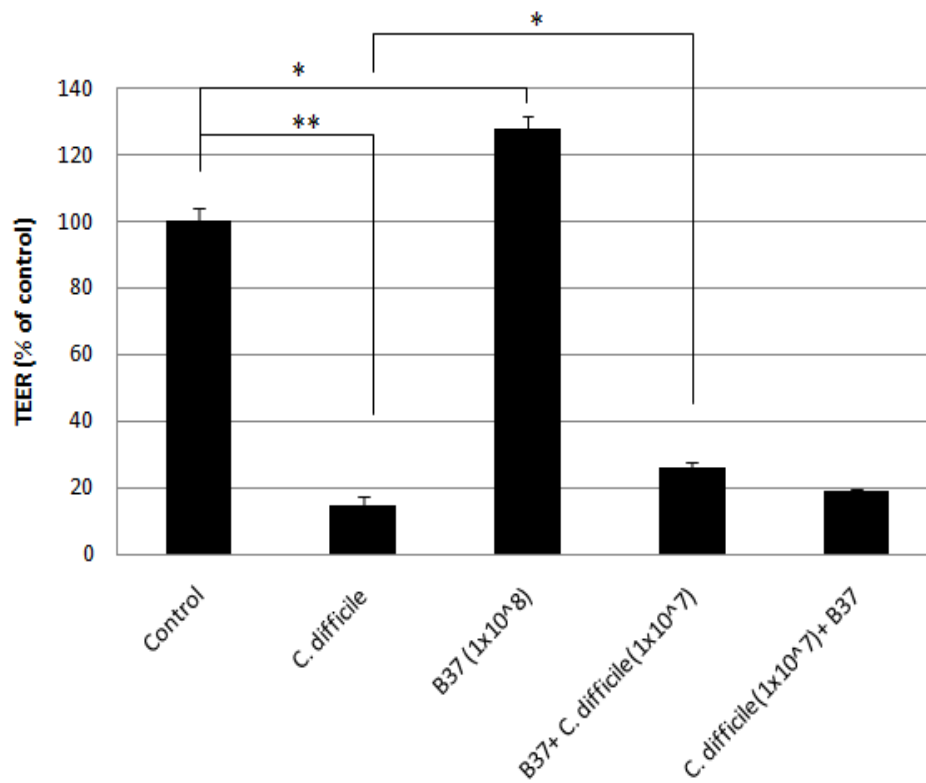
Figure 20. The enhancement effects of LS-B37 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, **$p<0.01$

Table 15. 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.

Identity	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
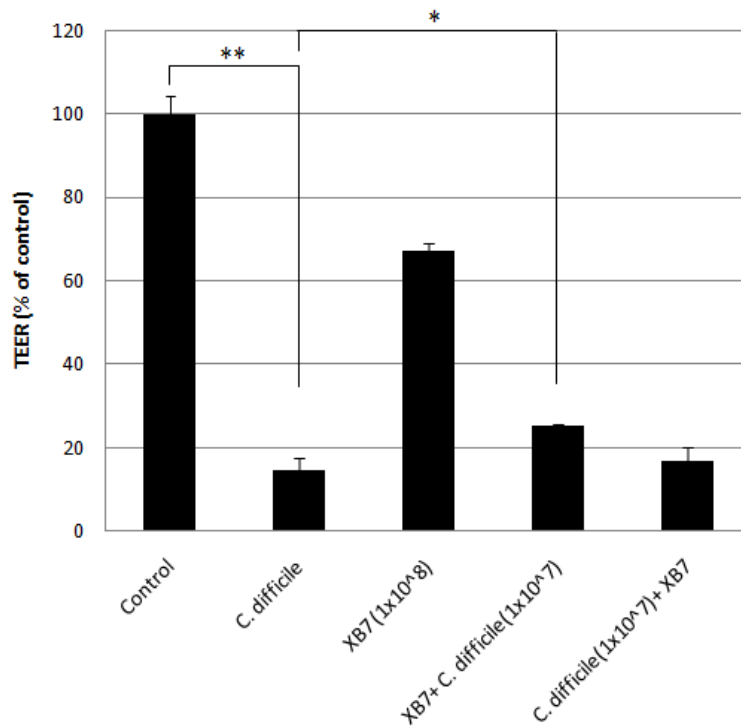
Figure 21. The enhancement effects of LF-XB7 on transepithelial electrical resistance (TEER) when added before or after *Clostridium difficile* in Caco-2 human colorectal adenocarcinoma cells. DMEM, Caco-2 media control; SD, standard deviation; significantly different from DMEM, Caco-2 media control, *$p<0.05$, **$p<0.01$

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

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 16-19 and Figures 22-25. 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.

