Screening Enteric Bacteria with Inhibitory Effects Against Pathogenic *Clostridium difficile* and Its Spore

Chonticha Romyasamit

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences
Prince of Songkla University
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Major Program	Biomedical Sciences

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การคัดกรองเชื้อแบคทีเรียในลำไส้ที่ต่อต้านเชื้อก่อโรคและสปอร์ของเชื้อ *Clostridium difficile*
ผู้เขียน นางสาวชลธิชา รมยะสมิต
สาขาวิชา ชีวเวชศาสตร์
ปีการศึกษา 2562

บทคัดย่อ

Clostridium difficile เป็นสาเหตุสำคัญของโรคติดเชื้อ *C. difficile* และโรคท้องร่วงที่เกี่ยวข้องกับยาปฏิชีวนะที่มีอัตราการเสียชีวิตเพิ่มสูงขึ้น การรักษาการติดเชื้อ *C. difficile* แบบมาตรฐานคือการใช้ยาปฏิชีวนะ อย่างไรก็ตาม ผู้ป่วยจะกลับมาติดเชื้อซ้ำภายใน 2 เดือน และเสี่ยงต่อการกลับเป็นซ้ำสูงขึ้นถึงร้อยละ 50–65 หลังจากติดเชื้อในครั้งที่ 2 หรือมากกว่า ปัจจุบัน การรักษาทางเลือกสำหรับการป้องกันการติดเชื้อ *C. difficile* และการกลับเป็นซ้ำ ประกอบด้วย การใช้โมโนโคลนอลแอนติบอดี วัคซีน toxin neutralization การปลูกถ่ายจุลชีพประจำถิ่นในอุจจาระ และโปรไบโอติก วัตถุประสงค์ของการศึกษาครั้งนี้คือ ระบุเชื้อแบคทีเรียในลำไส้สายพันธุ์ใหม่ที่ต่อต้านเชื้อ *C. difficile* และสปอร์ของเชื้อ และพัฒนาสูตรที่เหมาะสมในการแปรรูปเชื้อที่มีศักยภาพเป็นโปรไบโอติก เชื้อ *Enterococcus faecalis* จำนวน 9 ไอโซเลท ประกอบด้วย PK1003 PK1201 PK1202 PK1301 PK1302 PK1801 PK2003 PK2004 และ PK2502 ที่แยกได้จากอุจจาระของทารกที่ได้รับนมมารดา สามารถยับยั้งเชื้อ *C. difficile* ได้โดยทั้ง 9 ไอโซเลท มีคุณสมบัติของโปรไบโอติก คือ ทนต่อค่าความเป็นกรดต่ำ (pH 2) รวมทั้งทนต่อเปปซิน และเกลือน้ำดี ดีกว่า *Lactobacillus plantarum* ATCC 14917 ซึ่งเป็นโปรไบโอติกสายพันธุ์อ้างอิง เชื้อเหล่านี้สามารถเกาะติดกับเซลล์มะเร็งลำไส้ชนิด HT-29 ได้อย่างมีประสิทธิภาพ และยังไวต่อยา แอมพิซิลลิน เพนนิซิลลิน อิมมิพีแนม และแวนโคมัยซิน แต่ดื้อต่อยาเจนตามัยซิน และเชื้อเหล่านี้ไม่มียีนที่ดื้อต่อยาแวนโคมัยซินทดสอบโดยวิธีปฏิกิริยาลูกโซ่พอลิเมอเรส นอกจากนี้การทดสอบฟีโนไทป์ของเชื้อทั้ง 9 ไอโซเลท พบว่า ไม่ผลิตฮีโมไลซิน เจลาติเนส และไม่ย่อยมิวซิน ปัจจัยความรุนแรงของเชื้อ *E. faecalis* ทดสอบโดยใช้หนอน *Galleria mellonella* ซึ่งพบว่าทุกไอโซเลท ไม่ส่งผลต่อการรอดชีวิตของหนอน *G. mellonella* ในเวลาห้าวัน เมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับเชื้อ *E. Faecalis* อีกทั้งเชื้อ

เหล่านี้ลดความเป็นพิษของเชื้อ *C. difficile* ต่อเซลล์ HT-29 โดยป้องกันเซลล์ HT-29ไม่ให้เปลี่ยนแปลงรูปร่างเป็นทรงกลม รักษาโครงสร้างของ F-actin และ tight-junctions ระหว่างเซลล์ที่อยู่ติดกัน และเชื้อ *E. faecalis* ที่แยกได้เหล่านี้สามารถยับยั้งการงอกของสปอร์และการสร้างสปอร์ของเชื้อ *C. difficile*

นอกจากนี้ได้พัฒนาหาสูตรที่เหมาะสมต่อการแปรรูปเชื้อที่มีศักยภาพเป็นโปรไบโอติก พบว่าเชื้อ *E. Faecalis* ทั้ง 9 ไอโซเลทที่ละลายอยู่ใน 10% skim milk สามารถป้องกันความเสียหายของเซลล์ในระหว่างกระบวนการทำแห้งแบบแช่เยือกแข็งและการเก็บรักษาในระยะยาว หลังจากกระบวนการทำแห้งแบบแช่เยือกแข็ง *E. faecalis* ยังรักษาจำนวนเซลล์ที่รอดชีวิตอย่างเพียงพอ และยังคงคุณสมบัติของโปรไบโอติกในการยับยั้งเชื้อ *C. difficile* โดยสรุปเชื้อ *E. faecalis* ทั้ง 9 สายพันธุ์แสดงศักยภาพในการเป็นโปรไบโอติกที่สามารถยับยั้งเชื้อ *C. difficile* และสปอร์ของเชื้อได้อย่างมีประสิทธิภาพ ดังนั้น *E. faecalis* ทั้ง 9 สายพันธุ์เหมาะกับการนำไปพัฒนาเป็นอาหารชนิดใหม่หรือผลิตภัณฑ์ยา แต่ยังต้องการทดสอบฤทธิ์และความปลอดภัยในสัตว์ทดลองขนาดใหญ่ขึ้นต่อไป

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ABSTRACT

Clostridium difficile is a major cause of *C. difficile* infection (CDI) and antibiotic-associated diarrhea with the increasing rate of mortality. The standard treatments of CDI are antibiotics. However, patients relapse from the infection within 2 months with the risk of recurrences increasing to 50–65% after two or more episodes of the infection. Recently, alternative treatment for the prevention of CDI and relapse of CDI has included monoclonal antibodies, vaccination, toxin neutralization, fecal microbiota transplantation, and probiotics. Thus, the aims of this study are to identify new enteric bacteria as probiotic against *C. difficile* and its spore as well as to developed and evaluate the formula of potential probiotics. Nine *Enterococcus faecalis* including PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 isolated from breast-fed infant stools could inhibit *C. difficile*. These strains exhibited characteristics of probiotic including showed higher levels of viability in acid condition (pH 2) as well as tolerance to pepsin and bile salts than *Lactobacillus plantarum* ATCC 14917 a reference probiotic strain. All isolated strains could effectively adhere to HT-29 human colon cancer cell lines. They were susceptible to ampicillin, penicillin, imipenem, and vancomycin but resistant to gentamicin. In addition, Polymerase chain reaction demonstrated that all isolated strains were not carried vancomycin-resistant genes. Moreover, phenotypic assays showed that none of the isolated strains exhibited hemolysin production, gelatinase activity, and mucin degradation except 3 isolates could produce hemolysin. The effect of virulence factors of *E. faecalis* were tested by animal model (*Galleria mellonella*). Nine isolated strains did not affect the five-day survival of *G. mellonella* larvae when compared to that of negative control. These strains were reduced the Clostridial toxic effect upon HT-29 cell lines. They prevent HT-29 cell rounding, preserving the *F*-actin microstructure and

tight-junctions between adjacent cells. Furthermore, these *E. faecalis* isolated strains could inhibit the spore germination and sporulation of toxigenic *C. difficile* strains.

Mpreover, the appropriate formula of potential probiotic were determined. Nine *E. faecalis* suspended with 10% skim milk can prevent cell damage during freeze-drying process and long-term storage. After freeze-drying process, *E. faecalis* sustain and retain adequate viability in appropriate storage conditions with probiotic properties. Nine *E. faecalis* exhibit characteristics of probiotic. Overall, all 9 strains of *E. faecalis* showed potential probiotics that had ability to inhibit *C. difficile* and its spore. Thus, these probiotic candidates are very attractive for developing novel foods or pharmaceutical products. The efficiency of these potential probiotics to inhibit CDI *in vivo* and safety should be further evaluation *in vivo*.

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Chonticha Romyasamit

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS AND SYMBOLS

AAD	=	Antibiotic-associated diarrhea
ATCC	=	American type culture collection
BA	=	Blood agar
BHI	=	Brain heart infusion
CaCo-2	=	Caucasian colon adenocarcinoma cell
CCFA	=	Cycloserine Cefoxitin Fructose Agar
CCNA	=	Culture cytotoxicity neutralization assays
CDAI	=	Clinical Disease Activity Index
CDC	=	Centers for Disease Control and Prevention
CDI	=	*Clostridium difficile* infection
CFU	=	Colony forming unit
DAPI	=	4′,6-diamidino-2-phenylindole
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DMST	=	DMST Culture Collection
EDTA	=	Ethylenediaminetetraacetic acid
EIAs	=	Enzyme immunoassays
EFSA	=	European Food Safety Authority
EtOH	=	Ethanol
FAO	=	Food and Agriculture Organization of the United Nations
FBS	=	Fetal bovine serum
FDA	=	Food and Drug Administration
FMT	=	Fecal microbiota transplantation
g	=	Gram
GDH	=	Glutamate dehydrogenase
GI	=	Gastrointestinal
GIT	=	Gastrointestinal tract
GRAS	=	Generally Recognized as Safe

GSH	=	Glutathione
h	=	Hour
HCCA	=	Alpha-cyano-4-hydroxycinamic acid
HeP2 cells	=	HeP2 cells
HT-29	=	Colorectal adenocarcinoma cell
Ig	=	Immunoglobulin
IL	=	Interleukin
IV	=	Intravenous
Kb	=	Kilo pasepair
l	=	Litter
LAMP	=	Loop-mediated isothermal amplification
MALDI-TOF	=	Matrix-assisted laser desorption/ionization- time-of-flight mass spectrometer
mg	=	Milligrams
ml	=	Milliliter
mm	=	Millimeter
MRS	=	DeMan, Rogosa and Sharpe
NaCl	=	Sodium chloride
NFkB	=	Nuclear factor kB
PaLoc	=	Pathogenicity locus
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PCDAI	=	Pediatric Crohn's Disease Activity Index
RCDI	=	*Clostridium difficile* infection recurrence
rpm	=	Revolution per minute
WBC	=	White blood cell
WHO	=	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background and rational

Clostridium difficile infection (CDI) is implicated as a major cause of antibiotic-associated diarrhea (AAD) in hospitals worldwide. Its incidence and severity have been substantially increased for the last decade leading to high morbidity and mortality (1, 2). The pathogenesis of CDI is mediated by toxins (Toxin A, Toxin B, and/or binary toxin). These toxins inactivate the Rho family GTPases leading cause of morphological changes, inhibition of cell division, membrane trafficking, inflammatory reactions in the intestine, and cell death (3). The spectrum of disease ranges from mild diarrhea, pseudomembranous colitis to death (4, 5). Most cases of CDI have been associated with exposure and resistance to broad spectrum antimicrobial agents, especially fluoroquinolones and clindamycin that are commonly used to treat other bacterial infections (1, 6, 7). *C. difficile* has spread throughout North America, Europe, and Asia that can infecting and killing more people wherever it spreads (7, 8). Therefore, *C. difficile* has been declared as urgent threat by the Centers for Disease Control and Prevention (CDC).

Standard treatments of CDI are antibiotics, ordinarily vancomycin and metronidazole. However, up to 24% of patients relapse from the infection within 2 months of first episode with the risk of recurrences increasing to 50–65% after two or more episodes of the infection (1, 3, 7). Risk factors for recurrent infection include prolonged duration of hospital stay, age older than 65 years, and low serum albumin concentration. Every recurrence, there is an increased risk of further recurrences, leading to multiple diarrheal episodes (1). New antibiotics for recurrence associated with *C. difficile* infection (RCDI) have recently been described for several new drugs, including rifaximin, nitazoxanide, and tolevamer (6, 9). In spite of these developments

some patients continue to manifest relapsing diarrhea after completed treatment with novel drugs. An alternative treatment for the prevention of CDI and relapse of CDI has included monoclonal antibodies, vaccination, toxin neutralization, fecal microbiota transplantation (FMT), and probiotics (10, 11). FMT has a reported 90% success rate, but its long-term consequences have not yet fully studied. In June 2019, two patients who received FMT as part of a clinical trial developed life-threatening infections from multidrug-resistant bacteria delivered in the transplants. One of the patients has dead (12). Therefore, safety issues associated with FMT are underappreciated.

Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (13-15). Several studies supported that maintenance of health gut normal flora by probiotics provides protection against gastrointestinal (GI) disorder including GI infections, *Helicobacter pylori* infection, and inflammatory bowel diseases (16). In addition, probiotics can be prevent or treat allergy, cancer, and improving the immune systems (17). FDA approved probiotic to treat or prevent some diseases such as allergy. They can used as alternative treatment for enteric infection or to reduce the symptoms of AAD. Probiotics especially, *Lactobacillus* spp, *Bifidobacterium* spp, and *Saccharomyces boulardii* are under evaluation for the control of CDI. A literature review, in clinical trial showed the most common indication (17%) was the prevention of AAD and 3% were specifically to prevent or treat CDI. Several clinical trials have assessed the usefulness of probiotics in the prevention of AAD. Although with low quality of evidence and recommendation (7, 15). Probiotic is easy to use, and breaks the cycles of repeated antibiotic use and adds potential cost savings (10, 18). Thus, the aim of this study is to identify new enteric bacteria as probiotic as well as develop and evaluate proper formula of potential probiotics.

1.2 Review of literatures

1.2.1 *Clostridium* spp.

Clostridium is a genus of gram-positive, spore-forming anaerobe bacteria belonging to the family Clostridiaceae. They are rod shaped and arranged in pairs or short chains. There are close to 200 species of *Clostridium*, at least 30 are associated with human diseases, for example, *C. difficile, C. perfringens, C. botulinum, C. septicum, C. novyi, C. hemolyticum, C. tetani,* and *C. baratii* (19). *Clostridium* produces toxins that cause symptoms and lesions. These toxins are associated with intestinal infection, foodborne and ADD. In recent year, AAD is a significant cause of morbidity and mortality, particularly in the hospitalized elderly. The most common species that is a caused of AAD are *C. difficile* (1, 2).

1.2.2 *Clostridium difficile*

C. difficile is a gram positive rod, spore-forming, anaerobic toxin-producing bacterium. It was firstly isolated from stools of healthy neonates in 1935 as *Bacillus difficile* by Hall and O'Toole (20, 21). Colonies of *C. difficile* are large, gray, flat and spread when anaerobically culture at 37 °C. The colony also displays fluorescence under ultraviolet light; yellow when grows on Cycloserine Cefoxitin Fructose Agar (CCFA), and chartreuse when grows on blood agar (22, 23). *C. difficile* produced potent toxins which are the most common cause of the CDI, AAD and pseudomembranous colitis worldwide. CDI is considered as a nosocomial infection that mostly affects elderly patients who receive broad-spectrum antibiotics including cephalosporin, clindamycin, amoxicillin, vancomycin, metronidazole and fluoroquinolones (3, 24-26).

1.2.2.1 Epidemiology

Today, incidence and mortality rates of CDI have been increasing worldwide (27, 28). Epidemiological reports from the USA show that *C. difficile* has replaced methicillin-resistant *Staphylococcus aureus* as the most common cause of the

healthcare-associated infection (29). Many reports from Europe and Canada, the incidence of CDI has increased by 2-4 fold in the past decade, especially in the elder patients. For example, Quebec experienced a large outbreak of CDI and 4 fold increase in CDI in 1998 to 2004 (30). In the USA (2016), *C. difficile* is the leading cause of hospital-associated infection, with an estimated 14,000 deaths each year. Approximately 4–10% of patients are colonized with toxigenic *C. difficile* on admission to a health-care facility. The estimation of health care costs imputed in CDI of $9,000 to $13,000 for each case (31). In Thailand, a total cases diarrhea of 1,042,266 cases was reported from 77 provinces (morbidity rate was 1600.42/100,000 population) in 2015 by Bureau of Epidemiology, Department of Disease Control, Thailand (32). Most cases of CDI have been associated with exposure and resistance to broad spectrum antimicrobial agents, especially fluoroquinolones and clindamycin that are commonly used to treat other bacterial infections (1, 6, 7). Moreover, during the past decade, the *C. difficile* strains, that is restriction endonuclease analysis group BI, pulse-field gel electrophoresis type NAP1, and polymerase chain reaction ribotype 027, are designated BI/NAP1/027 has emerged in North America, Europe and some part of Asia (33). BI/NAP1/027 strain show ability to produce high concentrations of toxins, high transmissibility, high sporulation, production of binary toxins (*C. difficile* transferase CDT), high level of resistance to fluoroquinolone due to the mutations in *gyrA*, and variation in the *tcdC* repressor gene (increased toxin A [6-fold] and toxin B [23-fold]). Furthermore, the polymorphisms in *tcdB* could result in improved toxin binding (34). The incidence of BI/NAP1/027 strain is increasing in Eastern Europe. Recently, BI/NAP1/027 strain has been reported in Hong Kong, Japan, South Korea, Singapore, and China that can infecting and killing more people wherever it spreads (Figure 1) (30, 31, 35). Hence, *C. difficile* has been declared an urgent threat by the Centers for Disease Control and Prevention (CDC).

Figure 1. Countries in which the NAP1/B1/027 strain of *C. difficile* has been isolated (35).

1.2.2.2 Pathogenesis

The pathogenesis of CDI is mediated by pathogenicity locus (PaLoc) in genome of pathogenic *C. difficile* (Figure 2a) (26), which comprises of five genes that are divided into two groups. The first group is composed of regulatory genes including *tcdR*, *tcdE*, and *tcdC* genes. The *tcdR* gene encodes an option sigma factor (*TcdR*) that responsible for the transcription of toxin genes. The *tcdC* gene encodes an anti-sigma factor. It suppresses toxin production by direct interacting with TcdR or TcdR-containing RNA polymerase. The *tcdE* encodes a protein which shares similarity to phage holin proteins, and may be involved in toxin secretion. The second group includes toxin genes (*tcdA* and *tcdB* genes). The pathogenicity of *C. difficile* is determined by production of toxin(s) including enterotoxin (Toxin A; TcdA) and/or cytotoxin (Toxin B; TcdB) encoded by *tcdA* and *tcdB* genes, respectively (3, 26). TcdA (308 kDa) and TcdB (270 kDa) are carrying three functional domains; an amino-terminal catalytic domain, a central translocation domain and a carboxy-terminal cell-

binding domain. Toxins A and B are the primary determinants of virulence and pathogenicity. TcdA and TcdB toxins disrupt the actin cytoskeleton and tight junctions via inactivation the Rho family GTPases such as Rho, Cdc42 and Rac leading to decrease epithelial barrier function, fluid accumulation and destruction of the intestinal epithelial cells. A consequence of the toxin secretion is the releasing of inflammatory cytokines from the intestinal epithelial cells, mast cells and macrophages. The effect of these signaling molecules is an influx of inflammatory cells which further amplifies the inflammatory response, inhibition of cell division, pseudomembranous, and cell death eventually (Figure 3) (3, 26, 36).

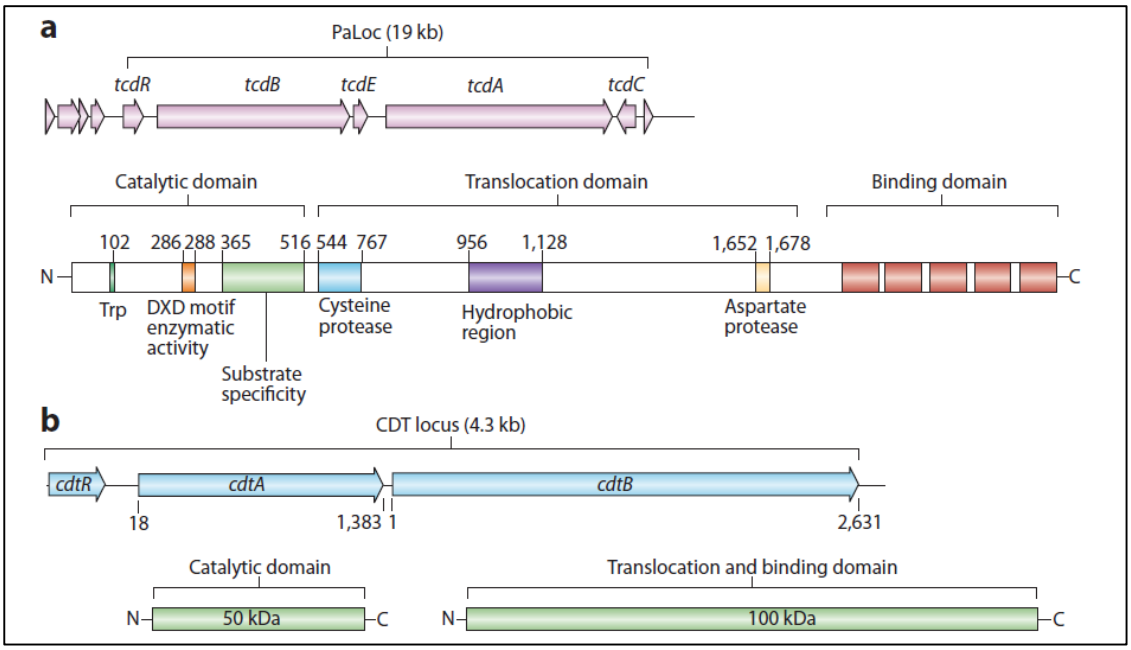


Figure 2. Toxins produced by *C. difficile*. (a) Two large toxins, toxin A and toxin B (TcdA and TcdB), are encoded on the pathogenicity locus (PaLoc). Both toxins are single-chain proteins, and several functional domains and motifs have been identified. In non-toxigenic strains, this region is replaced by a short 115 bp sequence. TcdB is shown in detail below the PaLoc. (b) The binary toxin or CDT (*C. difficile* transferase), is encoded on a separate region of the chromosome (CdtLoc) and comprises three genes (26).