Table 16. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *C. 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 ($\Omega.cm2$)	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*
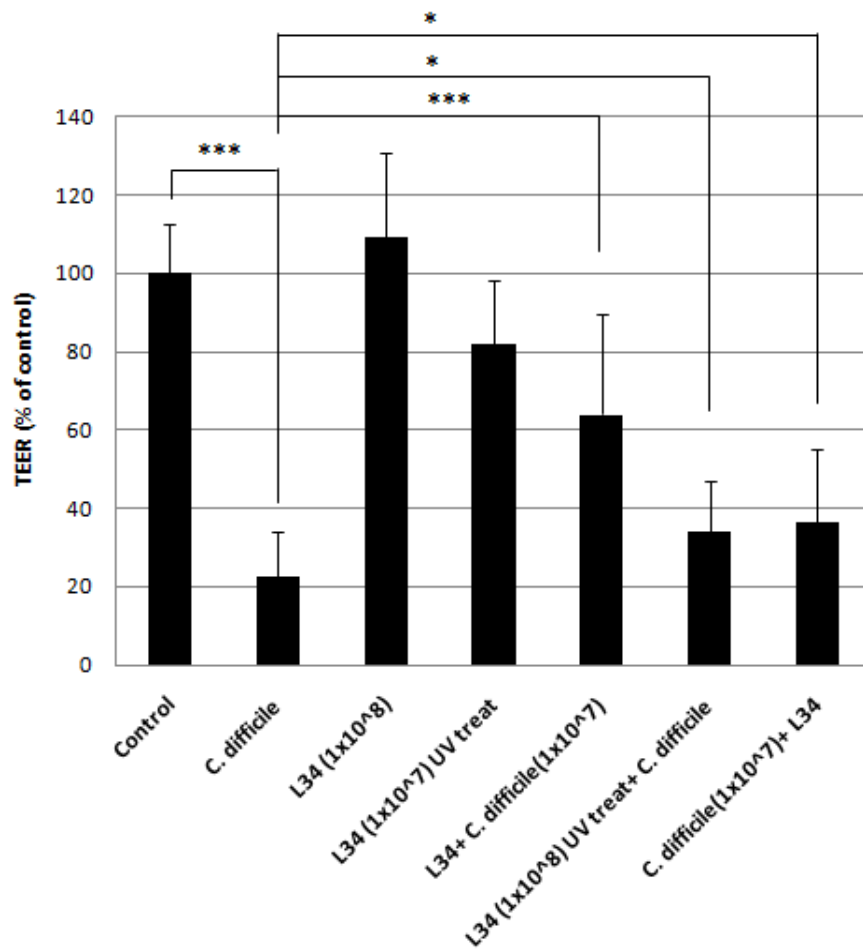
Figure 22. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *C. difficile* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed four times in duplicate. *$p<0.05$, ***$p<0.001$

Table 17. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *V. 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 ($\Omega.cm2$)	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
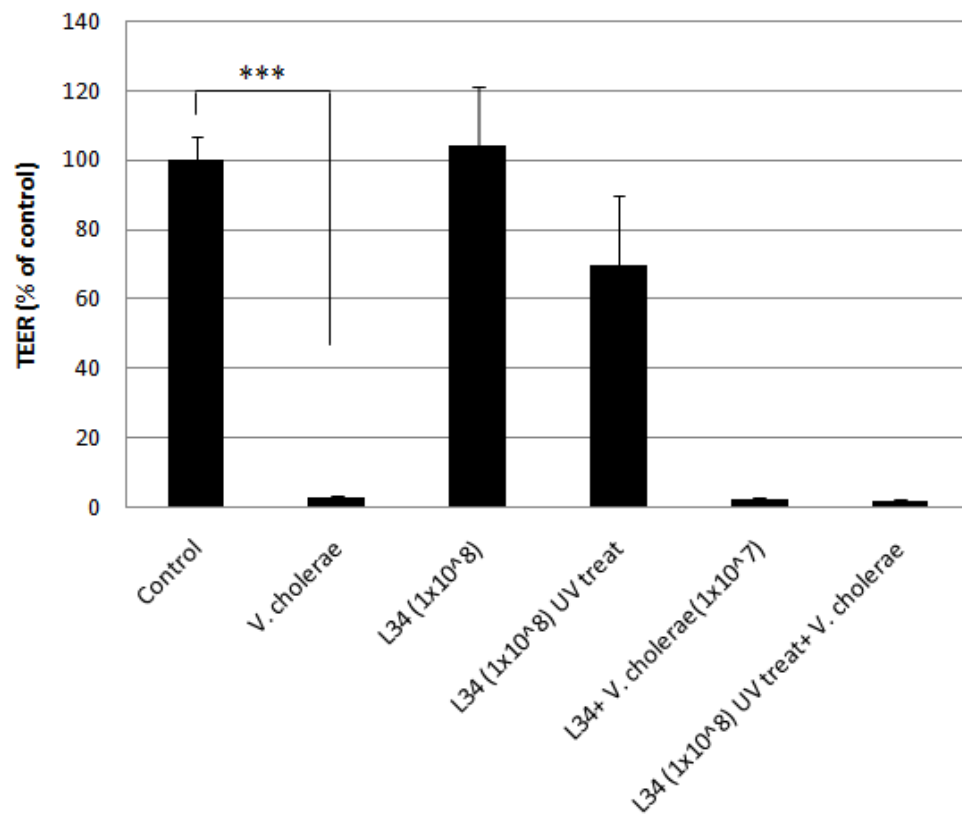
Figure 23. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *V. cholera*e O1 Inaba in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate. ***$p < 0.001$