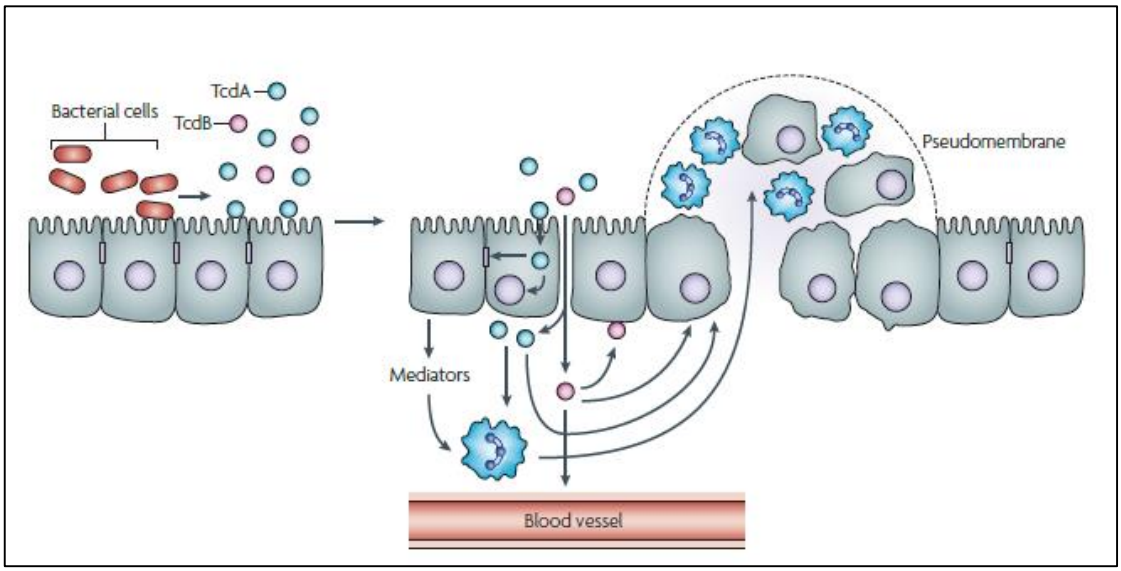


Figure 3. *C. difficile* pathogenesis. *C. difficile* colonizes the intestine following disruption of the host gut normal flora. *C. difficile* adheres to the gut mucosa and toxigenic strains secrete toxins. The toxins insert into the membrane and then self-cleave releasing the catalytic (enzymatic) amino-terminal portion of each toxin into the cytoplasm. The toxins cause disruption of the tight junctions leading to cell death or the production of inflammatory mediators (26).

Another *C. difficile* toxin is binary toxin, associated with increased severity of the symptom, composed of CdtA and CdtB components. The binary toxin genes encode for *cdtA* and *cdtB* genes harbored by CdtLoc in chromosome (Figure 2b) (26). The CdtA is an enzymatic component containing ADP-ribosyltransferase, whereas the CdtB is the binding and translocation component. The CdtB binds to host cells in order to translocate *CdtA* into cytosol to exert its toxicity. The pathogenic of the binary toxin is suggested by modifying actin molecules, trigger microtubule protrusion, thereby increasing the adherence of *C. difficile* to gut epithelium (37, 38).

1.2.2.3 Risk factors

Several factors may increase the risk of developing CDI. The major factor are antibiotic therapy in advanced age (more than 65 years) patient, exposure to acute or chronic care facilities and immunodeficiency due to AIDS or chemotherapy. Use of

ampicillin, amoxicillin, cephalosporins, fluoroquinolones, and clindamycin, which are common drugs to treat other infections, are the highest incidence of CDI (Figure 4) (26).

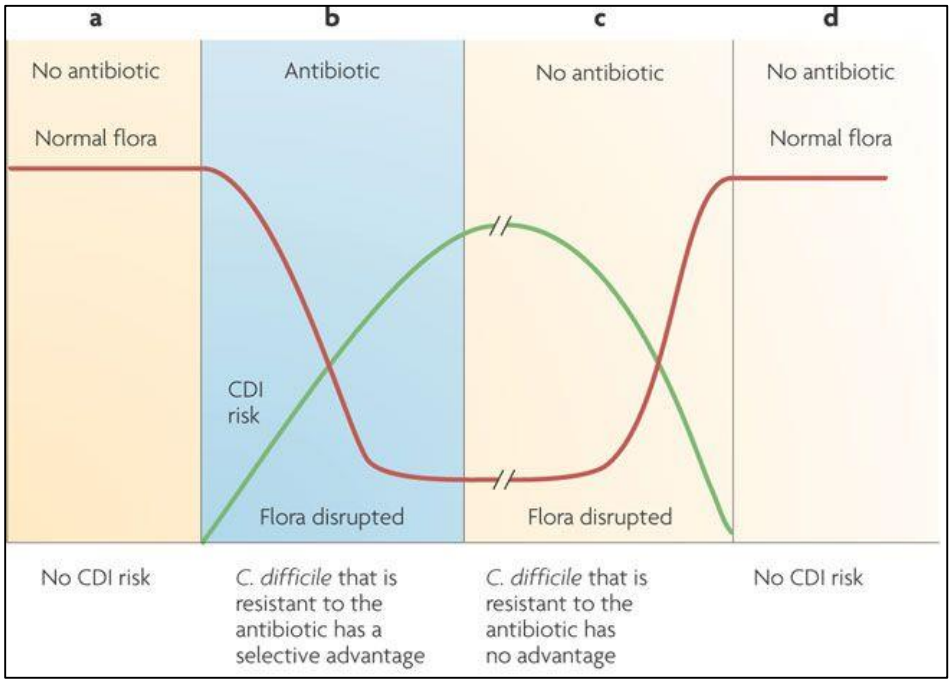


Figure 4. The effect of antibiotics on the gut normal flora and the risk of CDI. (26).

1.2.2.4 Sign and symptom

CDI can range from mild to life threatening. Symptoms of mild cases include watery diarrhea, three or more times a day for consecutively several days, with abdominal pain or tenderness. Symptoms of more severe CDI include: watery diarrhea, up to 15 times per day, severe abdominal pain, loss of appetite, fever, blood or pus in the stool, weight loss, pseudomembranous colitis, sepsis and death (3-5).

1.2.2.5 Diagnosis of CDI

CDI can be diagnosed by various techniques following enzyme immunoassays (EIAs), glutamate dehydrogenase (GDH), anaerobic stool culture, cell culture cytotoxicity neutralization assays (CCNA), toxigenic culture (TC) (39), and by nucleic acid amplification tests (3, 31, 40, 41). Several laboratories use EIAs or GDH for screening of *C. difficile* toxin. EIA and GDH are rapid, feasible, and inexpensive methods but they are lacks of sensitivity and specificity (31). The gold standard methods for detection of *C. difficile* are TC and CCNA by detection of changing in morphologies of effect of toxin(s). Mammalian cell lines used in these test such as HeP2, fibroblasts, CaCo-2, HT-29 or Vero cells. These methods showed high sensitivity but they are time-consuming, expensive and require specialist knowledge (31, 40, 41). The nucleic acid-based methods have been largely performed due to its high sensitivity and specificity (3, 31). Recently, Food and Drug Administration (FDA) approved that loop-mediated isothermal amplification (LAMP) detecting *tcdA* gene and real-time PCR detecting *tcdB* gene are standard approaches for *C. difficile* diagnosis (Figure 5) (3).

Methods/assays	Performance characteristics	
	Sensitivity (%, range)	Specificity (%, range)
Toxigenic anaerobic culture	N/A	N/A
Enzyme immunoassays	31–99	84–100
Cell culture neutralization assays	67–86	97–100
Glutamate dehydrogenase	71–100	76–98
Nucleic acid amplification tests		
BD-GeneOhm™	84–96	94–100
Prodesse ProGastro™	77–92	95–99
GeneXpert™	94–100	93–99
illumigene™	99	98

Figure 5. Performance characteristics of various methods for CDI diagnosis (3)

1.2.2.6 CDI Treatment

1.2.2.6.1 Conventional antimicrobial therapy

Medical treatment of CDI varies based on a graded severity scale (Table 1) (40). Guidelines recommend the use of metronidazole 500 mg orally three times per day for 10–14 days for initial mild to moderate disease, and vancomycin 125 mg orally three times per day for 10 days for mild-to-moderate disease. Vancomycin 125 mg orally four times per day for 10 days treats for severe disease. Vancomycin 500 mg orally four times a day and metronidazole 500 mg IV every 8 h treats for severe and complicated diseases (40, 42). Between 10% and 58% of patients experience recurrent infection, with higher proportions in those aged 65 years and older. For every recurrence, there is an increased risk of further recurrences, leading to multiple diarrheal episodes and further treatment with vancomycin or metronidazole. There is a high risk of recurrence associated with CDI (RCDI). After an initial episode of treatment, 10 – 20% of patients showed chance of RCDI within 8 weeks is When patient has one recurrence, rates of further recurrences increased to 40 – 65% (40, 42). Recurrence comprises both episodes of relapse infection by the current strain and reinfection by a new strain, and it remains difficult to distinguish between both infections. Recurrences may be due to an impaired immune response and/or alteration of the colonic microbiota. Patients with recurrent CDI should be treated with metronidazole or vancomycin pulse regimen. Helping combat the increasing burden of recurrence, the FDA approved fidaxomicin for the

treatment of CDI in 2011. Patients treated with fidaxomicin show lower rates of recurrence, lower rates of hospital readmission, and shorter hospital stays resulting in an overall saving of US$ 3047 per patient (6). However, current costs of fidaxomicin may limit the widespread use (43) and the reduction in recurrence of the BI/NAP/027 strain was not observed (44).

1.2.2.6.2 Surgery

Surgical intervention for patients with severe fulminant colitis who are not responding to aggressive antibiotic treatment can increase the chances of survival. The surgery is to prevent complications as bowel perforation and stool peritonitis and avoid high morbidity and mortality. However, the mortality associated with surgery case is high due to the ill health of patients (45, 46).

Table 1. CDI severity scoring system and summary of recommended treatments (40)

Severity	Criteria	Treatment
Mild-to-moderate disease	Diarrhea plus any additional signs or symptoms not meeting severe or complicated criteria	Metronidazole 500 mg orally three times a day for 10 days. If unable to take metronidazole, vancomycin 125 mg orally four times a day for 10 days
Severe disease	Serum albumin < 3 g/dL plus ONE of the following: WBC ≥ 15,000 cells/mm^3 or abdominal tenderness	Vancomycin 125 mg orally four times a day for 10 days
Severe and complicated disease	Any of the following attributable to CDI: Admission to intensive care unit for CDI, hypotension with or without required use of	Vancomycin 500 mg orally four times a day and metronidazole 500 mg IV every 8 h, and vancomycin per rectum

	Vasopressors,fever ≥ 38.5 °C, ileus or significant abdominal distention, mental status changes, WBC ≥ 35,000 cells/mm^3 or < 2,000 cells/mm^3, serum lactate >2.2 mmol/l	(vancomycin 500 mg in 500 mL saline as enema) four times a day Surgical consultation suggested
Recurrent CDI	Recurrent CDI within 8 weeks of completion of therapy	Repeat metronidazole or vancomycin pulse regimen or FMT
CDI, *Clostridium difficile* infection; FMT, fecal microbiota transplant; IV, intravenous; WBC, white blood cell.		

1.2.2.6.3 Immunotherapy and vaccines

New therapeutic approaches for CDI such as passive immunotherapy and active immunization through vaccination have been used to target the major toxin A and toxin B. Previous studies with a vaccine containing toxoid A and toxoid B have been shown to resolve recurrent diarrhea with increase in serum IgG levels against both toxins (47). Another paper, oral immunization of hamsters with spores of *Bacillus subtilis* expressing the cell-binding domains of toxin A and toxin B was found to confer protection from infection (39). Previous studies have now shown that passive immunization is suitable for treatment of chronic relapsing CDI in humans (39). In the study of Lowy et al, examined 200 patients received an intravenous dose of anti-toxin A/B showed a significant reduces in CDI recurrence. The relapse rate was 7% in treated patient and 25% in patients receiving a placebo (10, 48). Recently, monoclonal antibodies targeting toxin A and toxin B, namely actoxumab and bezlotoxumab are currently being introduced into the clinical practice (49, 50). Nowadays, no effective vaccine against CDI is available on the market. Moreover, clinical trial on Clover vaccine (Pfizer®) and Cdiffense vaccine (Sanofi®) are in phase III, but results have not been yet published (51).

1.2.2.6.4 Fecal microbiota transplantation (FMT)

FMT is the administration of a solution of fecal matter from a donor into the intestinal tract of a recipient in order to directly change the recipient's microbial composition and confer a health benefit (11). The process usually involves first selecting a healthy donor. The stools prepare by mixing with water or normal saline. Then, filtrate to remove any particulate matter. The mixture can be administered through a nasogastric tube, nasojejunal tube, esophagogastroduodenoscopy, colonoscopy, or retention enema (11). A systematic literature review 317 patients treated across 27 case series reports that FMT treatment for recurrent CDI and pseudomembranous colitis was highly effective and showed disease resolution in 92% of cases (52). However, effectiveness varied by route of instillation, relationship to stool donor, volume of FMT given, and treatment before infusion. In June 2019, two patients who received FMT as part of a clinical trial developed life-threatening infections from multidrug-resistant bacteria delivered in the transplants. One of the patients has dead (12). This is an important consideration which the legal, regulatory and safety issues associated with FMT are underappreciated.

1.2.2.6.5 Probiotics

FAO/WHO definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (13-15). The most frequently used microorganisms are *Lactobacillus* spp, *Bifidobacterium* spp, and *Saccharomyces boulardii*. Probiotics are available in a wide variety of formulations ranging from tablets and powders to yogurts, milk, ice cream, and juices. Physicians tend to recommend tablets and powders (53). Probiotic is easy to use, and breaks the cycles of repeated antibiotic use, which in turn reduces the risk of antibiotic associated resistance and adds potential cost savings when compared to repeated antibiotic administration and hospitalizations (54). The mechanism of probiotics is normalization of microbiota, regulation of intestinal transit, gut barrier reinforcement, colonization resistance, acid and short-chain fatty acid production, vitamin synthesis, bile salt metabolism, enhance innate and adaptive immunity. Probiotics are shown to be

beneficial in the treatment and prevention of acute gastroenteritis, eczema, allergies and AAD. FDA approved probiotic to treat or prevent some disease such as allergy. They can used as alternative treatment for enteric infection or to reduce the symptoms of AAD (10, 55). The interference of normal gut microbiota leads to CDI, its restoration is important, especially following antibiotic treatment. Probiotics reach the small intestine and colon. They can colonize in gastrointestinal tract (GIT) and restore normal bacterial gut flora as well as effect the functioning of the GIT by a variety of mechanisms (56). A wide variety of probiotics have been tested and used to prevent or treat CDI. The best studied probiotic agents in CDI are *Saccharomyces boulardii*, *Lactobacillus* GG (LGG) and other lactobacilli, and probiotic mixtures (10, 57).

1.2.2.7 Prevention

Two main aspects prevention of CDI are the elimination of the infection source and the restriction on the use of antibiotics. Isolation of patients who were infected with *C. difficile*. Use of gloves and gowns by medical staff are effective barrier methods. Hand hygiene as hand washing are also an important. Appropriate and accurate use of one single antimicrobial in patients reduces risk of CDI (40).

1.2.3 Probiotics

1.2.3.1 History of probiotics

Probiotics come from the Greek language ‘pro and bios’ that means ‘for life’. The history of probiotics starts with the history of man by consuming fermented foods such as cheese and fermented milk. In 1908 (58), the relationships of probiotics and health benefits were made at the turn of the century when Ellie Metchnikoff, Russian researcher who is the Nobel Prize winner studied the effects of probiotic microorganisms (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) on human health. The result suggests that Bulgarians who receive fermented milk product are healthy and long-lived because the fermented milk product consists of rod-shaped bacteria. Thus, these bacteria affect the gut micro-flora positively and decrease the

pathogenic microorganisms (59, 60). Moreover, the evaluation of various probiotic research studies supports knowledge about microorganisms that affect human and animal health as follows:

In 1885 Escherich (60) studied the bacteria in the normal feces and the GI tract in order to understand physiology, pathology and treatment of intestinal diseases of microbial origin.

In 1900, Tissier (61) was able to firstly isolate *Bifidobacterium bifidum* from breast-fed infants. This isolated bacteria can help to prevent diarrhea in infants. Moreover, Moro (61) also isolate *Bacillus acidophilus* from the oral cavity and GI tract of breast-fed infant that resistant to low pH.

In 1920, Rettger and Cheplin (60) reported that mice and human who receive milk or lactose led to a modification of the intestinal micro-flora resulting in predominance of acidophilus and bifidus type culture. These findings stimulated commercial interest in products fermented by *L. acidophilus*.

In 1930, Shirota (60) isolate and culture *Lactobacillus* strain that can survive in GI tract condition. The culture identified as *L. casei* that was used for the production of the fermented dairy product called ‘‘Yakult’’.

In the period 1930s to 1950s, the research in this scope lost its pace likely due to the war. The attention in the intestinal human micro-flora was seen in the late 1950s and early 60s that led to the introduction of the probiotic concept.

1.2.3.2 Definition of probiotics

The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances that stimulate the growth of other microorganisms. After this year the word ‘probiotic’ was used in a different meaning (60). For FAO/WHO definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (13-15). Some definitions are listed in Table 2 (60-62).

Table 2. Some of the definitions of probiotics commonly cited over the years. Modified from Vasiljevic T, et al. 2008, Schrezenmeir J et al. 2001 and Kechagia M, et al. 2013 (60-62)

Year	Description	Source
1953	Probiotics are common in vegetable food as vitamins, aromatic substances, enzymes and possibly other substances connected with vital processes	Kollath
1954	Probiotics are opposite of antibiotics	Vergin
1955	Deleterious effects of antibiotics can be prevented by probiotic therapy	Kolb
1965	A substance secreted by one microorganism which stimulates the growth of another	Lilly and Stillwell
1971	Tissue extracts which stimulate microbial growth	Sperti
1973	Compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms in vitro	Fujii and Cook
1974	Organisms and substances that contribute to intestinal microbial balance	Parker
1992	Live microbial feed supplement which beneficially affects the host animal by improving microbial balance	Fuller
1992	Viable mono- or mixed culture of live microorganisms which, applied to animals or man, have a beneficial effect on the host by improving the properties of the indigenous microflora	Havenaar and Huis int'Veld
1996	Live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host	Salminen
1996	Living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition	Schaafsma
1999	Microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host	Salminen, Ouwehand, Benno and Lee
2001	A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effect in this host	Schrezenmeir and de Vrese
2002	Live microorganisms that when administered in adequate amount confer a health benefit on the host	FAO/WHO

Probiotics are challenging for industrial applications that relevant for human and animal health. Probiotic products consist of different vitamins, enzymes, capsules or tablets, and some fermented foods as milk and cheese contain microorganisms which have beneficial effects on host. They can contain one or several species of microorganism. Probiotics are available in a wide variety of formulations ranging from tablets and powders to yogurts, milk, ice cream, and juices. Physicians tend to recommend tablets and powders (53, 63). Probiotics are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. In the Table 3 show main of probiotic strains used in commercial applications (64-66).

Table 3. Main of probiotic strains used in commercial applications.

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other spp.**
acidophilus	*pseudocatenulatus*	*Streptococcus intermedius*
johnsonii	*catenulatus*	*S. salivarius*
plantarum	*bifidus*	*S. cremoris*
rhamnosus	*infantis*	*S. lactis*
delbruecki	*longum*	*S. intermedius*
reuteri	*thermophiles*	*Aspergillus nigerlactis*
fermentum	*adolescentis*	*A. oryzae*
brevis		*Leuconostoc mesenteroides*
lactis		*Pediococcus acidilactici*
cellobiosus		*Enterococcus faesium*
paracasei		*E. faecalis*
helveticus		*Lactococcus lactis*
		Saccharomyces boulardii
		Propionibacterium freudenreichii

1.2.3.3 Selection criteria for probiotics

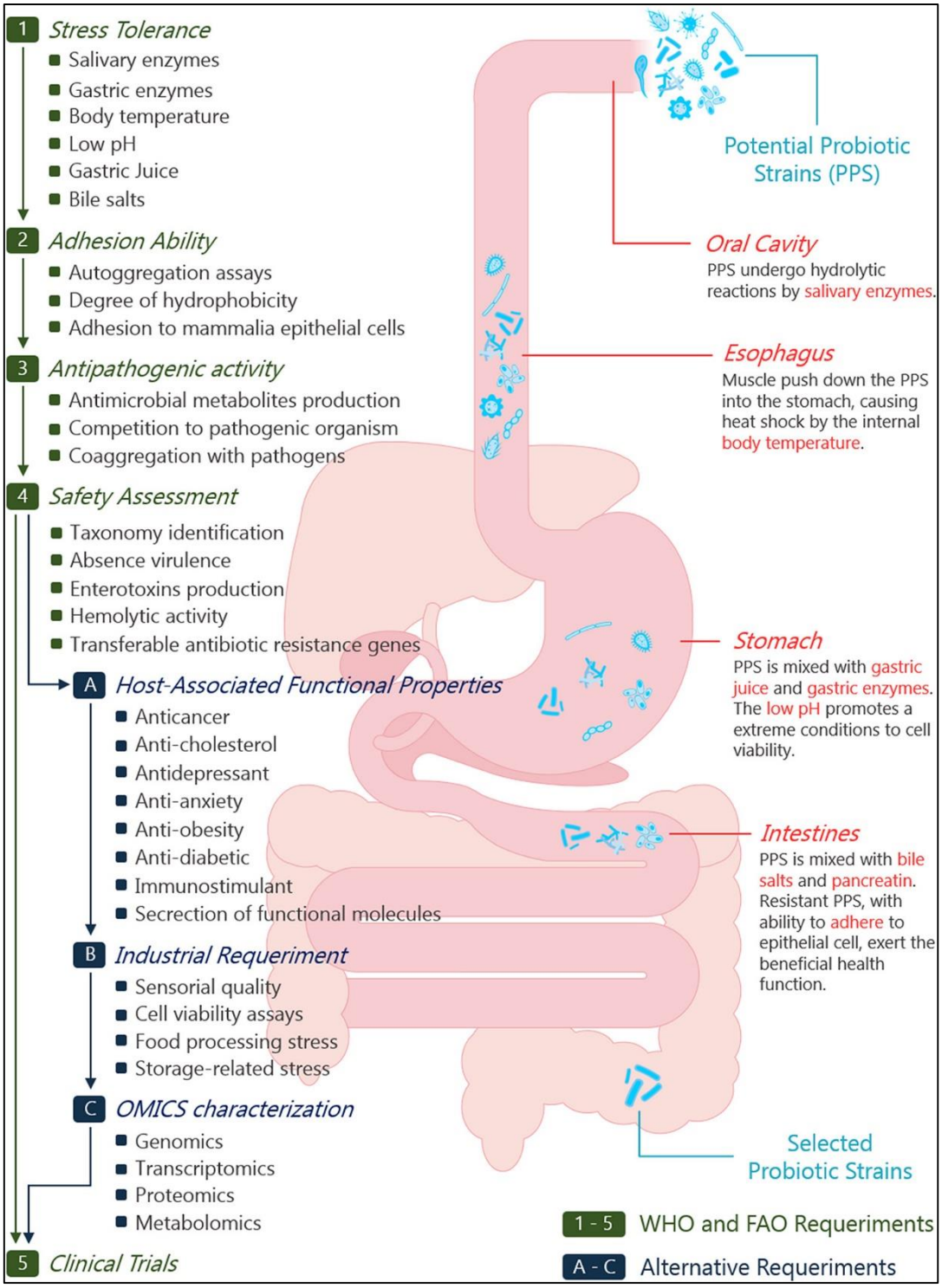


Figure 6. Screening approaches used for characterization of probiotic strains according to WHO/FAO

According to the suggestions of the WHO, FAO, and EFSA (the European Food Safety Authority) (67, 68), probiotic strains must safety and functionality criteria, as well as those related to their technological usefulness (Figure 6) (67, 68). The safety of a strain is defined by its origin, the absence of association with pathogenic cultures, and the antibiotic resistance profile. It must survive passage through the GI tract their immunomodulatory effect. It must be able to function in the gut environment. The functional requirements of probiotics include tolerance to human gastric juice and bile, adherence to epithelial surfaces, persistence in the human GI tract, immune stimulation, antagonistic activity toward intestinal pathogens, and the capacity to stabilize and modulate the intestinal microbiota (69, 70). The selection criteria can be categorized into four basic groups including (69):

Appropriateness:	- Accurate taxonomic identification - Normal inhabitant of the species targeted: human origin for human probiotics - Nontoxic, nonpathogenic, generally recognized as safe (GRAS) status
Technological suitability	- Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution - Viability at high populations - Stability of desired characteristics during culture preparation, storage, and delivery - Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes - Genetically stable - Genetically amenable
Competitiveness	- Resistant to bile - Capable of survival, proliferation, and metabolic activity at the target site *in vivo* - Resistant to acid

	- Able to compete with the normal microflora, including the same or closely related species; potentially resistant to bacteriocins, acid, and other antimicrobials produced by residing microflora - Adherence and colonization potential preferred
Performance & functionality	- Able to exert one or more clinically documented health benefits (e.g. lactose tolerance) - Antagonistic toward pathogenic/cariogenic bacteria - Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds) - Immunostimulatory - Antimutagenic - Anticarcinogenic - Production of bioactive compounds (enzymes, vaccines, peptides)

Probiotic have these criteria used in order to get effective on health and functional probiotic. Probiotics are using the criteria in Figure 3. Proposed the properties of probiotics in three basic groups as; functionality, technological and safety. Some main of selection criteria will be discussed in details.

1.2.3.3.1 Acid and bile tolerance

Probiotics bacteria are joined in the food system with ingested to the lower GI tract via the mouth. In this food system, probiotic bacteria have to resistant to enzymes such as lysozyme in the oral cavity. After that, the probiotic bacteria is going to the stomach and enter the upper GI tract that contains bile salt. These strains should have the ability to resist the digestion processes. Probiotics strains must resist the low pH conditions of the stomach (pH 1.5-3.0) and the upper intestine that contain bile salt (69, 70). Probiotic strains would reach to the lower intestine and maintain it over there. Because the first criteria are investigated probiotic strains that are resistant to acid and bile salt. Bile salt is synthesized in the liver from cholesterol and sent to the gall bladder

and secreted into the duodenum in the conjugated form. In the large intestine, probiotic strains can modified some chemical molecule (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) that show antimicrobial activity (69).

1.2.3.3.2 Antimicrobial activity

Antimicrobial activity is important criteria of probiotics, especially targeting to the enteric pathogens. Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (acetic, propionic acids, lactic, and other aroma compounds), diacetyl, hydrogen peroxide, antimicrobial substances, and bacteriocins (69, 71, 72). Nowadays, there are many researches showing that different species of microorganism produce different antimicrobial substances (67, 73). Bacteriocins are heat-stable and small peptides that have antimicrobial properties against different groups of microorganisms (74). Bacteriocins were divided into four classes. The class I bacteriocins comprise of 19-50 amino acid that is a small peptide (<5 kDa) and include nisin and other lantibiotics. The class II bacteriocins are small (<10 kDa) heat-stable proteins, non-modified peptides. The class III bacteriocins are large, heat-labile (>10 kDa) protein bacteriocins. The class IV bacteriocins are complex bacteriocins containing lipid or carbohydrate moieties (75). Moreover, Lactic acid bacteria mainly produce two classes of bacteriocins (74, 75). For example, *L. lactis* produce lacticin 3 l 4 7 and class I bacteriocin, nisin A (76, 77), *S. boulardii* produce protease, *E. feacalis* DS16 produces a class I bacteriocin cytolysin; *L. plantarum* produces plantaricin S, *L. acidophilus* produces a class III bacteriocin acidophilucin A. Moreover, bacteriocins are highly affected by the factors of not only the species of microorganisms, but also ingredients, pH of medium, and incubation temperature and time (78).

1.2.3.3.3 Safety aspects of probiotics

Traditional probiotic dairy strains of lactic acid bacteria such as *Lactobacillus* and Bifidobacteria have a long history of safe use for more than 70 years and are often recognized as “food grade” or generally recognized as safe (GRAS) microorganisms

(15, 79). Some LAB strains such as *Lactococcus* and *Enterococcus* have been consumed daily since humans started to use fermented milk as food. Nowadays, there is evidence that probiotic strains used as commercial bacteria are safe to use in applications. The safety of the probiotic products is evaluated with the phenotypic and genotypic characteristics and the statistics of used microorganisms. Safety aspects of probiotic bacteria include the following requirements: (79-81)

- Study on the natural occurrence and presence of probiotic strains in the human gastrointestinal tract.
- Study on the intrinsic properties of the strains.
- Studies on the pharmacokinetic properties of strains (survival, activity in the intestine, dose–response relationships)
- studies searching the interactions between the strain and the human host

1.2.3.4 Mechanisms of probiotic actions

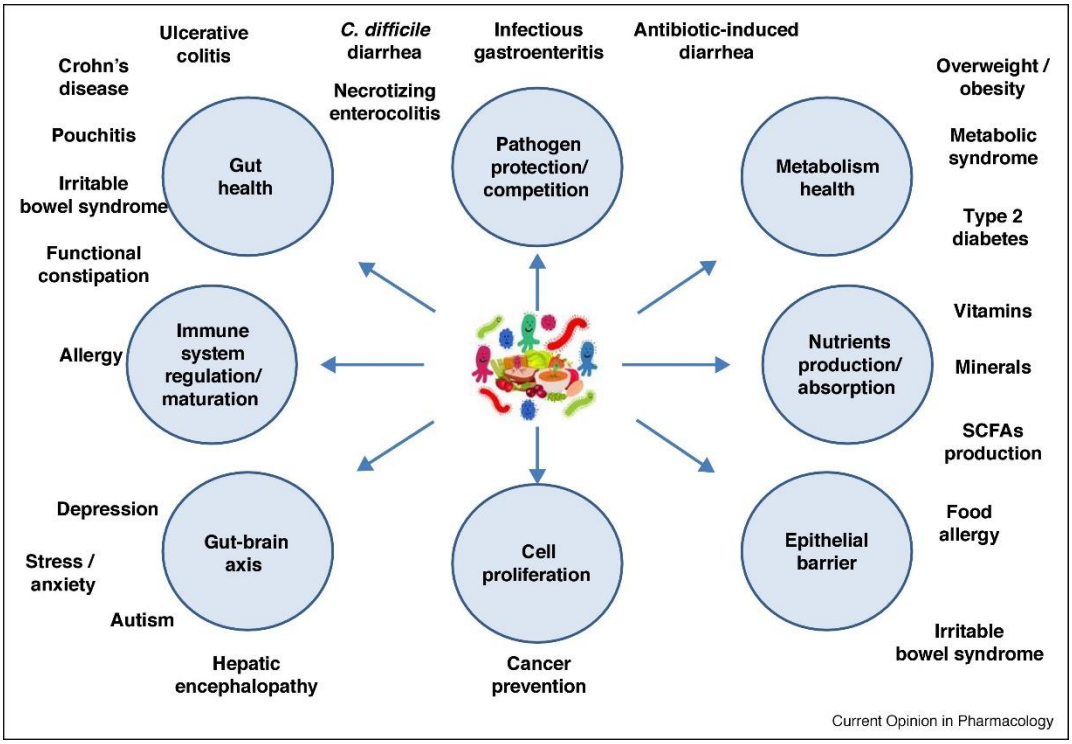


Figure 7. Schematic representation of the main mechanisms probiotics exert their health effects and potential clinical target (82).

There are many studies on the evaluation of probiotics for health benefits (83).While a number of reported effects have been only partially established. The effect of probiotic may include modulation of host immune response, enhanced antibody production, enhanced natural killer cell activity, modulation of dendritic cell phenotype, function, modulation of nf-kb and ap-1 pathway, altered cytokine release, inhibition of proteasome activity, modulation of apoptosis, enhanced epithelial barrier function, enhanced tight junction protein phosphorylation, upregulation of mucous production, enhanced epithelial cell glycosylation, antimicrobial effects, decreased luminal pH, stimulation of defensin secretion, secretion of antimicrobial peptides, inhibition of pathogenic bacterial invasion, blockade of bacterial adhesion to epithelial cells (Figure 7) (82).

Depend on these mechanisms, probiotics have many protective effects against many diseases as diarrhea, inflammatory disease, irritable bowel syndrome, cancer, bacterial infections, and several others (Table 4) (73, 84).

Table 4. Effects of probiotics on different diseases. This table modified from A Ahmad et al, 2018. (84)

Diseases	Study Subjects	Observed Effects
Traveler's diarrhea	Humans	Reduced relative risk and duration of diarrhea in travelers; useful in conjunction with rehydration
Acute infectious diarrhea	Humans (children)	Reduced risk and average duration of diarrhea; reduced risk of rotavirus infection
Antibiotic-associated diarrhea	Humans (children)	Beneficial for treatment of AAD; reduction in diarrheal episodes and *C. difficile* infections
Cancer	Humans/rats	Reduced postoperative infectious complications in CRC; anticarcinogenic properties

Diabetes	Humans/rats	Effective for prevention and management of T1D and T2D; reduces serum CRP levels and increases GSH levels in T2D patients
Obesity	Humans	Regulates body weight and represents a viable treatment option for obesity; may facilitate alleviating metabolic syndrome
Lactose intolerance	Humans	Reduced effects of lactose intolerance; DDS-1 L. acidophilus strain was safe to use in acute lactose intolerance
Ulcerative colitis	Humans	Probiotic treatment was more effective than placebo in controlling UC
H. pylori infection	Humans (children)	No strong evidence for eradication of *H. pylori* through probiotic supplementation
irritable bowel syndrome	Humans	Single probiotic at a low dose and with short treatment is more effective
Crohn's disease	Humans (children)	Effect on PCDAI was to lower by 73% from baseline; CDAI fell from 217 to 150

CDAI, Clinical Disease Activity Index; GSH, glutathione; PCDAI, Pediatric Crohn's Disease Activity Index.

1.2.3.4.1 Mechanism of probiotics to control CDI

Mechanisms of probiotics against CDI divide into two groups (Figure 8) including direct and indirect potential protective CDI (85). For direct effects on *C. difficile* or its toxins, probiotics may suppress germination, colonization and adhesion of *C. difficile* spores or vegetative cells such as Bifidobacteria produce antimicrobials and acids that inhibit *C. difficile* growth and adhesion to intestinal epithelial cells (85). *L. lactis* subsp produce lacticin 3147 that inhibits *C. difficile. B. thuringiensis* capable of producing a Thuricin CD which was shown to be as effective as metronidazole at inhibiting *C. difficile*. Probiotics can produce bile salt hydrolase (BSH) enzymes that

transformation of bile acids form conjugated bile acid to unconjugated bile acid that can inhibit spore germination of *C. difficile. S. boulardii* produce protease protein which hydrolyzed TcdA and TcdB toxins and inhibited their binding to their respective intestinal brush border receptors (85, 86). For indirect effects on *C. difficile* or its toxins that stimulate immune function, probiotics can have an anti-inflammatory effect via stimulation of the innate immune response such as *S. boulardii* can up-regulate specific anti-TcdA secretory IgA expression of CDI and can produce soluble anti-inflammatory factor that inhibits NF-kB-mediated IL-8 gene expression (86-88). *L. plantarum* ATCC 14917 (JCM 1149) and other LAB, can prevent AAD and CDI (89, 90). Moreover, *E. faecium* NM1015, *E. faecalis* NM815, and *E. faecalis* NM915 can inhibit *C. difficile in vivo* test (91). *E. faecium* and *L. lactis* were able to decrease the expression level of TNF-α and IL-8 induced by the cell-free supernatant of *C. difficile* 630 (92).

Several bacterial and fungal species have been use to test against CDI either as single probiotic agents or in combination with other agents including single agent formulations; S.*boulardii, L. rhamnosus* GG, *L. plantarum* 299v, *C. butyricum* M588, *C. difficile* VP20621, *L. plantarum*, other LAB, combination agent formulations; Bio-K + CL1285 (*L. casei* and *L. acidophilus*), Actimel (*L. casei, L. bulgaricus,* and *S. thermophiles*), Florajen3 (*L. acidophilus* and *B. bifidum*) (88-90, 93-95). Another bio-therapy is to induce colonization by a non-toxigenic *C. difficile*. Administration of a non-toxigenic *C. difficile* after antibiotic treatment have been an effective treatment in patients might be complicated by ongoing antimicrobial treatment and the susceptibility of the non-toxigenic *C. difficile* to it (96). Systematic review of Johnson et al. since 1976 to 2010 found 11 studies about CDI outcome of adult patients who had been received antibiotics and were randomized receive either a probiotic or placebo outcome. Only two probiotics, *S. boulardii*, and Bio-K + CL1285 reduced CDI than placebo group (94). These probiotics have been mainly used for patients with RCDI and have been found to be highly effective and safe. However, there are risk of fungaemia and bacteremia in some case (56). Canadian Agency for Drugs and Technologies in Health (CADTH) report is to review the evidence-based guidelines regarding the use of probiotics for the prevention, management, and treatment of AAD and CDI (97). Few studies have actually documented survival of probiotic as it transits the gut and different probiotic species and genus may showed have different immunological and

physiological effects in CDI states. The method of delivery probiotic such tablets, powders, yogurts, milk, ice cream, and juices may have an impact on the viability and number of bacterial colonies (56, 94). These formula of probiotics are developed for the oral administration. The traditional products such as yogurts, milk, ice cream, and juices show limited stability of probiotics. The lyophilized formula has been widely used for delivery of probiotics to the GIT because it can preserve viability of probiotics (98, 99). So, in this study is to screen enteric bacteria as probiotic against *C. difficile* and its spore and to develop the alternative treatment of *C. difficile* infection

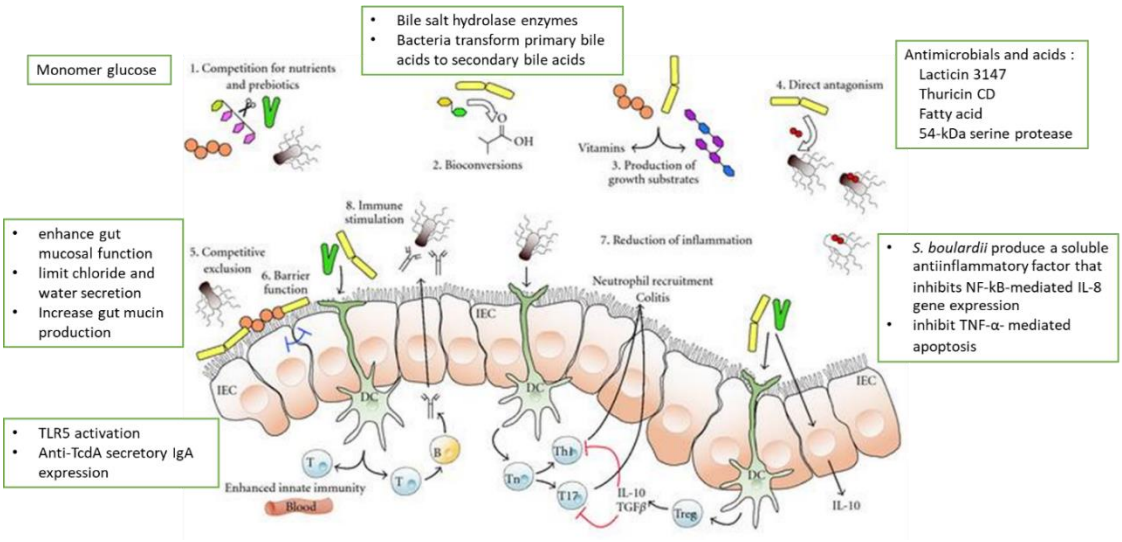


Figure 8. The mechanisms of probiotic to control pathogens (85)

1.2.3.4.2 Using probiotics to treat other diseases

Probiotics could improve health or control pathogenic infections, help in treatment diseases and they may treat inflammatory enteral conditions, including ulcerative colitis, Crohn's disease, and non-specific ileitis (67). There are many reports on the application of probiotics in the treatment of diseases such as *L. rhamnosus* GG reduced number of respiratory tract infections, *L. rhamnosus* GG, *L. reuteri*, *L. casei* Shirota, and *B. animalis* Bb12 can shorten the duration of acute rotavirus diarrhea (62), and *L. rhamnosus* GG can modulate the immune response and prevent onset of allergic diseases (17). Moreover, there is evidence that the consumption of probiotics-containing dairy products results in the reduction of blood cholesterol, which may be

helpful in the prevention of obesity, diabetes, cardiovascular diseases, and cerebral stroke (17, 67). *B. pseudocatenulatum* SPM 1204, *B. longum* SPM 1205, *B. longum* SPM 1207, and *L. plantarum* ATCC 14917 were shown to reduce body weight and adiposity, reduced cholesterol (total, HDL-cholesterol and LDL-cholesterol), TAG, glucose, leptin and hepatic enzyme (aspartate aminotransferase, alanine aminotransferase and lipase) levels in plasma, suggesting that beneficial effects of this strain mixture protect obesity and type 2 diabetes (100-102). Moreover, *E. faecalis* CECT7121 can modulate the immune response that induces a strong dose-dependent activation of dendritic cells (DCs) and secretion of high levels of IL-12, IL-6, TNFα, and IL-10 (103). *E. faecium* M-74 showed a clear cholesterol-lowering effect, specifically of LDL-cholesterol, whereas no change could be detected in HDL-cholesterol, and triglycerides (104).

1.2.4 Formula of probiotic

Probiotics formula divided into two groups including diary probiotic products and non-diary probiotic products (53). Non-diary probiotic products consist of three groups: fermented vegetable and fruit, grains, and meat (63, 105, 106). Traditionally, fermentation of probiotics is one of keeping shelf life and were particularly important before refrigeration but limited shelf life. Currently, probiotics may be incorporated in dietary supplements that are expected to have up to 2 years of stability at room temperature and humidity. Moreover, physicians tend to recommend tablets and powders. The main technologies were involved in fermentation, encapsulation, drying, rehydration, and storage developed and applied to use in probiotics but there are still many technological challenges (Figure 9) in producing and preserving probiotic foods (53, 107).

Bacterial survival circumstance	Technique	Existing challenge	Strategy
Fermentation	One step fermentation;	Temperature	Control of fermentation temperature/ time and pH;
	Continuous fermentation;	Oxygen content;	Addition of antioxidant
	Membrane bioreactors;	Acidification;	
	Immobilized cell fermentation	Undesirable change of sensory	
Encapsulation	Extrusion	Production capacity;	Alginate;
		Particle size	Alginate with starch/ chitosan/ calcium chloride/ poly-amino acids;
		Water-soluble capsuled particle;	Whey protein;
	Emulsion	Choice of solvents	Proteins derived from legumes /milk;
			Pectin;
			Milk;
			κ-carrageenan;
			Sodium carboxymethyl cellulose (NaCMC);
			Cellulose acetate phthalate (CAP);
Drying	Freeze drying	Crystal formation;	Cryo-, lyoprotectants (skim milk,
	Freeze-vacuum drying	Dry cake;	whey protein, glucose,
		Osmotic stress;	maltodextrine, trehalose);
		Mechanical stress	Freeze drying temperature/ rate;
			Pre-treatment to sub-lethal stress;
			Fermentation condition
	Spray drying	Heat stress;	Protectants (disaccharides);
	Two-step spray drying	Shear stress;	Outlet/inlet temperature;
	Spray freeze drying	Osmotic stress; Oxidative	Feed rate;
	Spray chilling	stress	Moisture content in the powder;
			Pre-treatment to sub-lethal stress
	Fluidized-bed drying	Low yields of probiotic cells	Protectants;
			Process parameters;
			Pre-treatment to sub-lethal stress
	Other drying	High cost	Alginate combined with other coating materials
	(hybridization; impinging aerosol technology; Electrospinning)	Probiotic survival is species dependent	
Rehydration	Dissolving	Rehydration media (osmotic stress, pH, composition and volume)	Increase buffering capacity
			Supply nutrients
			Optimize pH
			Proper cell density
Storage	Frozen;	Food ingredients	Optimal formulation of probiotics;
	Chilling;	Oxygen content	Addition of antioxidant;
	Room temperature	Water activity	Control of storage temperature and humidity;
		Storage temperature	Package
		pH and titratable acidity	

Figure 9. Technological challenges associated with survival of probiotics during processing and storage.