Table 18. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *S.* 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 (1×10^8)	87.29	0.70	93.96	0.16864
UV-treatedL34 (1×10^8)	89.43	7.93	96.27	0.33819
Live L34(1×10^8) + *S.* Typhimurium(1×10^7)	30.53	1.63	32.86	0.11589
UV-treated L34 (1×10^8) + *S.* Typhimurium(1×10^7)	28.55	1.17	30.73	0.50000
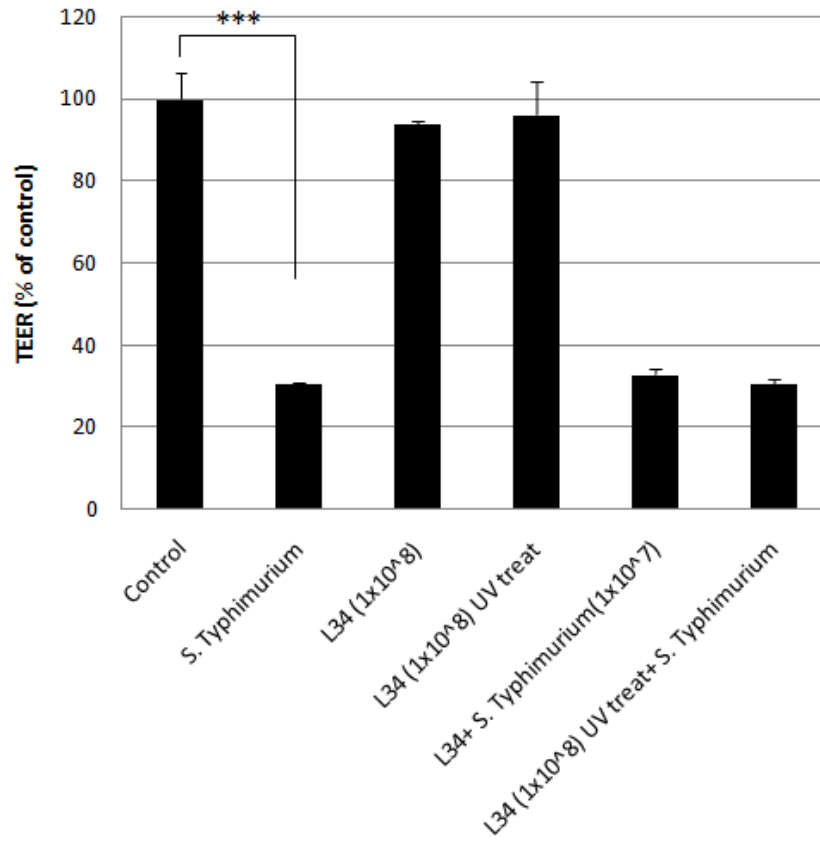
Figure 24. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *S.* Typhimurium in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed two times in duplicate. ***$p < 0.001$