1.2.4.1 Drying techniques of probiotic

Recent years, the commercial probiotic products such as food industry, cosmetic, and drug that are increasing but the regulatory scheme is still lacking clarity (108). The probiotics formulation are loss of viability after processing and storage. A lot of factors have an influence on the probiotic viabilities such as dosage formulation, pressure, temperature, oxygen level, and moisture level. Enhancing or preserving longterm stability of probiotics that is key to preserve probiotics in a dry formulation, in which the water is reduced (109, 110). Cryopreservation is one of the standard techniques to preserve probiotic starter cultures over a long time. But, it have many disadvantages, for example, the need for below zero transportation and storage temperatures, expensive and cellular damage to microorganism. So, the drying

technique for probiotics is preferred. Several techniques can be successful such as spray drying, vacuum drying, freeze drying, and fluidized bed drying. The main characteristics of the different drying techniques summarized in the table 5 (111).

Table 5. Overview of the main characteristics of different drying techniques (111).

	Spray drying	Freeze drying	Vacuum drying	Fluidized bed drying
Process type	continuous	batch	batch	continuous/ batch
Control of particle charachteristics	yes	no	no	yes (to some extent)
Knowledge/experience	increasing	well-known, well-described	limited	limited
Extra processing steps to obtain separate powder particles	no	micronization step is necessary to break up the dried cake into separate particles	micronization step is necessary to break up the dried cake into separate particles	granulate material is necessary
Process conditions				
a) Time	Seconds-minutes	hours-days	hours-days	hours
b) Temperature	high (up to 200 °C)	low (< 0 °C)	mild	mild
c) Pressure*	limited	high vacuum (≤ 10 mbar)	low vacuum (≥ 10 mbar)	limited

*Pure vacuum = 0 mbar.

1.3 Objectives

General objective

To identify probiotic from enteric bacteria against *C. difficile* and its spore

Specific objectives

1.3.1 To screen bacteria from stool of breast-fed infant donor that possess activity against *C. difficile* and its spore

1.3.2 To identify and characterize the properties of bacteria as probiotic

1.3.3 To evaluate activity of probiotic against pathogenic *C. difficile* and its spore

1.3.4 To develop and evaluate formula of potential probiotics using fermented milk and freeze-dried powder

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Material and Equipment

Table 6. Materials

Materials	Manufacturers
C. difficile ATCC 630	ATCC, USA
C. difficile ATCC 43255	ATCC, USA
C. difficile DMST 16662	DMST, Thailand
HT-29 cells	ATCC, USA
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, USA
DMSO	Amresco, USA
Fetal bovine serum (FBS)	Gibco, USA
Trysin-EDTA	Gibco, USA
Cycloserine Cefoxitin Fructose Agar	AnaeroGRO™ , USA
Blood agar base (infusion agar)	Himedia, India
De Man, Rogosa and Sharpe agar	Himedia, India
De Man, Rogosa and Sharpe broth	Himedia, India
Thioglycolate broth 500g	Himedia, India
BD™ brain heart infusion agar 500g.	Himedia, India
BD™ brain heart infusion broth 500g	Himedia, India
Glucose	Merck Millipore, Germany
Fuctose	Merck Millipore, Germany
Peptone	Himedia, India
Glycerol	Merck Millipore, Germany
NaCl	Merck Millipore, Germany
Tris-EDTA	Merck Millipore, Germany
Taurocholic acid	Merck Millipore, Germany
Ethanol	Merck Millipore, Germany

L-cysteine	Merck Millipore, Germany
Gas pack	MGC, Japan
Anaerobic jar	MGC, Japan
PBS, pH 7.4	Invitrogen, USA
Antibiotic Antimycotic	Gibco, USA
Vancomycin	Siam Bheasach, Thailand
PCR polymerase	Invitrogen, USA
dNTP	Invitrogen, USA
Primer	Macrogen, Korea
Lysozyme	Amresco, USA
Pepsin	Merck Millipore, Germany
Pancreatin	Sigma Aldrich, USA
Bile salts	Merck Millipore, Germany
Mucin type iii	Sigma Aldrich, USA
Amido black	Merck Millipore, Germany
Acetic acid	Merck Millipore, Germany
Xylene	Invitrogen, USA
Proteinase K	Amresco, USA
0.22 uM filters	Corning, USA
Tetramethylbenzidine	Merck Millipore, Germany
Peroxidase	Invitrogen, USA
Taurodeoxycholic acid	Merck Millipore, Germany
Alexa Fluor™ 488 Phalloidin 300 U.	Invitrogen, USA
4', 6-DIAMIDINO-2-PHENYLIN, 10 MG.	Invitrogen, USA
Acetone	Invitrogen, USA
TritonX-100	Merck Millipore, Germany
Skimed milk	Khotcher, Thailand
Tip	Corning, USA
PCR tube	Corning, USA
1.5 microcentrifuge tube	Corning, USA
15 ml tube	Corning, USA

50 ml tube	Corning, USA
Coolcell Freezing container	Corning, USA
96-well plate	SPL Lifesiences, Korea
24-well plate	SPL Lifesiences, Korea
12-well plate	SPL Lifesiences, Korea
Cell cuture flask	SPL Lifesiences, Korea
Petri dish	SPL Lifesiences, Korea
Serogical pipatte	Eppendorf, USA
Agarose	Invitrogen, USA
100 bp DNA ladder	Thermo Scientific, USA
1 Kb DNA ladder	Thermo Scientific, USA
Gram stain set	M&P IMPEX, Thailand
Syringe-driven Filter Unit	Merck Millipore, Germany

Table 7. Equipment

Equipment	**Manufacturers**
CO_2 incubator	Shel lab, USA
Incubator	Shel lab, USA
Microplate reader	Molecular device, USA
Biological safety cabinet class II	Esco, Singapore
CCD camera	Vilber, France
Thermal Cycler	Bio-rad, USA
Microwave	Sharp, Japan
Autoclave	Hirayama, Japan
Water bath	Memmert, Germany
Centrifuge	Eppendorf, USA
Spectrophotometer	Shimadzu, Japan
Refrigerator	Toshiba, Japan
Freezer	Haier, Japan
Inverted Microscopy	Olympus, Japan

2.2 Methods

2.2.1 Bacteria culture and cell culture condition

2.2.1.1 Bacteria culture

Toxigenic *C. difficile* strains were stored at -80°C in thioglycolate broth with 30% glycerol until testing. Toxigenic *C. difficile* strains were cultured on Cycloserine Cefoxitin Fructose Agar (CCFA) and the agar plates were incubated at 37°C for 48 h under anaerobic conditions.

2.2.1.2 Cell culture condition

The intestinal cell line HT-29, from human colon adenocarcinoma stored at Dulbecco's Modified Eagle Medium (DMEM) with 10% DMSO under liquid N_2. DMEM supplemented with 10% fetal bovine serum (FBS), 3 mM L-glutamine and a mixture of antibiotics (50 μg/mL streptomycin-penicillin, 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B) were used for HT-29 cells cultivation. This cell line was performed under standard conditions, at 37°C 5% CO_2 atmosphere, in a CO_2 incubator.

2.2.2 To screen bacteria from stool of breast-fed infant donor for possess activity against *C. difficile* and its spore

2.2.2.1 Collection of stool from breast-fed infant donors

The sample size (n) were calculated by

$$\frac{Z^2_{1-\alpha/2}\ P(1-P)}{d^2}$$

$Z^2_{1-\alpha/2}$ = confidence interval = 1.96

d = desired precision = 0.15

P = estimated proportion = 0.68

(following the prevalence from Rodríguez E et al 2012)(112)

$$n = [1.96 \times 0.68(1-0.68)] \div (0.15)^2$$

$$n = 38 \text{ samples}$$

38 stool samples were collected from breast-fed infant donors. Volunteers were enrolled according to the following inclusion criteria for collecting stools from breast-fed infant donors in this study is as followed (112):

- Breast-fed infants donor younger than 6 months
- Healthy infants and women without present or past underlying conditions
- Infants and women had not been prescribed antibiotics for at least 3 months prior to the study or had taken any probiotics
- Normal, full-term pregnancy
- Absence of infant and/or maternal perinatal problems, including mastitis

The experiments were carried out after ethic had been approved from the Ethics Committees at Faculty of Medicine, Prince of Songkla University (REC.61-064-4-2).

2.2.2.2 Isolation and culture condition

Stool samples from breast-fed infant donors was put into transport anaerobic medium [brain heart infusion (BHI) broth] that were replaced with O_2-free CO_2 gas and transported immediately to a laboratory. The samples were culture on BHI agar, DeMan, Rogosa and Sharpe (MRS) agar and blood agar (BA). The agar plates were incubated at 37°C for 48 h under anaerobic conditions (112, 113). After incubation, each of isolate colony was stored at -80°C in BHI broth with 30% glycerol until testing.

2.2.2.3 Screen for bacterial strains from stools of breast-fed infant donors active against C. *difficile*

Agar well diffusion assay was used to test inhibitory activity of the isolated colonies from stool of breast-fed infant against the toxigenic *C. difficile* (93). Overnight cultures of toxigenic C. *difficile* strains were suspended in BHI broth to a density of 1 x 10^8 CFU/mL and spread on the BHI agar. Five wells, each 9 mm in diameter, was cut out of the agar, and 50 µl of 1 x 10^8 CFU/mL of each selected anaerobic bacteria from

breast-fed infant donors were dropped into each well and incubated at 37 °C for 48 h under anaerobic condition. After the incubation period, the diameter of the growth inhibition zones were measured. Phosphate buffered saline (PBS) was used as negative control while 5 µg/well vancomycin was used as positive control. This test was performed in triplicate. For interpreted result, if the selected anaerobic bacteria can inhibit toxigenic *C. difficile* strains, that isolate was used for next studies: Identification and characterization the properties of anaerobic bacteria as probiotic.

2.2.3 To identify the properties of bacteria as probiotic

2.2.3.1 Bergey's test and Biochemical test

The morphological and biochemical properties of each isolate were characterized according to Bergey's manual (114). Gram staining, cell morphology, catalase activity, salt tolerance, gas production, and biochemical carbohydrate fermentation patterns were assessed by Microbiology laboratory in Songklanagarind hospital. Afterward, the isolated colonies were confirmed by MALDI-TOF and 16S rRNA gene sequencing.

2.2.3.2 MALDI-TOF

Three to five colonies of bacterial were transfer into 300 µl sterile water, suspended and mix with 900 µL of ethanol (EtOH). After centrifugation, the pellets were suspended gently with formic acid and incubated at room temperature for 30 min. Then 100% acetonitrile was added, the supernatant were collected. Then, alpha-cyano-4-hydroxycinamic acid (HCCA) matrix solution (10 mg HCCA in 1 mL of OS (500 µl of 5% triflouro acetic acid and 500 µl of 100% acetonitrile)) were added to the supernatant and spot 2 µl of mixture on MALDI plate. Then, the matrix were dried at room temperature (not under air flow). Finally, the MALDI plate was inserted into the mass spectrometer and main spectra was generated by double-measurement with adequate quality control criteria. All spectra were analyzed by using BioTyper 2.0 software and flexAnalysis software. Pattern matching was determined by calculation of

a score, considering the proportion of matching peaks between the unknown spectrum and the reference spectra of the database as well as the consistency of the peak intensities between these spectra. The identification by MALDI-TOF MS is based on the score value released by the equipment (Figure 10).

Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	no reliable identification	(-)	red

Figure 10. Bruker's interpretations of the score values

2.2.3.3 16S rDNA gene sequencing

DNA used as template for PCR was direct extracted from bacterial cultures. Briefly, 3-5 colonies of each bacterial isolated were suspended into 50 µl of TE buffer. Then, cell suspension was heated at 94 °C for 5 min followed by cooling at 4 °C and centrifuge 10,000 rpm 1 min after that keep supernatant for PCR testing. PCR were performed in a total volume of 50 µl PCR reaction consisted of 1X PCR buffer (10mM Tris-HCl, 50 mM KCl, and pH 8.3), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1U of *Taq* DNA polymerase, 0.5 µM of 16s rDNA primer pair and 2 µl DNA template. The PCR condition was 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 55 s at 60 °C and 72 s at 70 °C, and a final extension of 5 min at 72 °C. The PCR products were held at 4 °C until subjected to agarose gel electrophoresis. Sequencing was conducted using the dideoxy chain termination method and sequence similarity was determined by the BLAST search tool (115).

2.2.4 Characterize the properties of bacteria as probiotic

2.2.4.1 Antibiotic susceptibility test

The safety of probiotic strains is importance. They do not carry transmissible antibiotic resistance genes and they are not resistant to gentamicin, ampicillin, cephalosporins, and vancomycin (116). So, the bacterial susceptibility of probiotic to clinically relevant antibiotics were screened by a disc diffusion assay. The antibiotic profile of the isolates were done by Kirby-Bauer method. Cultured isolates were suspended in BHI broth to a density of 1.0 McFarland. A sterile cotton swab was placed in the suspension. The inoculum were spread on surface of the plate. All susceptibility tests were performed on BHI agar. The antibiotics were selected for testing: ampicillin, penicillin, imipenem, vancomycin, gentanicin, erythromycin, tetracycline and cefotaxime (117). Plates were incubated for 24 h under anaerobic conditions. The zone of inhibition were measured and susceptibility reporting were carried out under CLSI 2007 guidelines (118).

Moreover, vancomycin resistant gene was determined by PCR and sequencing. Briefly, PCR was performed in a total volume of 50 µl PCR reaction consisted of 1X PCR buffer (10mM Tris-HCl, 50 mM KCl, and pH 8.3), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1U of *Taq* DNA polymerase, 0.5 µM each of primer pair (*VanA* and *VanB*) and 2 µl DNA template (Direct extraction DNA). The PCR condition was 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 55 s at 60 °C and 72 s at 70 °C, and a final extension of 5 min at 72 °C. The PCR products were held at 4 °C until subjected to agarose gel electrophoresis. It was resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.2.4.2 Survival under gastrointestinal tract (GIT) conditions

The potential of probiotic candidates, its ability to tolerance and survive under GIT conditions should first be investigated.

2.2.4.2.1 Lysozyme sensitivity test

Following the method by Rodríguez E, et al. 2012 (112) with slight modifications. Isolated strains were cultured on BHI broth at 37 °C for 24 h. Isolated strains were harvested by centrifugation at 10,000g for 5 min, and washed twice with PBS (pH 7.2). Cultured isolates were suspended in BHI broth to a density of 1 x 10^8 CFU/mL. Suspensions were inoculated at 10 µl/ml of BHI broth with or without 25 µg/ml lysozyme. The cultures were incubated at 37 °C for 5 min and plated on BHI agar. Viable counts were determined after 48 h incubation under anaerobic condition. The experiment were carried out in triplicate.

2.2.4.2.2 Resistance to low pH

Resistance to low pH were tested as described previously by Rodríguez E, et al. 2012 (112). In brief, bacterial cells from overnight cultures were harvested by centrifugation at 10,000g, for 5 min, washed twice with PBS (pH 7.2) and re-suspended in PBS adjusted to pH 2.0, 3.0 and 4.0. Resistance to low pH were assessed in terms of viable colony counts and enumerated on BHI agar plates after incubation at 37 °C for 0 and 120 min.

2.2.4.2.3 Tolerance to simulated gastric and pancreatic digestion

Selected strains were tested as describe by Monteagudo-Mera A et al. 2012 (117) with slight modifications. Briefly, simulated gastric juice were prepared by suspending pepsin in NaCl to a final concentration of 3 mg/mL and adjusting the pH to 2.0 with HCl. Simulated pancreatic juices were prepared by suspending pancreatin in NaCl to a final concentration of 1 mg/ml and adjusting the pH to 8.0 with NaOH. 0.5 ml of 1 x 10^8 CFU/mL bacterial cell suspensions mixed with 1 mL of pepsin or pancreatin. Tolerance were assessed in terms of viable colony counts on BHI agar plates and enumerated after incubation at 37 °C for 0 and 180 min with pepsin, and 0 and 240 min with pancreatin.

2.2.4.2.4 Bile salts tolerance

This method was performed according to Monteagudo-Mera A et al. 2012 (117) with slight modifications. Briefly, 10^8 CFU/mL of selected strains were inoculated in 10 ml of BHI broth supplemented with 0.3%, w/v bile salts. The mixture were incubated at 37 °C for 4 h without agitation. Then, aliquots of 0.1 mL were removed periodically for determination of total viable count following the spread-plate method. The experiment were performed in triplicate.

2.2.4.2.5 Consecutive exposition under gastric and intestinal conditions

Selected strains were tested as describe by Monteagudo-Mera A et al. 2012 (117). Isolated strains were cultured on BHI broth at 37 °C 24 h. Isolated strains were harvested by centrifugation at 10,000g, for 5 min, and washed twice with PBS (pH 7.2). Cultured isolated were suspended in PBS to a density of 1 x 10^8 CFU/mL. Suspension were inoculated at 10 µl/mL of PBS with 25 µg/mL lysozyme. The cultures were incubated at 37 °C for 5 min. Then harvested by centrifugation at 10,000g, 5 min, re-suspended in PBS adjusted to pH 3.0 with 3 mg/mL of pepsin. Suspension were incubated 37 °C for 180 min and harvested by centrifugation at 10,000g, for 5 min. Then, re-suspend in pancreatin in NaCl to a final concentration of 1 mg/mL and adjusting the pH to 8.0 with NaOH and 0.3% bile salt. Suspension were then incubated 37 °C for 240 min. Viable cell population was determined by plate counting. The experiment were performed in triplicate.

2.2.4.3 Adhesion ability

Adhesion of probiotic strains to the intestinal surface has been suggested as important prerequisite for probiotic action. Hydrophobicity assay and adhesion to mammalia epithelial cells were tested in this study.

2.2.4.3.1 Hydrophobicity assay

The hydrophobicity were determined by xylene extraction according to VLKOVÁ E et al. 2008 with minor modifications (119). Ten ml of overnight cultures were harvested by centrifugation, washed in potassium phosphate buffer, re-suspended in 5 mL of the same buffer into 1 OD_{600} of cell suspension. One mL of xylene was added and the suspension were vortexed for 2 min, the phases were allowed to separate, and OD_{600} of the aqueous phase was measured. % Hydrophobicity (H%) was calculated according to the formula

H% = [(A0 – A)/A0] × 100, where A0 and A are absorbance values measured before and after xylene extraction.

This method can be used for screening potentially probiotic bacteria adhere to cells. The high percentage of hydrophobicity, probiotic can adhere and colonize in GI tract.

2.2.4.3.2 Adhesion to mammalia epithelial cells

This method was modified from Monteagudo-Mera A et al. 2012 (117). Briefly, for each adhesion assay, 1 mL of 10^8 CFU/mL of selected bacterial suspension were added to each monolayer of cells at 80% confluence placed in 24-well tissue culture plates, which incubated at 37 °C in 5% CO_2. After 2 h of incubation, the monolayer were washed four times with sterile PBS in order to remove unbound bacteria. Then, 200 µl of trypsin (2.5%, w/v) was added to detach the monolayer cells. The adhered bacteria were recovered by repeated pipetting with 800 µl of sterile water and enumerated by plating serial dilutions on BHI agar. Adhesion values (%) was calculated as follows:

% Adhesion = $(V_1 \times 100)/V_0$

V_0 is the initial viable count of bacteria tested, and V_1 is the viable bacteria count obtained from the HT-29 cells, at the end of the experiment.

The adhesion ability to the intestinal epithelial cell is key for probiotic colonization. If the probiotic show a high percentage of adhesion ability, the probiotic can strongly adhere to the intestinal epithelial cell.

2.2.4.4 Antimicrobial activity

Overnight cultures of bacterial strains (Table 8) were suspended in BHI broth to a density of 1 x 10^8 CFU/mL and spread on the BHI agar. Five wells, each 9 mm in diameter, was cut out of the agar, and 50 µl of 1 x 10^8 CFU/mL of each selected anaerobic bacteria from breast-fed infant donors were dropped into each plate and incubated at 37 °C for 48 h. After the incubation period, a clear zone of inhibition around the well was considered positive. PBS was used as negative control. This test was performed in triplicate.

Table 8. Bacteria strains for antimicrobial activity test

No.	Bacteria strains
1	Clinical *C. difficile*
2	*Clostridium perfringens* ATCC 13123
3	*Clostridium sordellii* ATCC 9714
4	*Clostridium sporogenes* ATCC 19404
5	*Salmonella typhimurium* DMST 22842
6	*Shigella flexneri* DMST 44237
7	*Acinetobacter baumannii* ATCC 17978
8	*Acinetobacter calcoaceticus* DMST 26590
9	*Listeria monocytogenes* DMST 17303
10	*Staphylococcus aureus* ATCC 6538
11	*Escherichia coli* O157:H7 DMST 2743
12	*Vibrio cholerae* nonO1/nonO139 DMST 2873
13	*Vibrio parahaemolytica* DMST 15285
14	*Haemophilus influenza* ATCC 49247
15	*Stenotrophomonas maltophiliam* DMST 19079
16	*Bacillus cereus* DMST 11098
17	*Salmonella enteritidis* DMST 15676

18	*Bacillus subtilis* DMST 7988
19	*Campylobacter coli* ATCC 11353
20	Clinical *Klebsiella pneumoniae*
21	Clinical *Haemophilus influenza*
22	Clinical *Stenotrophomonas maltophilia*
23	Clinical *Shigella sonnei*
24	Clinical *Shigella flexneri*

2.2.4.5 Safety assessment

The safety of probiotic strains is important. They do not carry transmissible antibiotic resistance genes and virulence factor genes that encode different protein including:

- *Agg;* aggregation protein involved in adherence to eukaryotic cells, cell aggregation, and conjugation
- *gelE;* toxin; extracellular metalloendopeptidase, hydrolyzes gelatin, collagen, hemoglobin, and other bioactive compounds
- *cylM*; pttranslational modification of cytolysin (hemolysin-bacteriocin, which lyses a broad range of eukaryotic and gram-positive cells)
- *cylB*; transport of cytolysin
- *cylA;* activation of cytolysin
- *espfs*; cell wall–associated protein involved in immune evasion
- *efaAfs*; cell wall adhesins expressed in serum
- *cpd, cob, ccf, cad*; sex pheromones, chemotactic for human leukocytes; facilitate conjugation (120)

for *cylA, cylB* and *cylM* genes that are considered the main pathogenic factor of *E. faecalis* (121).

The safety assessment was determined by both genotype and phenotype tests. The virulence gene were screened by PCR while phenotype test were investigated by detection of gelatinase production, hemolysis production, and mucin degradation. In addition, the survival of animal model (*Galleria mellonella*) that is used as an infection

model to study virulence factors and pathogenesis of many prominent bacteria were also performed (120, 122, 123).

2.2.4.5.1 PCR

The multiplex PCR condition was modified from Reviriego C., et al. (2005) and Lempiainen H., et al. (2005) in order to simultaneously detect virulence gene carried by isolated strains (120, 124). Briefly, multiplex PCR were performed in a total volume of 50 µl PCR reaction consisted of 1X PCR buffer (10mM Tris-HCl, 50 mM KCl, and pH 8.3), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1U of *Taq* DNA polymerase, 0.5 µM each of primer pair (Table 9.) and 2 µl of DNA template (Direct extraction DNA). The PCR condition was 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 55 s at 60 °C and 72 s at 70 °C, and a final extension of 5 min at 72 °C. The PCR products were held at 4 °C until subjected to agarose gel electrophoresis. It was resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Then, the positive gene were confirmed by sequencing.

Table 9. The PCR primers for enterococcal virulence factor and product size used in this study

Gene	Primers	Sequence(5'→3')	Expected product (bp)	Ref.
Agg2	TE32	GTTGTTTTAGCAATGGGGTAT	1210	(110)
	TE33	CACTACTTGTAAATTCATAGA		
gelE	TE9	ACCCCGTATCATTGGTTT	419	
	TE10	ACGCATTGCTTTTCCATC		
cylM	TE13	CTGATGGAAAGAAGATAGTAT	742	
	TE14	TGAGTTGGTCTGATTACATTT		
cylB	TE15	ATTCCTACCTATGTTCTGTTA	843	
	TE16	AATAAACTCTTCTTTTCCAAC		
cylA	TE17	TGGATGATAGTGATAGGAAGT	517	
	TE18	TCTACAGTAAATCTTTCGTCA		

esp	TE34	TTGCTAATGCTAGTCCACGACC	933	
	TE36	GCGTCAACACTTGCATTGCCGAA		
cpd	TE51	TGGTGGGTTATTTTTCAATCC	782	
	TE52	TACGGCTCTGGCTTACTA		
espfs	TE34	TTGCTAATGCTAGTCCACGACC	933	
	TE36	GCGTCAACACTTGCATTGCCGAA		
cob	TE49	AACATTCAGCAAACAAAGC	1405	
	TE50	TTGTCATAAAGAGTGGTCAT		
ccf	TE53	GGGAATTGAGTAGTGAAGAAG	543	
	TE54	AGCCGCTAAAATCGGTAAAAT		
cad	E42a	TTGCTTTGTCATTGACAATCCG	1299	
	TE43a	ACTTTTTCCCAACCCCTCAA		

2.2.4.5.2 Phenotype assay

The methods was modified from Reviriego C., et al. (2005) (120).

Gelatinase production: single colony of selected bacteria were streaked onto medium agar containing gelatin, grown overnight at 37°C, and placed at 4°C for 5 h before examination for zones of turbidity around the colonies. The turbidity zone indicate the activity of gelatinase in hydrolysis of gelatin.

Hemolysin production: selected bacteria were streaked onto blood agar plates and incubated for 24 h at 37°C. Zones of clearing around colonies indicated hemolysin production. For hemolytic activity tube test, briefly red blood cell (RBC) were washed with 0.9% NaCl 4 times and resuspension in 0.9% NaCl and then incubated with CFS of *E.faecalis* or 1% (v/v) tween80 after that incubated at 37 °C for 60 min. Hemolysis was monitored by measuring the absorbance wavelength at 595 nm by micro-plate reader. Percentage of hemolysis was calculated as follows:

% hemolysis = $[(h_1-h_0) \times 100]/ (h_2-h_0)$

h_0=OD of 0.9% NaCl, h_1=OD of test, h_2 =OD of tween80

Mucin degradation: the ability to degrade mucin is considered as one of the valuable indicators of potential pathogenicity and local toxicity of intestinal bacteria.

Partially purified pig gastric mucin type III was used to determine mucin degradation according to Monteagudo-Mera A et al. 2012 (117). Firstly, mucin was added to an basal medium (2 g Peptone, 2 g yeast extract, 0.1 g l-cysteine-HCl, 4 mL mineral solution-1 (0.78% K_2HPO_4 solution), 4 mL of mineral solution-2 (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2CO_4$, 0.12% $CaCl_2$, 0.25% $MgSO_4 \cdot H_2O$), 1.5% agar and 192 mL of distilled water) at a concentration of 0.5% (w/v) with 3% (w/v) glucose or without glucose. Ten µl of bacterial cultures were inoculated by spotting on agar plates. The plates were incubated at 37 °C for 2 days under anaerobic condition. After incubation, the plates were stained with 0.1% (w/v) amido black in 3.5 M acetic acid for 30 min. Then, they were washed with 1.2 M acetic acid until the mucin lysis zone around the colony of positive control culture appeared. Positive control is fecal flora grown overnight in BHI medium. This assay was performed in triplicate.

2.2.4.5.3 *Galleria mellonella* survival test

The method was modified from Yang HF., et al. (2017) (125). Ten larvae (200 – 300 mg each) per group and each larvae was injected 10 µl of 5×10^8 CFU/mL selected bacteria, 10 µL of 5×10^8 CFU/mL *E. faecalis* DMST 4736, or 10 µl of PBS into the last left pro-leg into the haemocoel using insulin syringes after that incubated in the dark at 37°C for 5 days. The number of dead larvae was recorded daily. Survival rate were plotted using the Kaplan–Meier method. The experiment were performed in triplicate.

2.2.5 To identify mechanisms of probiotic against pathogenic *C. difficile*

Probiotic can inhibit pathogenic bacteria via production of antimicrobial substances production such as bacteriocin, hydrogen peroxide and bile salt hydrolase. So, we identified antimicrobial substance production by spot-on-lawn method, hydrogen peroxide production test, and bile salt hydrolase (BSH) activity test, respectively.

2.2.5.1 Spot-on-lawn method

This method was modified from Han SK et al. 2014 (115), which widely used to screen bacteriocin production of bacteria. 10^8 CFU/mL bacteria cells were centrifuged (7000g for 10 min), after that kept the supernatants and adjusted to pH 6.5 with 1 N NaOH. Then, the neutralized supernatants were add with or without 1 mg/mL of proteinase K. Mixtures were incubated at 30 °C for 2 hours and then heated at 80 °C for 10 min to inactivate the enzymes. After that, tne mixture were filtered through 0.2 μm pore size membrane filters. The supernatants were dropped into each plate which had been spread with overnight culture of *C. difficile* onto the surface of BHI agar via sterile cotton swab and incubated at 37 °C for 48 h under anaerobic conditions. The plates were checked for inhibition zones. For interpreted result, if the selected bacteria can produce bacteriocin, smaller clear zone or no clear zone presented in well incubated with proteinase K when compared to that of wells did not treated with proteinase K.

2.2.5.2 Hydrogen peroxide production

Probiotic may be produce hydrogen peroxide that inhibit or kill *C. difficile.* The method to detect H_2O_2 production was modified from Song YL et al. 1999 (126). 10^8 CFU/mL (1 mL) of selected bacterial suspension H_2O_2 production were tested with BHI agar supplemented with 0.25 mg/mL of tetramethylbenzidine and 0.01 mg/mL peroxidase. Inoculated plates were anaerobically incubated for 2 days at 37 °C. After incubation, the plates were removed from the chamber and exposed to ambient air for 30 min to allow for color production. H_2O_2 production was ranked as strongly positive, weakly positive, or negative according to the intensity of blue color development.

2.2.5.3 Bile salt hydrolase (BSH) activity test

This method was modified from Wang Z et al. 2012 (127). Briefly, overnight cultures of selected anaerobic bacteria were streaked on BHI agar supplemented with or without 0.5% taurodeoxycholic acid. The plates were incubated for 2 days at 37°C under anaerobic conditions. The BSH activity was indicated by the formation

precipitated deconjugated bile acids surrounding the colonies on the bile salt-containing BHI agar plate.

2.2.6 To evaluate activity of probiotic against vegetative *C. difficile*

2.2.6.1 Co-culture probiotic with toxigenic *C. difficile* and cell lines

This method was modified from Valdés et al., 2016 (128). The selected anaerobic bacteria strains were cultured in BHI broth and incubated at 37 °C for 18 h under anaerobic conditions. Then, they were washed twice with PBS and re-suspended 10^8 CFU/mL in the HT-29 cultivation medium supplemented with 10^8 CFU/mL toxigenic *C. difficile* and then incubated for 1 h under anaerobic conditions with stirring at 300 rpm. Next, 20 µl of the mixtures were directly added to upon HT-29 monolayers which bad been seeded with 5×10^4 cells per well onto 96-well tissue culture microliter plates and were incubated at 37°C in 5% CO_2 until a confluent monolayer form in order to test their cytotoxicity upon HT-29 cells. The plates were incubated for 24 h at 37 °C in 5% CO_2. After that, morphological change of cells were examined under an inverted microscope. The cytopathic effect was indicated by more than 50% of rounded cells.

2.2.6.2 Immunofluorescence assay (F-actin)

Probiotic against cytotoxic effect of *C. difficile* on intestinal epithelial HT-29 cells were confirmed by *F*-actin detection (Immunofluorescence assay) The method was modified from Valdés et al., 2016 and Jafari NV et al., 2016 (128, 129). Briefly, the HT-29 monolayers cells submitted to different supernatants treatments were analyzed by confocal microscopy. For this, 8-well µ-Slide were seeded with 300 µl of 2×10^6 HT-29 cells/mL and incubated for 20 h to reach confluent state. Then, supernatant was removed and wells were filled with the same volume of each supernatant containing different selected anaerobic bacteria strains with toxigenic *C. difficile* or DMEM medium (negative control). Incubation continued for additional 24 hours. Then, supernatant of each well were removed and HT-29 monolayers cells were fixed with 300 µl of 3.7% formaldehyde for 15 min. Samples were washed twice with

PBS for 5 min and permeabilized with PBS containing 0.1% Triton 100x for 15 min. The nonspecific binding sites were blocked with FBS (25% in PBS) for 20 min and finally washed once with PBS. The Phalloidin-Alexa-Fluor-488 probe toward F-actin was added in 100 µl of PBS (final concentration 1:40) and samples was incubated 1 h at 4°C in darkness. After washing three time with PBS, HT-29 nucleus was stained with DAPI probe used at 1:1000 (final dilution in PBS) and incubated 20 min. Finally, samples was washed and added to 50 µl of antifade mountants previous visualization under confocal laser scanning microscope ZEISS LSM 800 with airyscan.

2.2.7 To evaluate activity of probiotic against *C. difficile* spore

C. difficile spore play a main role in infection and disease transmission. Some probiotic can inhibit sporulation and germination of *C. difficile* spore. So, we was evaluated inhibition activities of probiotic against *C. difficile* spore by inhibitory germination test and inhibitory germination test.

2.2.7.1 Spore purification

This method was modified from Lawley TD et al., 2009 (13). *C. difficile* was grown on BHI agar overnight at 37 ^{o}C. Single colony from the BHI agar plate was inoculated in 10 ml of BHI broth with 0.5% yeast extract and 0.1% L-cysteine and incubated at 37 ^{o}C overnight under anaerobic conditions. One ml of BHI culture was sub-cultured into BHI agar with 0.1% L-cysteine incubated at 37 °C in an anaerobic jar for 7 days. After 7 days incubation at 37 ^{o}C sporulation efficiency was confirmed by phase-contrast microscopy and measured of heat-resistant CFU and spore crops harvested immediately or after overnight incubation at 4 ^{o}C. Spores were washed in PBS twice, then suspended in PBS containing 125 mM Tris, 200 mM EDTA, 0.3 mg/mL proteinase K and 1% sarcosyl, and incubated with gentle shaking at 37 ^{o}C for 2 h. Spores were centrifuged (6,500 g, 10 min) and pellets were resuspended in water and washed 10 times. After the final suspension in water, spores were heat-treated (60 ^{o}C, 20 min) to kill any residual cells. Spore supernatants were stored at 4 ^{0}C until testing. To calculate the spore CFU aliquots were serially diluted in PBS and plated

onto BHI agar supplemented with 0.1% sodium taurocholate. Plates were incubated for 48 h at 37 ^{o}C before CFU were enumerated.

2.2.7.2 Inhibitory germination test

This method is modified from Carlson PE et al., 2015 (130). Briefly, 15 µl of 5×10^6 spores/ml spore suspension were added to 96-well plates containing 135 µl of BHI broth and 0.01% taurocholate, with or without 10^8 CFU/mL selected anaerobic bacteria strains incubated anaerobically at 37 °C for 30 minutes. Germinated spores were enumerated by plating for CFU on BHI agar. Percent relative germination was calculated as [CFU post-assay/initial CFU] × 100. This method was performed in triplicate.

2.2.7.3 Inhibitory sporulation test

The method for inhibition of sporulation was modified from Carlson PE et al., 2015 (130). Spore formation was evaluated in broth cultures. Log-phase of *C. difficile* cultures in BHI were inoculated in tryptose yeast extract (TY) broth (3% tryptose, 2% yeast extract) at initial density of 1×10^6 CFU/mL with or without 10^8 CFU/mL selected anaerobic bacteria strains. After 48 h of culture, samples were analyzed for the presence of vegetative cells and spores using microscopic method. The percentage of sporulation were calculated by [number of spore/ (number of spore × number of vegetative cell] × 100.

2.2.8 To develop and evaluate formula of potential probiotic

2.2.8.1 Optimization of dairy probiotic product

The method for preparation of fermented milk was modified from Westerik N et al., 2016. Isolated *E. faecalis* was cultured in BHI broth incubated at 37 °C for 18 h. Then, 1 mL of the culture were inoculated into 5 mL of sterile BHI broth for 5 tube and incubated at 37 °C. Bacterial cells were harvested by centrifugation

(×6000 g, 10 min, 4°C), washed three time with sterile DI water and re-suspended in 1 mL sterile DI water. To prepare pasteurized milk, Ultra-high-temperature (UHT) processed milk was mixed with 5% sugar, and 3% skim milk from different company including Dumex, Anlene, Diary, and Skin Milk Replacer. Then, the mixtures were heated at 70°C for 30 min. One ml of 10^8 CFU/mL *E. faecalis* suspension was inoculated into 50 mL pasteurized milk incubated at 37 °C for 16 h. Finally, the final pH, viability of *E. faecalis*, and antimicrobial activity were determined.

2.2.8.2 Freeze-dried powder assay

The method for preparation of freeze-dried powder of selected bacteria strains was modified from Jalali M et al., 2012 (131). Specific anaerobic bacterial was cultured in BHI broth incubated at 37 °C for 18 h under anaerobic conditions. Then, 5 ml of the culture was inoculated into 200 mL of sterile BHI broth and incubated at 37 °C under anaerobic conditions. Cells in the early stationary phase of growth were harvested by centrifugation (×6000 g, 10 min, 4°C), washed twice with sterile DI water and re-suspended in 10% skim milk to obtain final bacterial cell concentration of 1×10^{11} CFU/mL. Three ml of suspensions were transferred into glass tube and frozen at -80°C. Frozen media was desiccated under vacuum for 24 h and freeze-dried powders were stored at 4°C.

2.2.8.3 Activity of potential probiotic formula

2.2.8.3.1 Viability test

The viability cell of selected bacteria strains was modified from Begum AA et al., 2015 (132). The content of the freeze-dried powders were resuspended in 3 ml sterile distilled water, while the fermented milk were serial dilution. After performing serial dilution, appropriately diluted solutions were then plated onto BHI agar. Plates from each dilution were incubated at 37°C and colonies were counted after 24 h incubation.

2.2.8.3.2 Antimicrobial activity (agar diffusion assay)

The antimicrobial activity of selected bacteria strains was modified from Schoster A et al., 2013 (93). Overnight cultures of toxigenic C. *difficile* strains were suspended in BHI broth to a density of 1 x 10^8 CFU/mL and spread on the BHI agar. 50 µl of 1 x 10^8 CFU/mL of probiotic were dropped into plate and incubated at 37 °C for 48 h under anaerobic conditions. After the incubation period, the diameter of the growth inhibition zones was measured. PBS was used as negative control while vancomycin was be used as positive control.

2.2.8.3.3 Hemolysis activity

The content of the freeze-dried powders were resuspended in 3 ml sterile distilled water while fermented milk was used directly. Then, were streaked onto blood agar plates after that incubated for 24 h at 37°C. Zones of clearing around colonies indicated hemolysin production. For hemolytic activity tube test, briefly red blood cell (RBC) were wash 4 time with 0.9% NaCl and resuspension in 4 mL of 0.9% NaCl and then incubated with freez-dried suspension or 1% (v/v) tween80 after that incubate 37 °C, 60 min. Hemolysis activity was monitored by measuring absorption at 595 nm by micro-plate reader. Percentage of hemolysis was calculated as follows:

% hemolysis = $[(h_1-h_0) \times 100]/ (h_2-h_0)$

h_0=OD of 0.9% NaCl, h_1=OD of test, h_2 =OD of tween80

2.2.8.3.4 *Galleria mellonella* Survival test

The method was modified from Yang HF., et al. (2017) (125). Five larvae (200 – 300 mg) each per group and each larvae was injected 10 µl of freeze-dried powder suspension, 10 µl of 5×10^8 CFU/mL *E. faecalis* DMST 4736, or 10 µl of PBS into the last left pro-leg into the haemocoel using insulin syringes after that incubated in the dark at 37°C for 5 days. The number of dead larvae was recorded daily. The experiment were performed in triplicate.

2.2.9 Statistical analysis

Values presented are the mean ± standard deviations. Differences in test group and control group were considered statically significant when $p < 0.05$ (student's t test). Survival cure were plotted using the Kaplan–Meier method.

CHAPTER 3

RESULTS

3.1 Isolation and screening for bacteria active against *C. difficile* from stools of breast-fed infant donors

Thirty-eight stool samples from breast-fed infant donors were collected and isolated. Eighty-nine isolated colonies from stool samples were screened for the inhibition activity against toxigenic *C. difficile* strains (*C. difficile* ATCC 43355, ATCC 630, clinical *C. difficile* 17 and 541) by agar well diffusion assay (Figure 11). This result showed that nine isolated strains (PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502) exhibited clear zone on agar well plate indicating that these isolates could inhibit toxigenic *C. difficile*. Therefore, all of nine isolates were selected for further experiments.

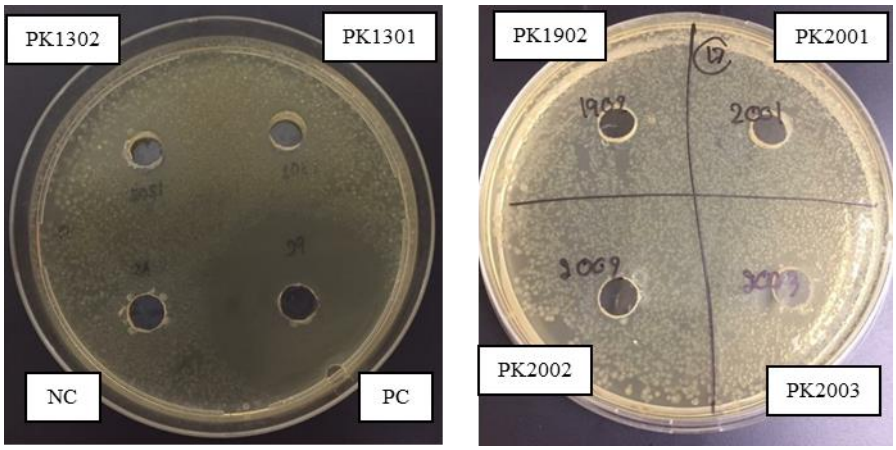
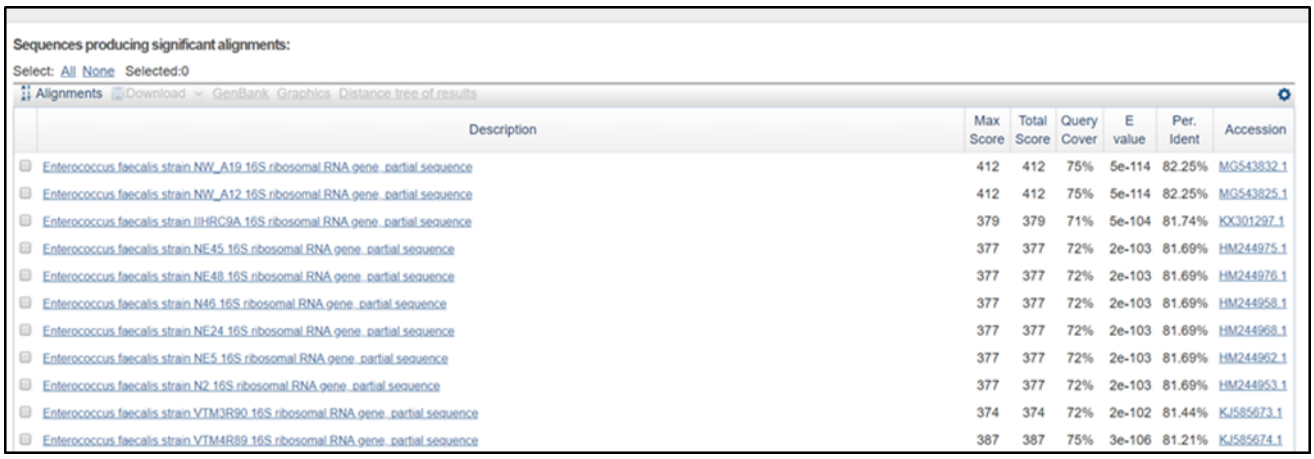


Figure 11. Agar well diffusion assay PC; vancomycin, NC; negative control, isolated strains are positive; PK1301, PK1302, and PK2003, isolated strains are negative; PK1902, PK2001, and PK2002.