Table 19. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *C. 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 ($\Omega.cm2$)	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**
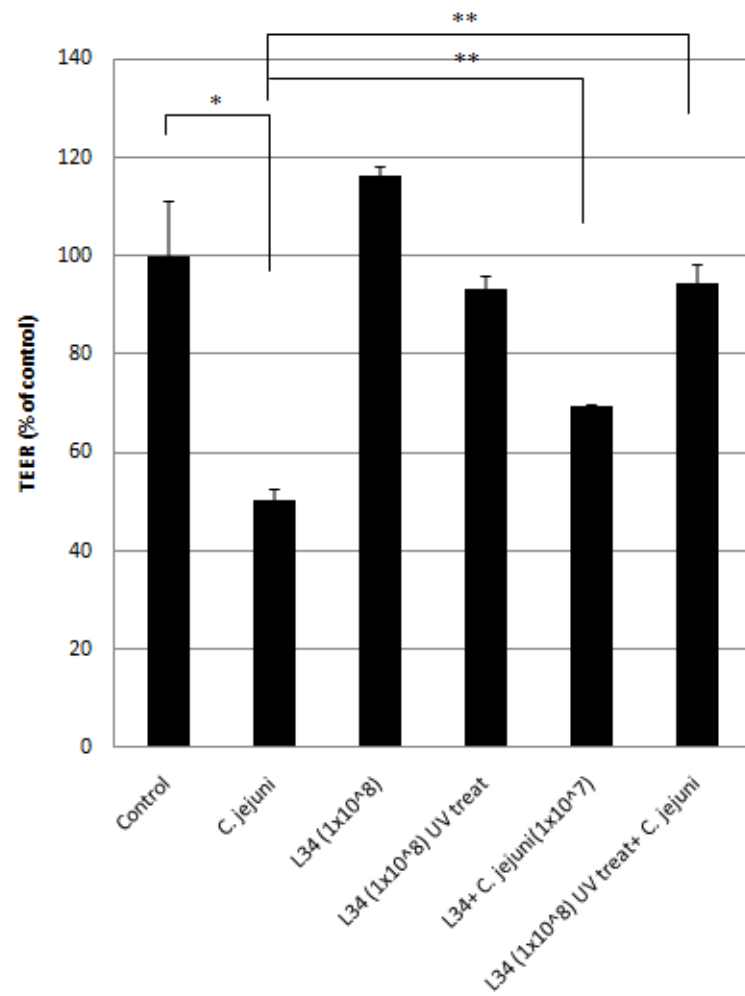
Figure 25. The enhancement effects of live or UV-treated LR-L34 on TEER when coculture with *C. jejuni* in Caco-2 human colorectal adenocarcinoma cells. The experiments were performed three times in duplicate. *$p<0.05$, **$p<0.01$

7.   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 26).