3.2 Identification of bacterial strains active against *C. difficile*

Nine strains were isolated from breast-fed infant samples. The morphological and biochemical properties of each isolate were characterized according to Bergey's manual. All isolates exhibited as Gram-positive, catalase-negative cocci, tolerance to 6.5% NaCl, and produce bile esculin, which were *Enterococcus faecalis*. After that the isolates were confirmed by 16s rDNA sequencing and MALDI-TOF MS/MS (Table 10). The nucleotide sequences of 9 isolated were analyzed by the BLAST search tool at NCBI by comparison to the 16S rDNA genes in the database. The results showed that nine isolates showed more than 82% homology to *E. faecalis* (Figure 12). Moreover, these isolated strains were identified by MALDI-TOF MS that match with *E. faecalis* with score value between 2.302-2.443 indicating that the identifications of species are reliable. Overall results indicated that all isolated strains are *E. faecalis*.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain NW_A19 16S ribosomal RNA gene, partial sequence	412	412	75%	5e-114	82.25%	MG543832.1
Enterococcus faecalis strain NW_A12 16S ribosomal RNA gene, partial sequence	412	412	75%	5e-114	82.25%	MG543825.1
Enterococcus faecalis strain IIHRC9A 16S ribosomal RNA gene, partial sequence	379	379	71%	5e-104	81.74%	KX301297.1
Enterococcus faecalis strain NE45 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244975.1
Enterococcus faecalis strain NE48 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244976.1
Enterococcus faecalis strain N46 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244958.1
Enterococcus faecalis strain NE24 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244968.1
Enterococcus faecalis strain NE5 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244962.1
Enterococcus faecalis strain N2 16S ribosomal RNA gene, partial sequence	377	377	72%	2e-103	81.69%	HM244953.1
Enterococcus faecalis strain VTM3R90 16S ribosomal RNA gene, partial sequence	374	374	72%	2e-102	81.44%	KJ585673.1
Enterococcus faecalis strain VTM4R89 16S ribosomal RNA gene, partial sequence	387	387	75%	3e-106	81.21%	KJ585674.1

Figure 12. 16s rDNA sequencing of isolated *E. faecalis*

Table 10. Bruker BioTyper Classification Results

Analyze No.	Organism (best match)	Score Value
PK1003	*Enterococcus faecalis*	2.427
PK1201	*Enterococcus faecalis*	2.302
PK1202	*Enterococcus faecalis*	2.430
PK1301	*Enterococcus faecalis*	2.385
PK1302	*Enterococcus faecalis*	2.443
PK1801	*Enterococcus faecalis*	2.320
PK2003	*Enterococcus faecalis*	2.313
PK2004	*Enterococcus faecalis*	2.411
PK2502	*Enterococcus faecalis*	2.326
L. plantarum ATCC 14917	*Lactobacillus plantarum*	1.994

3.3 Probiotic properties characterization of bacterial strains active against *C. difficile*

3.3.1 Antibiotic susceptibility test

Eight antibiotics were used to analyze antibiotic susceptibility of nine isolated strains and the results are shown in Table 11. All of the isolated strains were susceptible to ampicillin, penicillin, imipenem, and vancomycin; however, they were resistant to gentamicin. Moreover, *E. faecalis* PK1003, PK1301, PK2003, PK2004, and PK2502 were resistant to erythromycin and tetracycline, while *E. faecalis* PK1801 were resistant to tetracycline. These antibiotics (Gentamicin, erythromycin, and tetracycline) could be considered as intrinsic resistance. Vancomycin resistance in the genus enterococcus is significant concerned for Enterococcus safety (122). Interestingly, all of nine *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 were sensitive to vancomycin. All of nine *E. faecalis* strains were not carried *Van-A* and *Van-B* genes that were confirmed by PCR.

Table 11. Antibiotic susceptibility test for Enterococcus strains

strains	Ampicillin (10 µg)	Penicillin (10 µg)	Imipenem (10 µg)	Vancomycin (30 µg)	Gentamicin (10 µg)	Erythromycin (15 µg)	Tetracycline (30 µg)	Ciprofloxacin (5 µg)
PK1003	S	S	S	S	R	R	R	S
PK1201	S	S	S	S	R	I	S	I
PK1202	S	S	S	S	R	I	S	I
PK1301	S	S	S	S	R	R	R	S
PK1302	S	S	S	S	R	I	S	S
PK1801	S	S	S	S	R	I	R	I
PK2003	S	S	S	S	R	R	R	I
PK2004	S	S	S	S	R	R	R	I
PK2502	S	S	S	S	R	R	R	I

R: resistant and, I: intermediate, S: susceptible

The epidemiological cut-off values according to the CLSI 2017 guideline

3.3.2 Survival under gastrointestinal tract (GIT) conditions

Probiotic candidates must survive through the GIT, tolerance to low pH (pH 1.5-3), tolerance to simulated gastric (pH 2.0, 3 mg/mL pepsin, 3 h) and pancreatic digestion (pH 8.0, 1 mg/mL pancreatin, 4 h), and tolerance to bile salts (0.3%, w/v bile salts, 4 h). So, ability of all 9 isolates to tolerance low pH, pepsin, pancreatic, and bile salts were evaluated. *L. plantarum* ATCC 14917 was selected as a reference probiotic strain. The results of survival under GIT conditions are shown in Table 12.

All of nine *E. faecalis* strains have good survival in pH 2-4 during 2 h. All of the *E. faecalis* strains displayed higher levels of viability in the pH 2 and 3 than *L. plantarum* ATCC 14917. *E. faecalis* PK2004 showed the highest level of viability in the pH 2 (9.24 ± 0.28). However, it is not significantly different in tolerance to low pH when compared to other *E. faecalis* strains.

For tolerance to pepsin, the nine *E. faecalis* strains showed higher levels of viability than *L. plantarum* ATCC 14917 (5.22-2.47 vs. 0 log CFU/mL). *E. faecalis* PK2502 showed the highest levels of viability (5.22 ± 0.11), followed by *E. faecalis* PK2101 (5.20 ± 0.13), and *E. faecalis* PK1801 (4.86 ± 0.28).

For tolerance to pancreatin, all of *E. faecalis* strains and *L. plantarum* ATCC 14917 showed good survival in the presence of pancreatin (1 mg/mL pancreatin, 4 h) in medium (11.84-12.21log CFU/mL). This result was similar between all of *E. faecalis* and *L. plantarum* ATCC 14917.

Viability of nine *E. faecalis* strains and *L. plantarum* ATCC 14917 in (0.3%, w/v) bile salts medium were deceased after 4 h of incubation. However, viability of nine *E. faecalis* strains in tolerance to bile salts showed higher than *L. plantarum* ATCC 14917 (5.42-6.20 vs. 3.33 log CFU/mL).

For consecutive exposition under GIT, all of *E. faecalis* strains and *L. plantarum* ATCC 14917 showed slightly decreased of viability in GIT conditions. Viability of nine isolated enough (10^4 CFU/mL) for colonized in GIT.

These results suggested that *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 from breast-fed infant samples might be able to survive in the gastrointestinal tract and potential probiotic.

Table 12. Numbers of nine *E. faecalis* strains (log CFU/mL) to survival under GIT conditions

	Time (h)	**PK1003**	**PK1201**	**PK1202**	**PK1301**	**PK1302**	**PK1801**	**PK2003**	**PK2004**	**PK2502**	***L. plantarum* ATCC14917**
Tolerance to low pH	0	9.6 ± 0.24	9.6 ± 0.06	9.71 ± 0.03	9.61 ± .02	9.5 ± .05	9.45 ± .04	9.57 ± .04	9.6 ± 0.14	9.5 ± 0.04	9.83 + 0.02
pH = 7.2	2	11.57 ± 0.11	11.64 ± 0.32	11.45 ± 0.33	11.33 ± 0.19	11.55 ± 0.15	11.56 ± 0.18	11.59 ± 0.13	11.49 ± 0.20	11.61 ± 0.19	11.17 ± 0.10
pH = 2	2	8.18 ± 0.00	8.49 ± 0.28	7.96 ± 0.45	9.01 ± 0.62	8.68 ± 0.67	8.85 ± 0.13	8.37 + 0.62	9.24 ± 0.28	6.39 ± 0.22	0
pH = 3	2	11.78 ± 0.09	11.66 ± 0.46	11.81 ± 0.13	11.37 + 0.06	11.41 ± 0.28	11.88 ±0.20	11.89 ± 0.03	11.91 ± 0.04	11.85 ± 0.10	3.80 ± 0.17
pH = 4	2	11.9 ± 0.17	11.9 ± 0.24	11.9 ± 0.07	12.20 ± 0.03	11.67 ± 0.28	11.91 ± 0.23	12.02 ± 0.07	12.04 ± 0.24	11.17 ± 0.04	10.05 ± 0.34
Pepsin	0	9.74 ± 0.08	9.77 ± 0.10	9.76 ± 0.14	9.70 ± 0.04	9.71 ± 0.03	9.72 ± 0.08	9.61 ± 0.08	9.70 ± 0.12	9.66 ± 0.05	11.42 ± 0.09
	3	2.47 ± 2.14	5.20 ± 0.13	4.26 ± 0.31	3.80 ± 0.17	3.70 ± 0.00	4.86 ± 0.28	1.33 ± 2.31	4.51 ± 0.20	5.22 ± 0.11	0
Pancreatin	0	9.75 ± 0.08	9.91 ± 0.06	9.80 ± 0.02	9.90 ± 0.08	9.98 ± 0.11	9.90 ± 0.03	9.81 ± 0.16	9.82 ± 0.08	9.89 ± 0.09	11.11 ± 0.06
	4	11.88 ± 0.06	11.89 ± 0.06	11.84 ± 0.02	11.90 ± 0.08	12.01 ± 0.06	12.09 ± 0.05	12.00 ± 0.09	11.92 ± 0.00	11.91 ± 0.23	12.21 ± 0.07
Bile salts	0	8.21 ± 0.29	8.18 ± 0.28	8.19 ± 0.33	8.21 ± 0.33	8.19 ± 0.36	8.16 ± 0.28	8.49 ± 0.20	8.44 ± 0.13	8.18 ± 0.28	10.77 ± 0.02
	4	6.09 ± 0.23	6.19 ± 0.42	6.01 ± 0.62	5.52 ± 0.45	6.20 ± 0.79	5.45 ± 0.54	5.81 ± 0.49	5.52 ± 0.54	5.42 ± 0.49	3.33 ± 0.04
Consecutive exposition under GIT	0	9.80±0.20	9.72±0.04	9.92±0.10	9.69±0.09	9.72±0.05	9.68±0.12	9.89±0.28	9.80±0.17	9.76±0.24	10.56±0.02
	end	5.85±0.29	5.68±0.51	5.48±0.30	5.17±0.23	4.54±0.74	4.52±0.54	4.71±0.46	4.72±0.63	4.23±0.40	4.85±0.48

Final counts: log CFU/mL

3.3.3 Adhesion ability

The adhesion ability of probiotic strains to adhere to the intestinal epithelial cells contributes to their colonization and inhibits foodborne pathogen adhesion to intestinal epithelial cell. Many factors have been suggested for the adhesion of bacteria to epithelial cells such as surface proteins, cell-bound exopolysaccharides, and cell-wall-associated proteins (133). So, the adhesion ability of the nine *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 to HT-29 cells was evaluated. The adhesion ability of each *E. faecalis* strains is presented in Figure 13. Nine *E. faecalis* strains showed similar adhesion to HT-29 cells than the *L. plantarum* ATCC 14917. *E. faecalis* PK1801 showed the highest adhesion ability (78.83 ± 4.16%), followed by *L. plantarum* ATCC 14917 (76.27 ± 3.66%) and *E. faecalis* PK1201 (76.15 ± 2.66%).

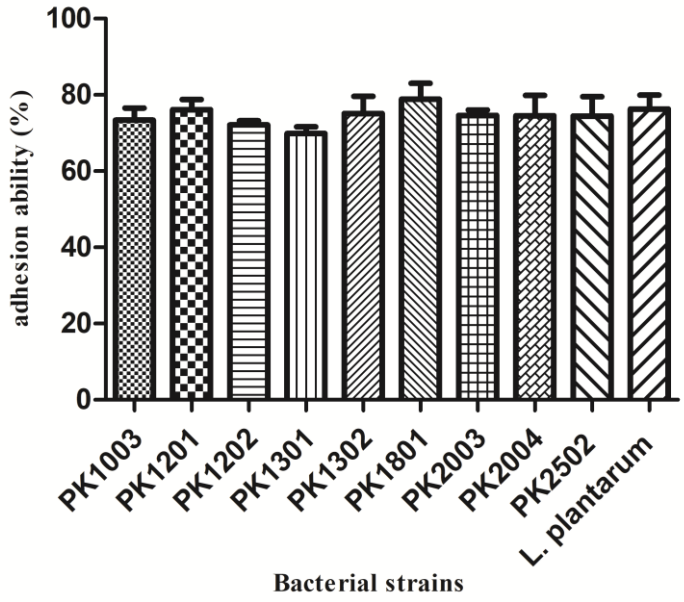


Figure 13. Adhesion ability of nine *E. faecalis* strains and *L. plantarum* ATCC 14917 to human intestinal HT-29 cells. Error bars indicate the standard deviation from three independent experiments.

The adhesion ability of the bacteria is associated with its hydrophobicity of bacterial cell surface. It is expedient for the maintenance of bacterial adhesion to the GIT. So, the hydrophobicity of nine *E. faecalis* from breast-fed infant that varied from 47.51 ± 3.02% to 85.00 ± 2.93% that is presented in Figure 14. All of isolated strains showed higher hydrophobicity than *L. plantarum* ATCC 14917 (41.08 ± 0.89%). Moreover, *E. faecalis* PK1003 showed the highest percentages of hydrophobicity (85 ± 2.93%). These results suggested their highly ability to adhere to the epithelial cells.

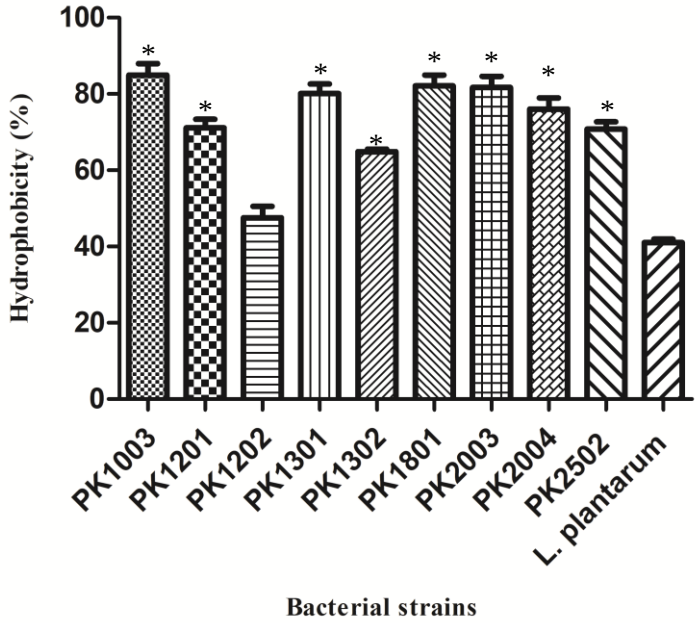


Figure 14. Hydrophobicity of nine *E. faecalis* from breast-fed infant samples and *L. plantarum* ATCC 14917. Error bars indicate standard deviation from three independent experiments. *; significant (p-value <0.05)

3.3.4 Antimicrobial activity

Some molecules are produced by probiotic bacteria, for instance, metabolites, organic acids, peptide, and bacteriocins that maybe contribute to the antimicrobial activity against bacterial pathogens (134). So, the antimicrobial activity of the isolated strains were evaluated using standard bacterial strains. All of isolated strains were able

to inhibit *C. coli* ATCC 11353, *C. difficile* DMST 16662, ATCC 630 and ATCC 43255, and clinical toxigenic *C. difficile* (Table 13). Surprisingly, all of nine isolates strains were not inhibit non-pathogenic strains that are normal flora in GIT of human.

Table 13. Inhibition of several foodborne pathogens by isolated strains from breast-fed infant stool

	PK1003	PK1201	PK1202	PK1301	PK1302	PK1801	PK2003	PK2004	PK2502	*L. plantarum* ATCC14917
V. cholerae DMST 2873[a]	-	-	-	-	-	-	-	-	-	-
V. parahaemolytica DMST 15285 [a]	-	-	-	-	-	-	-	-	-	-
E. coli O157:H7 DMST 2743 [a]	-	-	-	-	-	-	-	-	-	-
S. typhimurium DMST 22842 [a]	-	-	-	-	-	-	-	-	-	-
S. flexneri DMST 44237 [a]	-	-	-	-	-	-	-	-	-	-
L. monacytogenes DMST 17303 [a]	-	-	-	-	-	-	-	-	-	-
S. enteritidis DMST 15676 [a]	-	-	-	-	-	-	-	-	-	-
C. coli ATCC 11353 [a]	+	+	+	+	+	+	+	+	+	+
S. aureus ATCC 6538 [a]	-	-	-	-	-	-	-	-	-	-
C. perfringens ATCC 13123 [a]	-	-	-	-	-	-	-	-	-	-
C. difficile DMST 16662 [a]	+	+	+	+	+	+	+	+	+	+
C. difficile ATCC 630 [a]	+	+	+	+	+	+	+	+	+	-
C. difficile ATCC 43255 [a]	+	+	+	+	+	+	+	+	+	-
Clinical toxigenic *C. difficile* 17 [a]	+	+	+	+	+	+	+	+	+	-
Clinical toxigenic *C. difficile* 541 [a]	+	+	+	+	+	+	+	+	+	-
B. cereus DMST 11098 [a]	-	-	-	-	-	-	-	-	-	-

B. subtilis DMST 7988[b]	-	-	-	-	-	-	-	-	-	-
B. fragilis TISTR 447 [b]	-	-	-	-	-	-	-	-	-	-
B. subtilis TISTR 2057 [b]	-	-	-	-	-	-	-	-	-	-
L. lactis TISTR 1464 [b]	-	-	-	-	-	-	-	-	-	-
L. animalis TISTR 1115 [b]	-	-	-	-	-	-	-	-	-	-
E. faecalis TISTR 1285 [a]	-	-	-	-	-	-	-	-	-	-
L. casei subsp.*rhamnosus* TISTR 047 [b]	-	-	-	-	-	-	-	-	-	-

+; Inhibited, -; Not inhibited, [a]; Pathogenic strains, [b]; Non-pathogenic strain

3.3.5 Safety assessment

The safety of probiotic strains is important. They do not carry transmissible antibiotic resistance genes and virulence factor genes. Therefore, virulence gene and safety assessment were screened by genotype assay which was used PCR, phenotype assay, and *G. mellonella* survival test.

The results of the PCR screening for the presence of enterococcal virulence determinant genes showed different patterns that are summarized in Table 14. *ccf, cad* genes (sex pheromone determinants) were not presented in all of the nine *E. faecalis* strains, whereas $efaA_{fs}$ gene that is cell wall adhesins were carried by all strains. Moreover, *E. faecalis* PK1003, PK1202, and PK1301 carried *Agg* and *gelE* genes. *E. faecalis* PK1201 and PK1302 carried *gelE* gene. *E. faecalis* PK1801 carried *Agg* and *esp* genes. *E. faecalis* PK2003 carried *agg, gelE, cylM, cylB, cylA, and esp* genes. PK2004 carried *agg, cylM, cylB, cylA, and esp* genes. PK2502 carried *cylM, cylB, cylA, and esp* genes. For *Agg* gene is aggregation protein involved in adherence to eukaryotic cells, cell aggregation, and conjugation. *gelE* gene is extracellular metalloendopeptidase which hydrolyzes gelatin, collagen, hemoglobin, and other bioactive compounds. *cylM* gene is posttranslational modification of cytolysin. *cylB* gene is redpondsible for transport action of cytolysin and *cylA* gene is mediated the activation of cytolysin (120). The phenotype of virulence factor of *E. faecalis* showed none of the nine isolated strains produces mucin degradation, gelatinase activity and hemolysin production except *E. faecalis* PK2003, PK2004, and PK2502.

Moreover, we confirmed hemolytic activity by tube test. The result showed that, tween 80 as a positive control can lysed erythrocytes is 100%. The nine *E. faecalis* strains showed hemolysis activity (%) ranging from 1.68±0.31 to 2.51±0.97 which were not significant when compared to negative control (Figure 15). Thus, nine isolates strains are saved, when safety assessment was tested in vitro.

Table 14. Summary of PCR screening for virulence determinants of the nine *E. faecalis* strains from breast-fed infant samples

strains	**genes**										
	agg	*gelE*	*cylM*	*cylB*	*cylA*	*esp*	*cpd*	*efaA*$_{fs}$	*cob*	*ccf*	*cad*
PK1003	+	+	-	-	-	-	+	+	-	-	-
PK1201	-	+	-	-	-	-	+	+	+	-	-
PK1202	+	+	-	-	-	-	+	+	+	-	-
PK1301	+	+	-	-	-	-	+	+	-	-	-
PK1302	-	+	-	-	-	-	+	+	-	-	-
PK1801	-	+	-	-	-	+	+	+	-	-	-
PK2003	+	+	+	+	+	+	+	+	+	-	-
PK2004	+	-	+	+	+	+	+	+	+	-	-
PK2502	-	-	+	+	+	+	+	+	+	-	-

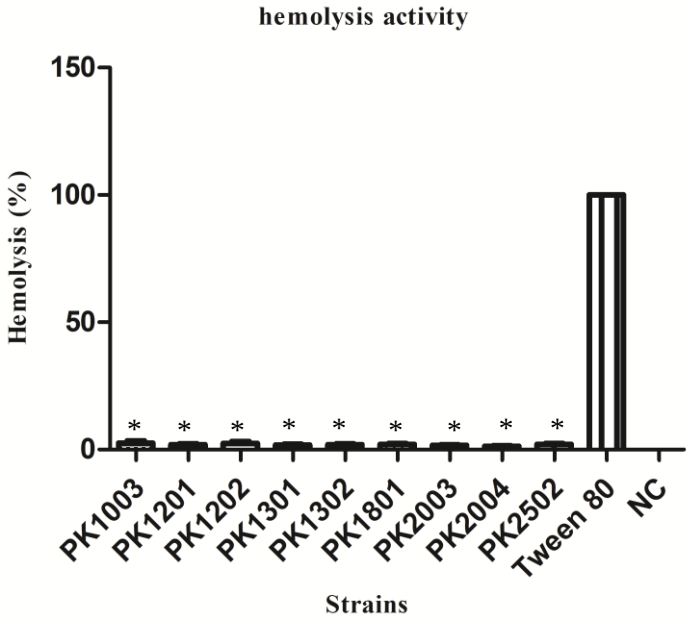


Figure 15. Hemolysis activity of nine *E. faecalis* from breast-fed infant samples and *L. plantarum* ATCC 14917. Error bars indicate standard deviation from three independent experiments. *; significant (p-value <0.05)

3.3.6 *Galleria mellonella* killing assay

The pathogenesis of nine *E. faecalis* strains were determined by *G. mellonella* killing assay. The effect of isolated strains and *L. plantarum* ATCC 14917 on *G. mellonella* infection were shown in Figure 16 in comparison with *E. faecalis* ATCC 4736 as positive control. *E. faecalis* ATCC 4736 induce 85 % death of *G. mellonella* larvae. While, control of PBS and no treated did not affect the five-day survival of *G. mellonella* larvae (survival rate 100% and 90%, respectively). The nine isolated *E. faecalis* strains and *L. plantarum* ATCC 14917 showed survival rate between 80%-100%. This result similar to negative control groups. Suggested that *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 are safe.

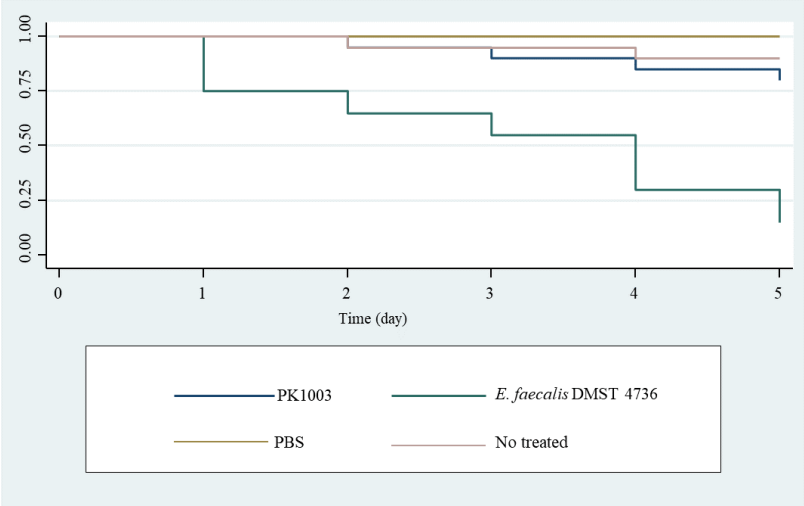


Figure 16. Survival plot of *G. mellonella* larvae infected with nine isolated *E. faecalis* strains, and *L. plantarum* ATCC 14917 compared with *E. faecalis* ATCC 4736, PBS and no injected.

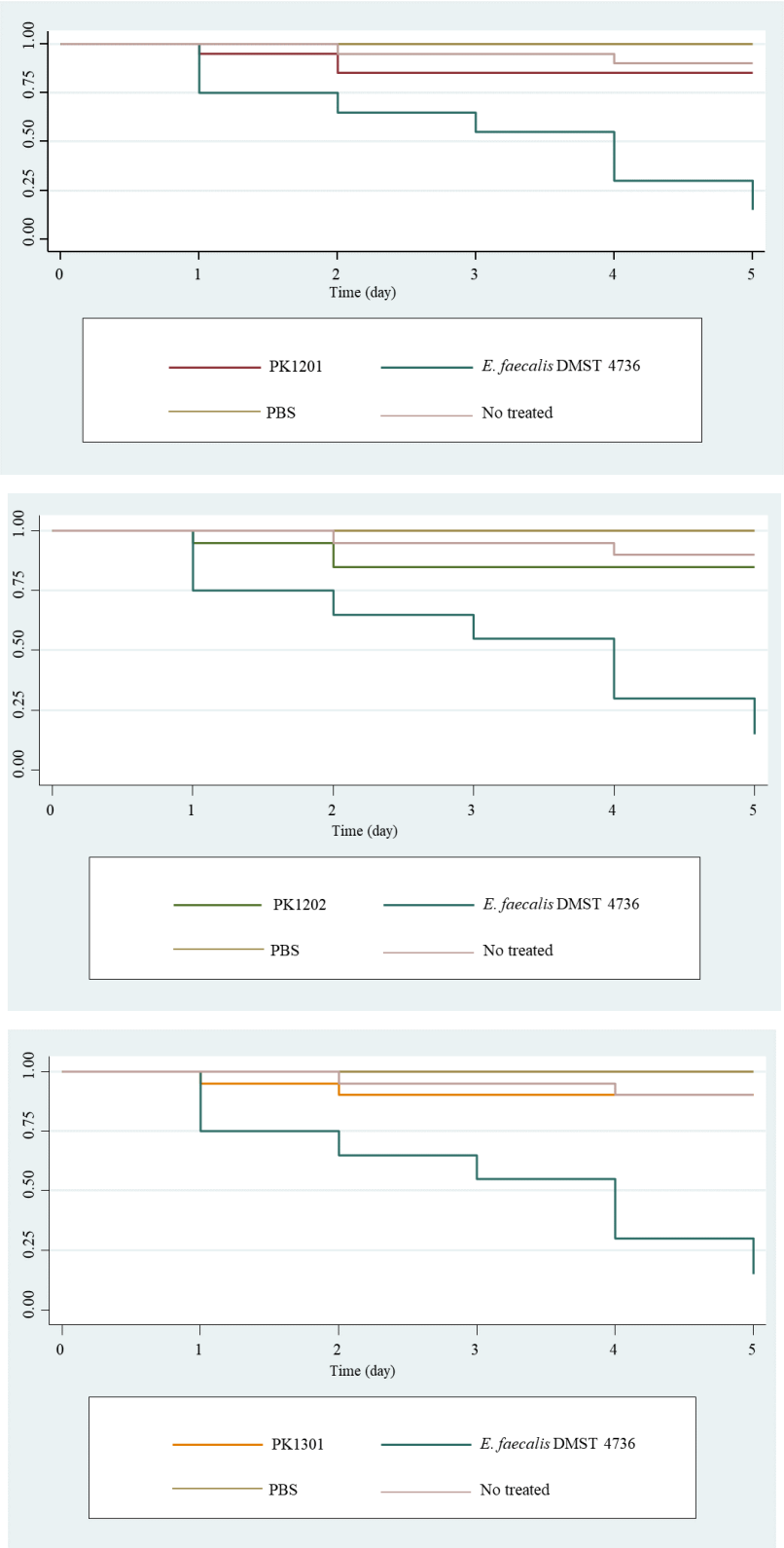


Figure 16. Survival plot of *G. mellonella* larvae infected with nine isolated *E. faecalis* strains, and *L. plantarum* ATCC 14917 compared with *E. faecalis* ATCC 4736, PBS and no injected. (cont.)

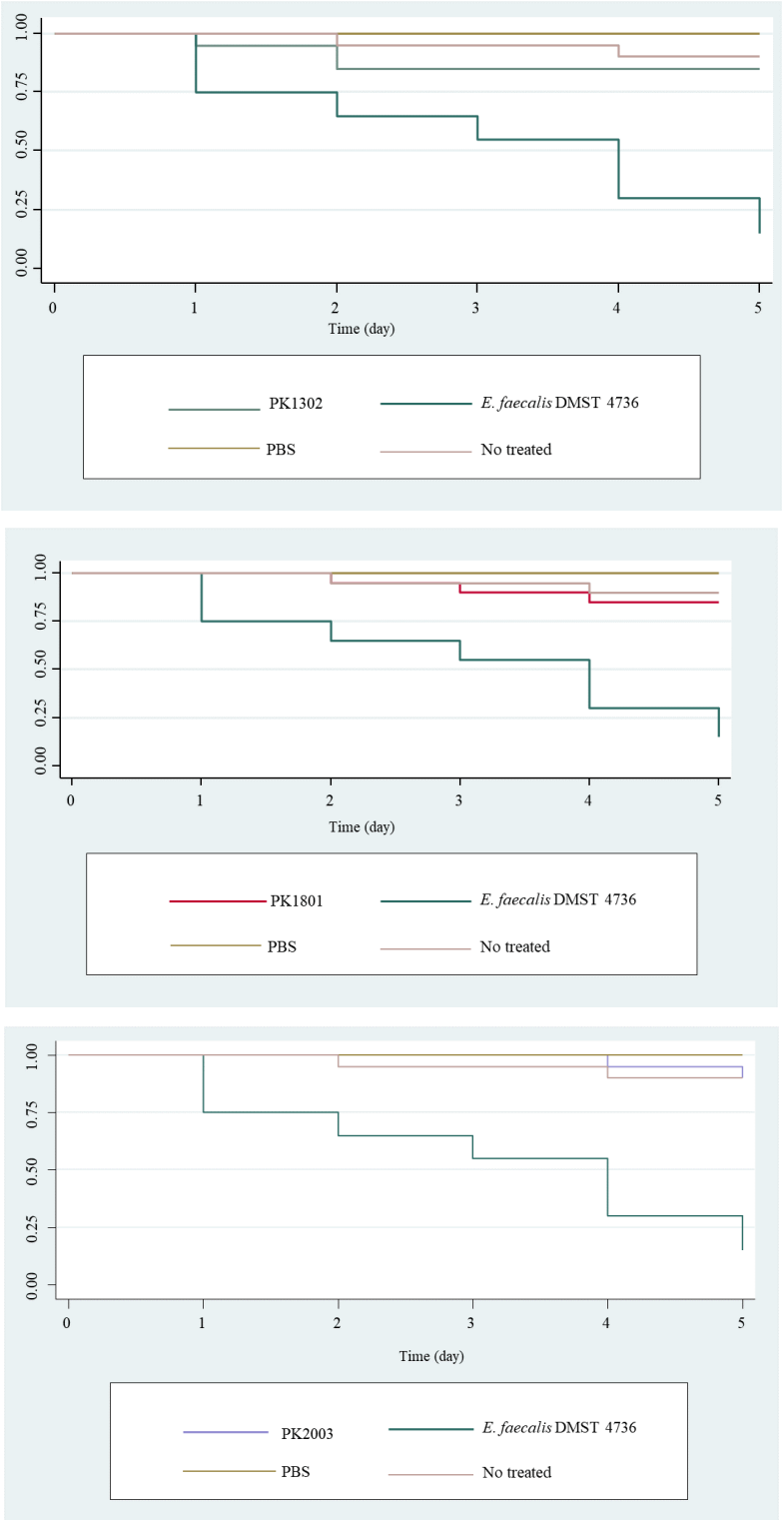


Figure 16. Survival plot of *G. mellonella* larvae infected with nine isolated *E. faecalis* strains, and *L. plantarum* ATCC 14917 compared with *E. faecalis* ATCC 4736, PBS and no injected. (cont.)

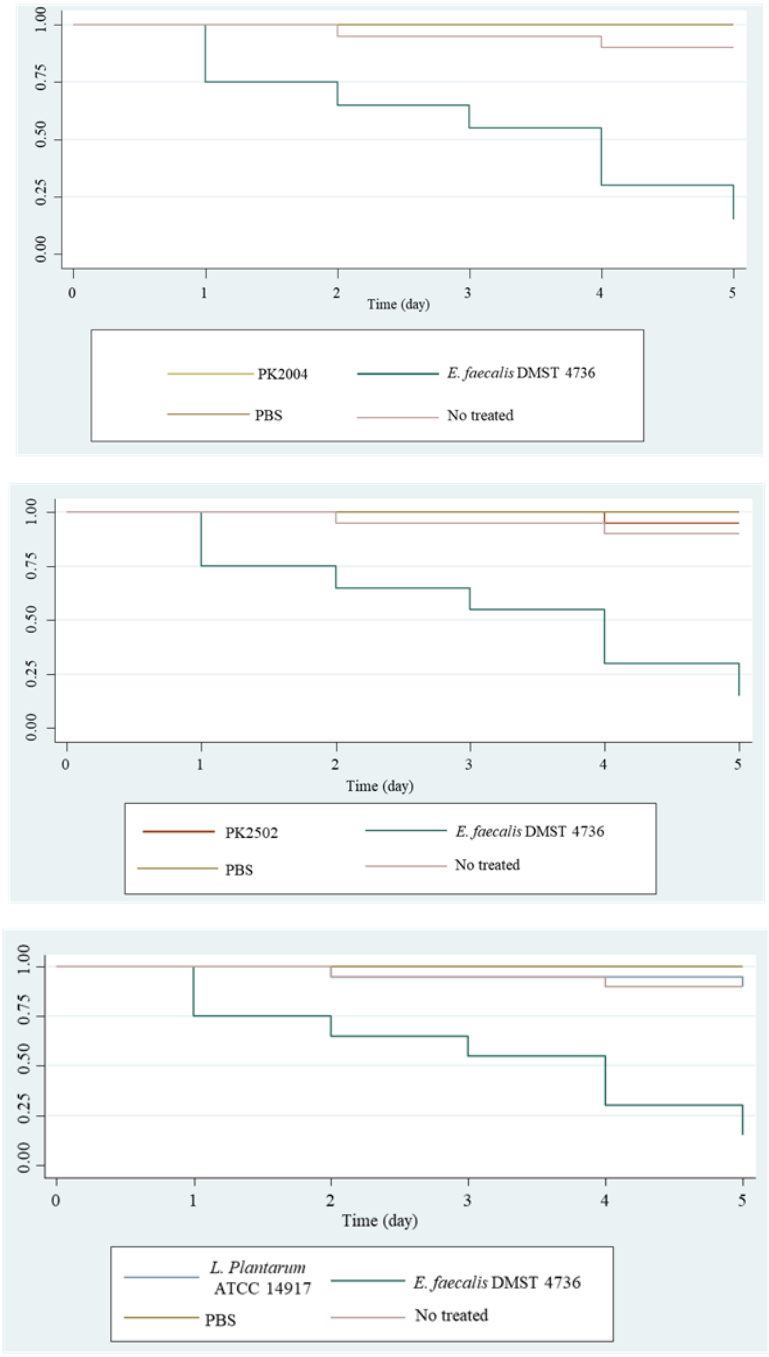


Figure 16. Survival plot of *G. mellonella* larvae infected with nine isolated *E. faecalis* strains, and *L. plantarum* ATCC 14917 compared with *E. faecalis* ATCC 4736, PBS and no injected. (cont.)

3.4 Identification of mechanisms of probiotic against pathogenic *C. difficile*

3.4.1 Spot-on-lawn method

Spot-on-lawn method widely used to screen protein production of bacteria. Cell-free supernatant from nine isolated *E. faecalis* strains incubated with proteinase K cannot inhibit pathogenic *C. difficile* which were exhibited by no present of clear zone. Antimicrobial agents in cell-free supernatant were completely inactivated by proteinase K. Thus, these result suggested that all of nine isolated *E. faecalis* strains may produce anti- *C. difficile* peptides such as bacteriocin.

3.4.2 Hydrogen peroxide production

Hydrogen peroxide produced by probiotics can inhibits the growth of pathogens and promotes epithelial restitution during colitis. Hydrogen peroxide production was tested in the nine *E. faecalis* strains from breast-fed infant samples and *L. plantarum* ATCC 14917. None of isolates strains and *L. plantarum* ATCC 14917 showed the ability to produce hydrogen peroxide.

3.4.3 Bile salt hydrolase (BSH) activity test

Guidelines for evaluation of probiotics described by WHO experts (17), BSH activity is a characteristic to be considered in the screening of probiotic. The expression of BSH protect system to allow the potential bacteria survive and/or colonize the intestinal cell after oral consumption and is associated with the reduction of serum cholesterol levels in mammals. So, BSH activity was screened in all the isolates *E. faecalis* strains and *L. plantarum* ATCC 14917. BSH activity was detected in *L. plantarum* ATCC 14917 and *E. faecalis* PK1003, PK1201, PK1202, PK1801, PK2003, PK2004, and PK2502 except PK1301 and PK1302.

Overall results suggested that nine *E. faecalis* may inhibit *C. difficile* by producing proteinous antimicrobial agents as bacteriocin and/or BSH.

3.5 Evaluate activity of probiotic against vegetative *C. difficile*

Cytotoxicity assay is one of the most sensitive methods and it is the gold standard method for detecting the effect of *C. difficile*. The cytopathic effect was indicated by more than 50% of rounded cells after incubation with toxigenic *C. difficile* or *C. difficile* toxin. This result showed that HT-29 cells treated with cell-free supernatant of *C. difficile* ATCC 630, ATCC 43255, and 2 clinical *C. difficile* (strains 17 and 541) were rounded more than 50% (Figure 17). HT-29 cells became spherical and the integrity of the monolayer was lost (Figure 18). The percent of cell rounding of HT-29 cells treated with cell-free supernatant of nine *E. faecalis* strains and *L. plantarum* ATCC 14917 were 5-10% that is similar to negative control. Moreover, HT-29 cells treated with co-culture of cell-free supernatant from nine *E. faecalis* inclubated with *C. difficile* DMST 16662 and clinical *C. difficile* 541 (toxin A and B positive) remained more stable (Figure 18) and percentage of cell rounding was significantly deceased (*C. difficile* 541; 73±5.77 – 10%, *C. difficile* DMST 16662; 18±2.89 – 10%) when compared with HT-29 cells treated with cell-free supernatant *C. difficile*. Moreover, % of HT-29 cells rounding was slightly decreased (Figure 17) when treated *C. difficile* ATCC 630, ATCC 43255 and clinical *C. difficile* 17 (toxin A, toxin B, and binary toxin positive) with cell-free supernatant prepared from nine isolated *E. faecalis*, although more than 50% of HT-29 cells were rounded.

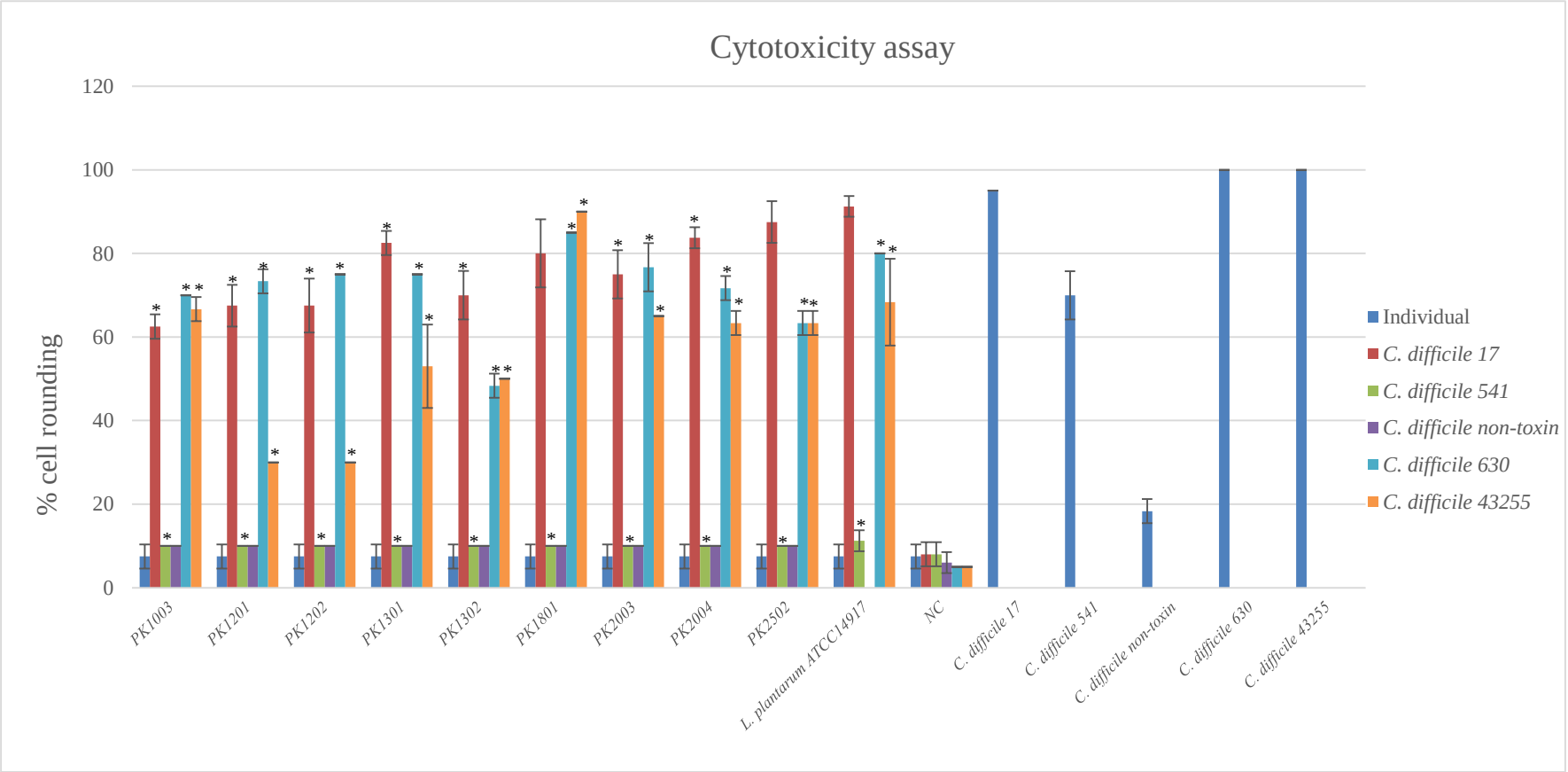


Figure 17. Percentage of cell rounding of cytotoxic effects of individual *C. difficile* strains, probiotic, and co-culture of probiotic with *C. difficile* on HT-29 cells. *; significant (p-value <0.05)