A

	24 Hours			
DMEM	+	+	+	+
Caco-2 cells	+	+	+	+
L. rhamnosus L34	-	+	+	-
C. difficile	-	-	+	+
Occludin					65 kDa
JAM-1					37 kDa
Claudin-1					20 kDa
β-actin					45 kDa
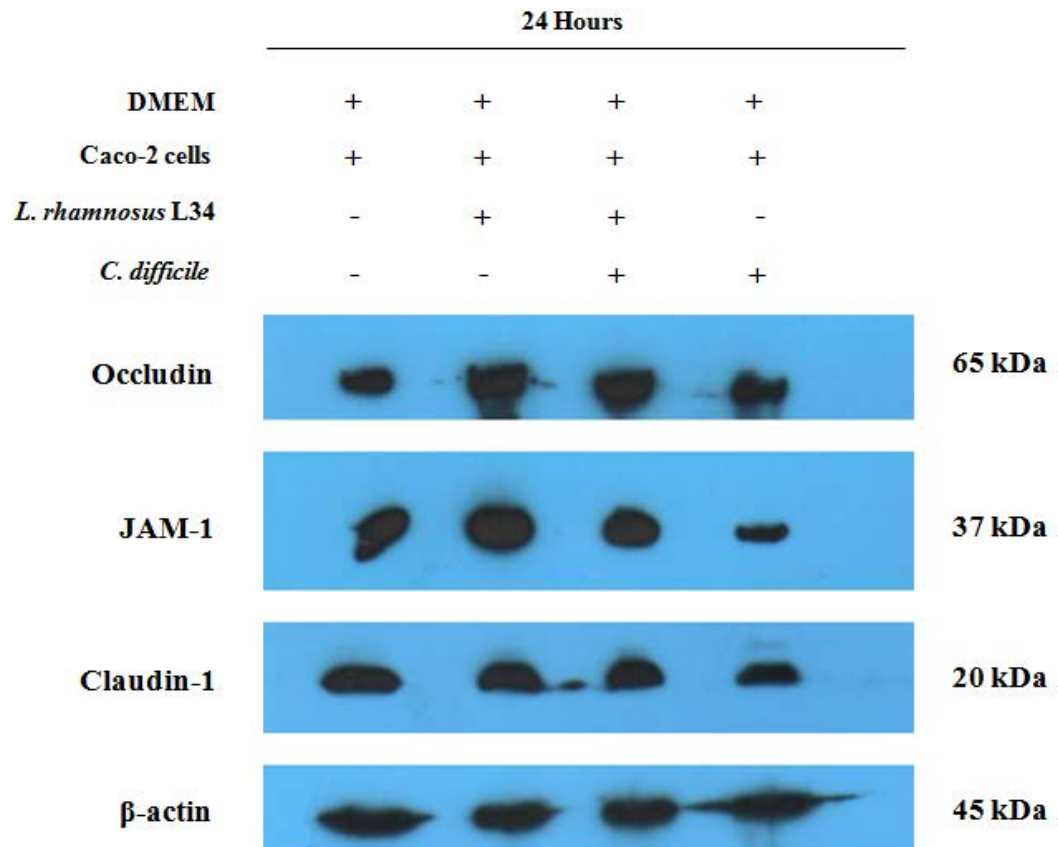
B
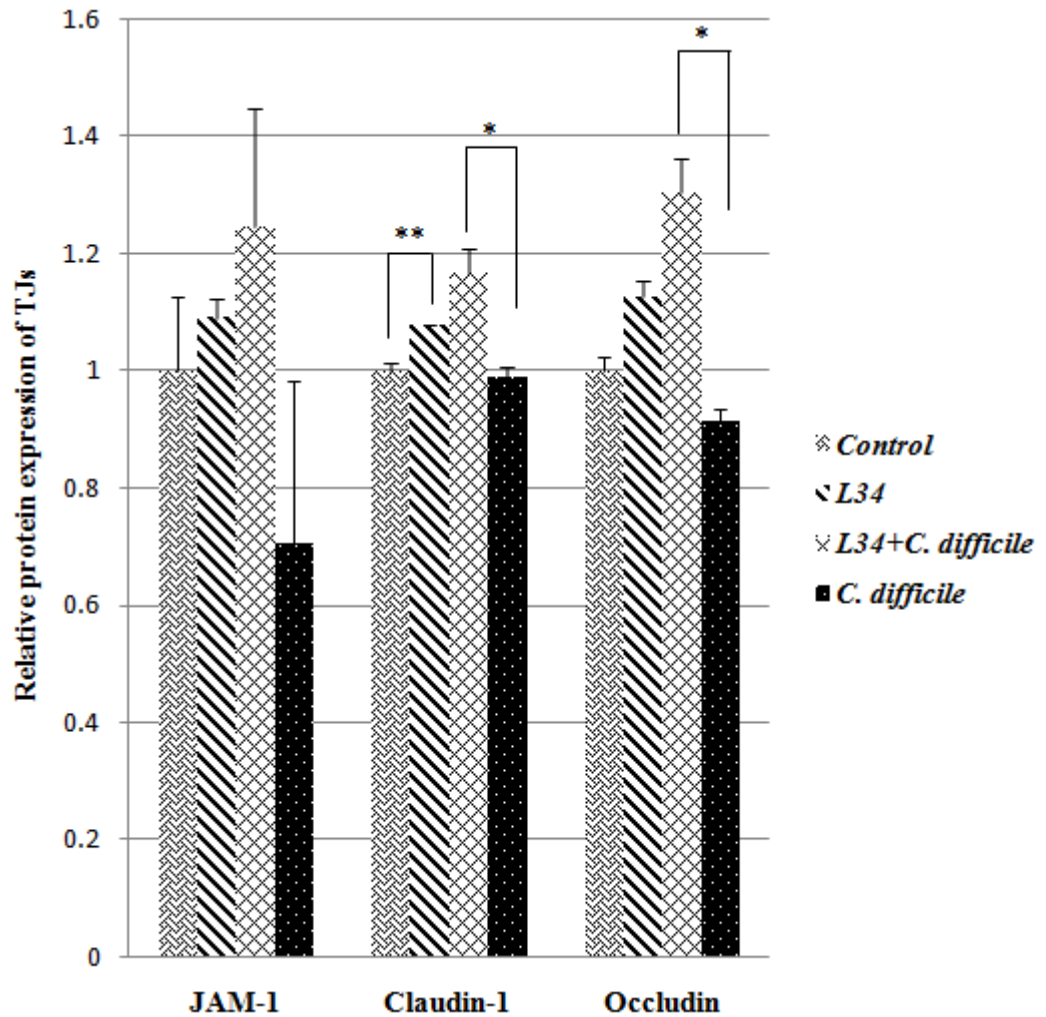
Figure 26. The expression of TJs proteins. A) Representative result of western blot analysis of JAM-1, Claudin and Occludin. B) Semi-quantitative analysis of western blot showed protein expression at different conditions. Values were calculated by Student's *t*-test.

CHAPTER V

DISCUSSION

The intestine contains normal flora about 10 trillion microbes with many different species, amounting to 1-2 kg in weight [61, 67, 74]. Some normal flora are probiotic bacteria which confer health benefit to host including 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 TJs and restore normal flora when host was encroached from pathogens [64, 74]. Furthermore, the efficacy of probiotics is strain-specific to each group of population [75]. 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. Recently, it has been reported that *L. plantarum* MB452 increases the integrity of TJs by increasing expression of genes involved in tight junction assembly [70] and oral-derived *L. plantarum* DSM 2648 increases TEER in high magnitude [70, 71].

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 [43, 76]. In addition, TJs can be destroyed by other factors such as pro-inflammatory cytokines and oxidative stress[77, 78]. Bruewer *et al.* demonstrated that IFN-γ and TNF-α can disrupt TJs proteins on T84 intestinal epithelial cells [79]. Current researches reported that *C. difficile, V. cholerae*, *S.* Typhimurium and *Campylobacter jejuni* can disrupt TJs proteins resulting in the decrease of TEER [10, 80-84]. 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 [61]. Most used probiotics include *Lactobacillus species*, *Bifidobacterium species*, *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 [85-87]. The ability of probiotics to protect the disruption of TJs by pathogens were previously reported. For examples, *L. rhamnosus* strain GG prevents the redistribution of TJs induced by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 [14]. *L. plantarum* prevents the changing of TJs induced by enteroinvasive *Escherichia coli* (EIEC) and enteropathogenic *Escherichia coli* (EPEC) [15, 16]. Furthermore, *L. plantarum* DSM 2648 enhances the integrity of TJs destroyed by enteropathogenic *Escherichia coli* (EPEC) O127:H6 (E2348/69) [71]. Both live *S. thermophilus* and *L. acidophilus* protect intestinal epithelial cells from the destruction of enteroinvasive *Escherichia coli* (EIEC 029: NM) in Caco-2 and HT-29 cells [88]. *Lactobacillus plantarum* (LBP)strain 299v increased TEER in Caco-2 cells when exposed to LBP alone and reduced secretory changing when induced by EPEC strain E2348/69 [89]. In addition, secreted bioactive factors from *B. infantis* increased the integrity of intestinal epithelial barrier by modulating TJs proteins redistribution and MAPK pathway [90]. Protease secreted from *S. boulardii* protects the destruction of epithelial epithelial barrier by *C. difficile* toxins A and B[57]. Our study showed that *L. rhamnosus* L34 at $1x10^7$ CFU per well increased TEER when cells were treated with L34 alone. L34 increased TEER significantly ($p< 0.001$) when cells were pretreated with L34 for 3 hours before the addition of *C. difficile* . UV-treated L-34 also increased TEER significantly ($p< 0.05$) which suggested that L34 cells hinder the binding of *C. difficile* toxins to initiate the activities. However, live L-34 increased TEER more than UV-treated L-34 which suggested that L34 secretes bioactive product capable of modulation of TJs production and redistribution.

L. plantarum prevented the damage of expression and rearrangement of claudin-1, occludin, JAM-1 and ZO-1 proteins induced by enteroinvasive *Escherichia*

coli (EIEC) [15]. Zhi-Hua Liu *et al.* demonstrated that *L. plantarum* also prevented the decrease in the expression of TJ proteins and F-actin in NCM460 cells induced by enteropathogenic *Escherichia coli* (EPEC) [16]. However, 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-significancly 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, 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.

Destruction of intestinal barrier function by *C. difficile* toxin A and toxin B was the modification and inactivation of Rho proteins via glucosylation. The glucosylation of Rho GTPases results in rearrangement of actin cytoskeleton and disruption of tight junctions [91-97]. Signaling pathways which regulate TJs proteins include protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and myosin light chain kinase (MLCK) [64]. It has been reported that *B. infantis* conditioned medium regulates the integrity of TJs proteins through MAPK pathway with increased level of phospho-ERK 1 and 2 [90]. It is thus interesting to investigate the influence of L34 on the expression of Rho proteins and signaling pathway associated with its expression.

CHAPTER VI

CONCLUSION

Eight *Lactobacillus* Thai isolates including *L. fermentum* L12 (LF-L12), *L. oris* NL49 (LO-NL49), *L. murinus* B57 (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*.

Lactobacillus rhamnosus 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 L34 had more effect than UV-treated L34. Live and UV-treated L34 had protection effect on the destruction of intestinal epithelial barrier by *C. jejuni* not *Vibrio cholerae* O1 Inaba and *Salmonella* Typhimurium ATCC 13311. 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. . 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-significantly increased as compared with cells infected *C. difficile* alone. LR-L34 is thus a probiotic strain with the ability to enhance TJs integrity, protect and improve the destruction of TJs by *C. difficile*.

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APPENDICES

APPENDIX A

MATERIALS AND EQUIPMENTS

Materials and reagents

- Man, Rogosa, Sharpe (MRS) broth (Oxoid, England)

- Man, Rogosa, Sharpe (MRS) agar (Oxoid, England)

- Brucella agar (Oxoid, England)

- Tryptone Soya agar (TSA) (Oxoid, England)

- Gaspak (AnaeroPack-Anaero, Mitsubishi, Japan)

- Dulbecco's modified eagle media (DMEM) (Gibco-Invitrogen, USA)

- HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) (Sigma, USA)

- Heat-inactivated fetal bovine serum (Gibco-Invitrogen, CA, USA)

- Caco-2 cells (ATCC HTB-37, Manassas, Virginia, USA)

- Trypan-Blue (Gibco-Invitrogen, CA, USA)

- Pierce® BCA protein assay kit (Pierce Biotechnology, USA)

- Polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA)

- Skim milk (Becton, France)

- Tris base (Sigma, USA)

- Sodium Dodecyl Sulfate (SDS) (Invitrogen, Japan)

- Tween 20 (Merck, Germany)

- Glycine (Sigma, Japan)

- Dithio threitol (DTT) (Ameresco, USA)

- Bromphenol Blue (Ameresco, USA)

- Ammonium Persulfate (APS) (Plusone®, Sweden)

- 40% Acrylamide/Bis Solution, 29:1 (3.3% C) (BioRad, USA)

- Triton X-100 (Sigma, USA)

- Glycerol (Merck, Germany)

- Methanol (Merck, Germany)

- HCl (Merck, Germany)

- TEMED (USB™, USA)

- Antibodies against ZO-1, Occludin, Cladin-1 and β-actin (Cell Signaling, USA)

- Antibodies against JAM-1 (Abcam, England)

- Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling, USA)

- SingnalFire™ ECL Reagent (Cell Signaling, USA)

- ColorPlus Prestained Protein Ladder, Broad Range (10–230 kDa) (BioLabs, England)

- Kodak® Developer GBX (Sigma, USA)

- Kodak® Fixer GBX (Sigma, USA)

- ORTHO CP-GU FILM (Agfa HealthCare, Belgium)

Equipments

- Anaerobic chamber (Camlab Memmert, Cambridge,UK)

- Autoclave (Hirayama, Japan)

- Deep Freezer (-80^0C) (Sanyo, Japan)

- Deep Freezer (-20^0C) (Sanyo, Japan)

- 96-well flat-bottom tissue culture plate (Nunclon$^®\Delta$, Roskilde, Denmark)

- 24-well flat-bottom tissue culture plate (Nunclon$^®\Delta$, Roskilde, Denmark)

- 6-well flat-bottom tissue culture plate (Nunclon$^®\Delta$, Roskilde, Denmark)

- 6.5mm Transwell® -COL Collagen-Coated 0.4µm Pore PTFE Membrane
  Insert, Sterile (Corning, USA)

- Centrifuge (Kubota, Japan)

- Autopipettes (Gilson, France)

- Hot air oven (Haraeus, Germany)

- Incubator (Binder, New York, USA)

- Light Microscope (Nikon, Japan)

- McFarland 6 standard (Merieux, France)

- Microcentrifuge (Eppendorf, USA)

- Mini-PROTEAN$^®$ 3 Cell (BioRad, USA)

- Mini Trans-Blot$^®$ Electrophoretic Transfer Cell (BioRad, USA)

- pH meter (Orion, USA)

- Vortex mixer (Scientific, USA)

- Water bath (Memmert, USA)

- Voltohmmeter

 (EVOM^2 Epithelial Tissue Voltohmmeter, WorldPrecision Instruments, FL)

Software and program

 - Program analyze Western Blot

  - ImageJ 1.46

APPENDIX B

PREPARATION OF MEDIA AND REAGENT

Media for lactobacilli

MRS agar

MRS agar	62	g
Distilled water	1,000	ml

MRS broth

MRS broth	52	g
Distilled water	1,000	ml

20% glycerol MRS broth

Glycerol	20	ml
Distilled water	40	ml
MRS broth	40	ml

(MRS 2.08 g + DW 40 ml)

20% glycerol MRS broth using for kept lactobacilli cell in deep freeze.

Media for *Clostridium difficile*

Brucella agar

Brucella agar

Distilled water

1.5M Tris-HCl pH 8.8

Tris base	181.71 g
Distrilled water	1,000 ml

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc.). Adjust volume with   DW to make 1,000 ml. Sterilize the solution by autoclaving at 121⁰C for 15 minutes.

0.5M Tris-HCl pH 6.8

Tris base	60.55	g
Distrilled water	1,000	ml

Dissolve in distilled water and adjust pH to 6.8 with HCl (conc.). Adjust volume with   DW to make 1,000 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4xTris HCl/SDS pH 8.8

Tris base	91.05	g
SDS	2	g

Dissolve 91.05 g Tris base in 300 ml distilled water and adjust pH to 8.8 with HCl   (conc.). Adjust the volume to 500 ml with distilled water. Pass the solution thought a 0.45 μm filter, and add 2 g SDS. Store at 4°C

10x TBS (50mM Tris 0.15M NaCl pH7.5)

Tris base	60.57	g
NaCl	87.66	g
Distilled water	800	ml

Adjust pH to 7.5 and adjust the volume to 1,000 ml with distilled water

1x TBST

10X TBS	100	ml
Distilled water	900	ml
Tween 20	1	ml

10x Blotting buffer

Tris base	30.3	g
Glycine	144	g
Distilled water	800	ml

Dissolve in distilled water for 1x blotting buffer 1,000 ml and add 200 ml methanol. Store at room temperature

5x Running buffer

Tris base	15.1	g
Glycine	72	g
SDS	5	g

Dissolve in distilled water and adjust volume to 1,000 ml. Store at room temperature

6x Sample buffer with Dithiothretol (DTT)

4xTris HCl/SDS pH 8.8	7	g
Glycerol	3	g
SDS	1	g
Dithiothretol (DTT)	0.93	g
Bromphenol Blue	1.2	mg

Store at room temperature

10% Ammonium Persulfate (APS)

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20$^{\mathbf{o}}$C

10% Sodium dodecyl sulfate (SDS)

SDS	1	g
Distilled water	10	ml

Mix the solution and store at room temperature

30% Acrylamide/0.8% Bisacrylamide

Acrylamide	30	g
Bisacrylamide	0.8	g

Dissolve in distilled water and adjust volume to 100 ml. Sterilize the solution by filtration. Store in the dark at room temperature

RIPA buffer (10 ml)

50 mM TrisHCl pH 7.4	1	ml
150 mM NaCl	1.5	ml
Triton X-100	100	µl
0.5% Na-DOC	1	ml
0.1% SDS	100	µl

12% SDS-PAGE (For 2 gels)

Distilled water	6.872	ml
1.5 M Tris HCl pH 8.8	4	ml
10% SDS	160	µl
10% APS	160	µl
40% Acrylamide/Bis Solution	4.8	ml
TEMED	8	µl

5% Stracking gel

Distilled water	5.4	ml
0.5 M Tris HCl pH 6.8	1	ml
10% SDS	80	µl
10% APS	80	µl
40% Acrylamide/Bis Solution	1.34	ml
TEMED	8	µl

BIOGRAPHY

Miss Waraporn Siriterm was born on July 21, 1987 in Uboratchathani, Thailand. She graduated with Bachelor of Science degree in Medical Technology (2nd Class Honours) from the Faculty of Allied Health Sciences, Chulalongkorn University in 2010. She was supported by H.M. King Bhumibol Adulyadej's 72nd Birthday Anniversary Scholarship for her Master's degree study at the Interdisciplinary Program of Medical Microbiology, Chulalongkorn University.