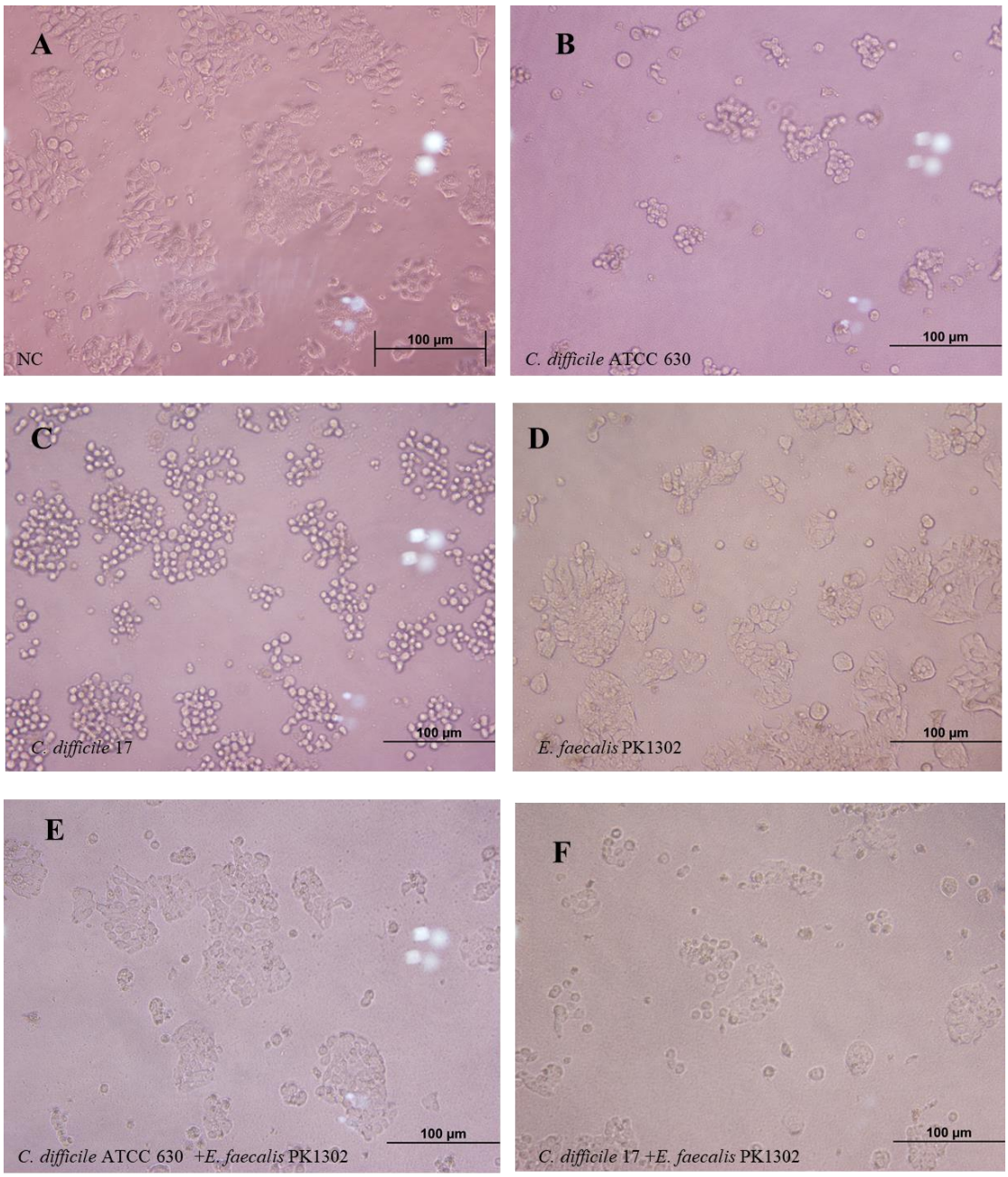


Figure 18. Comparison of cytotoxic effects of individual *C. difficile* strains, probiotic, and co-culture of probiotic with *C. difficile* on HT-29 cell line. (A) HT-29 cells monolayer; (B-D) HT-29 cells treated with cell-free supernatant from toxigenic *C. difficile* strain, and *E. faecalis*; (E-F) HT-29 cells treated with cell-free supernatant from co-culture of *E. faecalis* and *C. difficile.*

Furthermore, we performed immunofluorescence assay (*F*-actin detection) to confirm the cellular event under the different treatments. These results showed that HT-29 cells of control group is a typical *F*-actin cytoskeleton, nucleus is imbibed, and cells were connected. However, HT-29 cells treated with cell-free supernatant of individual *C. difficile* lost the interconnection of *F*-actin cytoskeleton and nucleus that was condensation seems to be the initial stage of apoptosis. HT-29 cells become rounding and tight junction was disrupted. The image from HT-29 cells treated with cell-free supernatant of *E. faecalis* PK1202, *E. faecalis* PK1302, and *L. plantarum* ATCC 14917 were similar to the control group. *F*-actin showed normal morphology (interconnection structure). Nucleus showed less intense DAPI staining comparable to control but more intense than HT-29 cells treated with cell-free supernatant of individual *C. difficile.*

The image from HT-29 cells treated with cell-free supernatant obtained after incubation of each *E. faecalis* PK1202, *E. faecalis* PK1302, and *L. plantarum* ATCC 14917 with each toxigenic *C. difficile* showed that, HT-29 cells were slightly damage when compared with HT-29 cells treated with cell-free supernatant of individual *C. difficile* (Figure 19). Some parts of *F*-actin cytoskeleton showed interconnected structure and nucleus was similar to control group that was normal morphology, but some nucleus of HT-29 was condensed.

All these result showed that nine *E. faecalis* can protected HT-29 cells become rounding, lost the interconnection of *F*-actin cytoskeleton and protect nucleus condensation that confirmed by immunofluorescence assay. These results suggested that nine *E. faecalis* can reduce effect of *C. difficile* toxins on HT-29 cells.

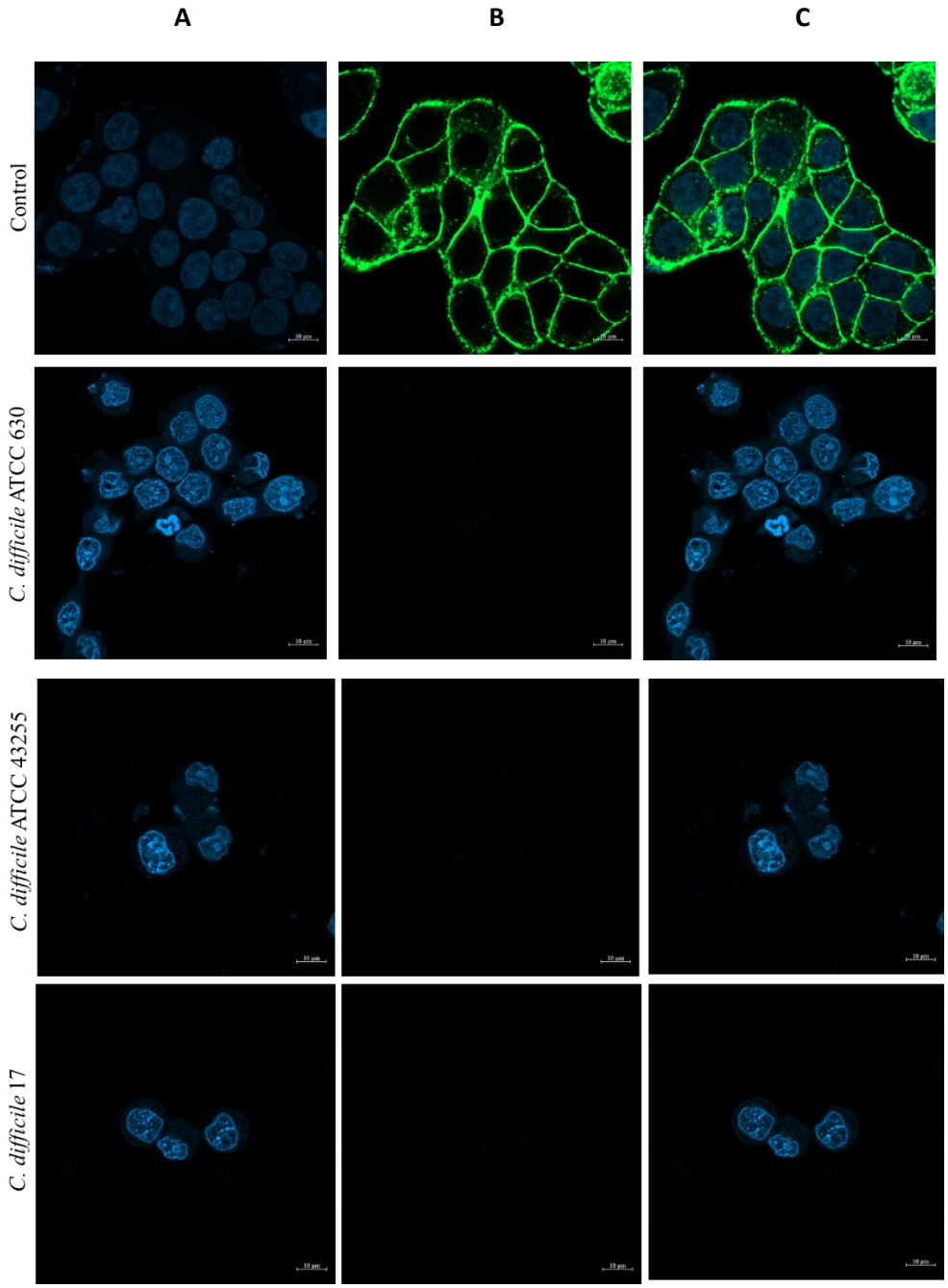


Figure 19. Immunofluorescence images obtained by confocal scanning laser microscopy of HT-29 cells after 24 hours incubation with cell-free supernatant of individual *C. difficile*, and probiotic, and with cell-free supernatant obtained after incubation of probiotic with *C. difficile.* A; DAPI-stained nucleus (blue, excited at 405 nm), B; *F-actin* stained with Phalloidin-Alexa-Fluor-488 probe (green, excited at 490 nm), C; combination of nucleus and *F-actin* stained

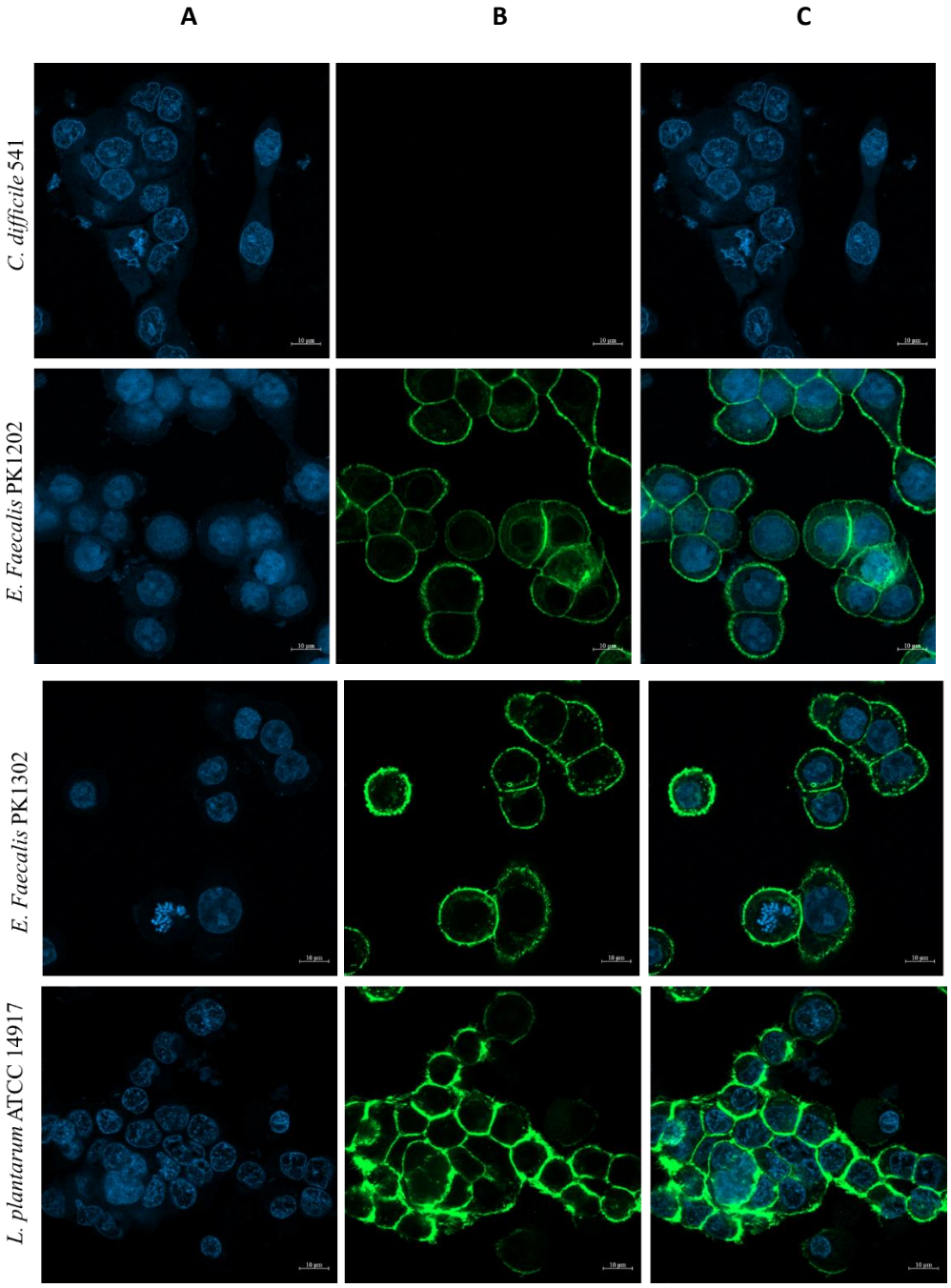


Figure 19. Immunofluorescence images obtained by confocal scanning laser microscopy of HT-29 cells after 24 h incubation with cell-free supernatant of individual *C. difficile,* and probiotic, and with cell-free supernatant obtained after incubation of probiotic with *C. difficile.* A; DAPI-stained nucleus (blue, excited at 405 nm), B; *F-actin* stained with Phalloidin-Alexa-Fluor-488 probe (green, excited at 490 nm), C; combination of nucleus and *F-actin* stained (cont.)

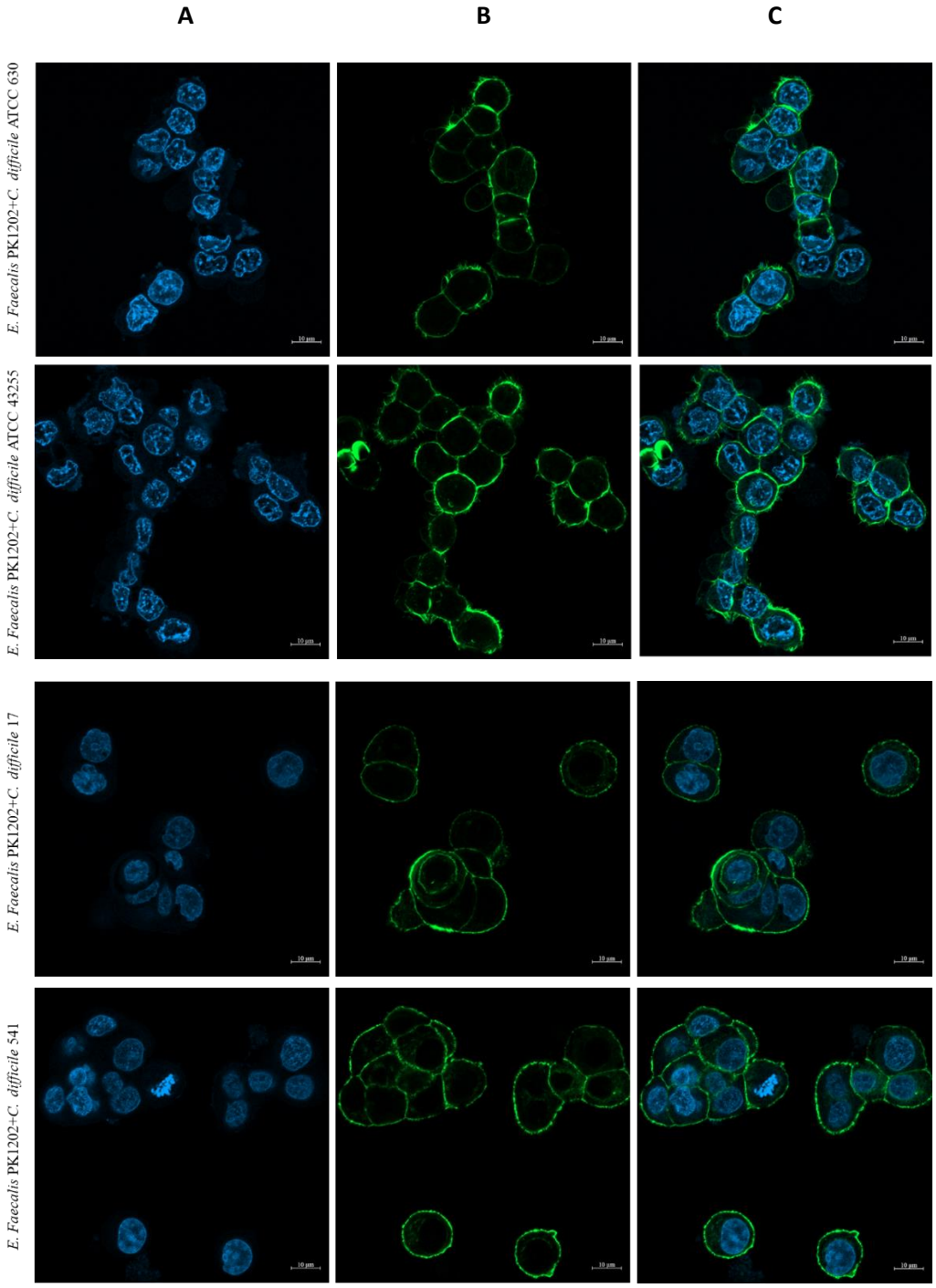


Figure 19. Immunofluorescence images obtained by confocal scanning laser microscopy of HT-29 cells after 24 h incubation with cell-free supernatant of individual *C. difficile*, and probiotic, and with cell-free supernatant obtained after incubation of probiotic with *C. difficile.* A; DAPI-stained nucleus (blue, excited at 405 nm), B; *F-actin* stained with Phalloidin-Alexa-Fluor-488 probe (green, excited at 490 nm), C; combination of nucleus and *F-actin* stained (cont.)

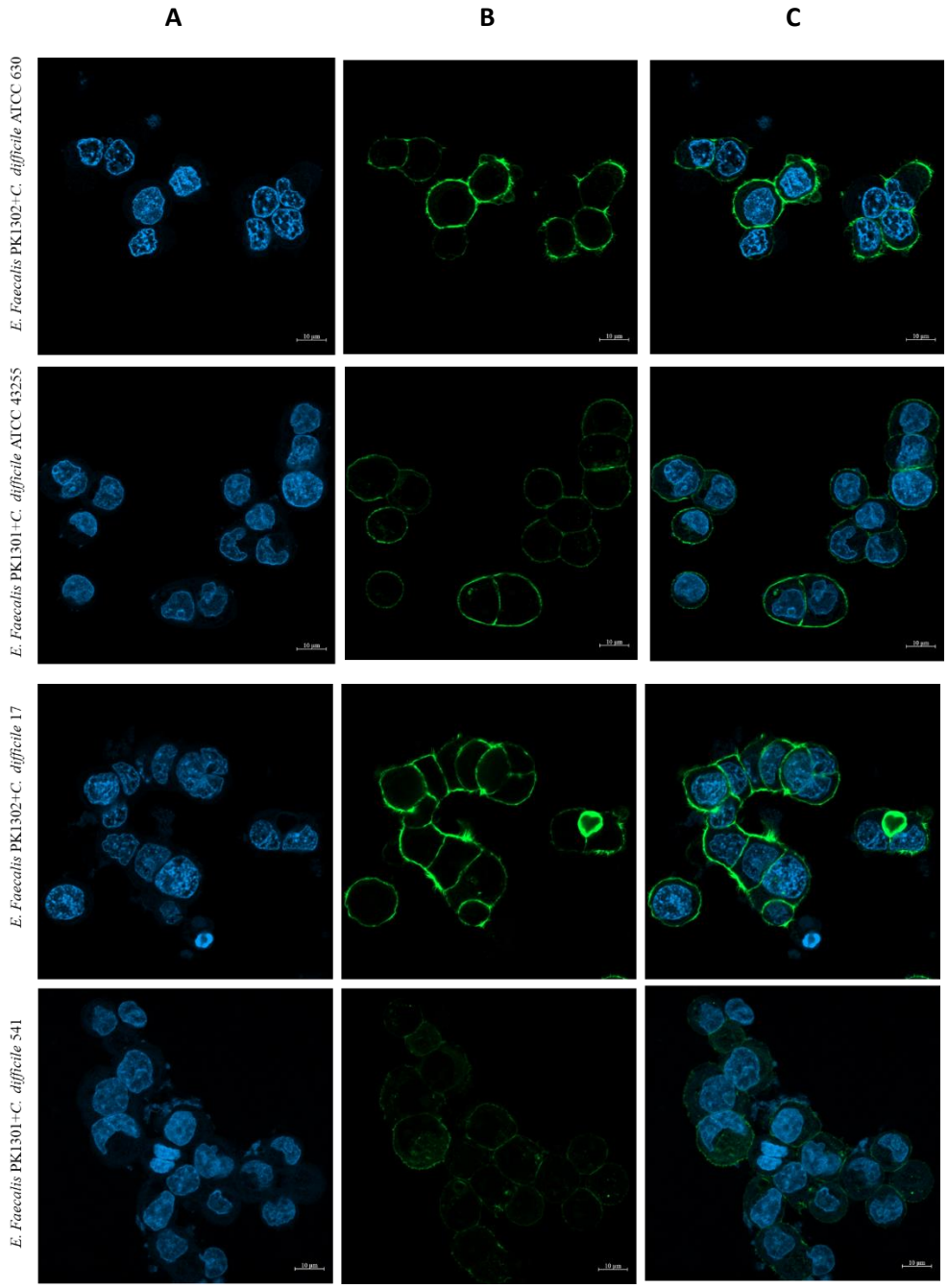


Figure 19. Immunofluorescence images obtained by confocal scanning laser microscopy of HT-29 cells after 24 h incubation with cell-free supernatant of individual *C. difficile*, and probiotic, and with cell-free supernatant obtained after incubation of probiotic with *C. difficile.* A; DAPI-stained nucleus (blue, excited at 405 nm), B; *F-actin* stained with Phalloidin-Alexa-Fluor-488 probe (green, excited at 490 nm), C; combination of nucleus and *F-actin* stained (cont.)

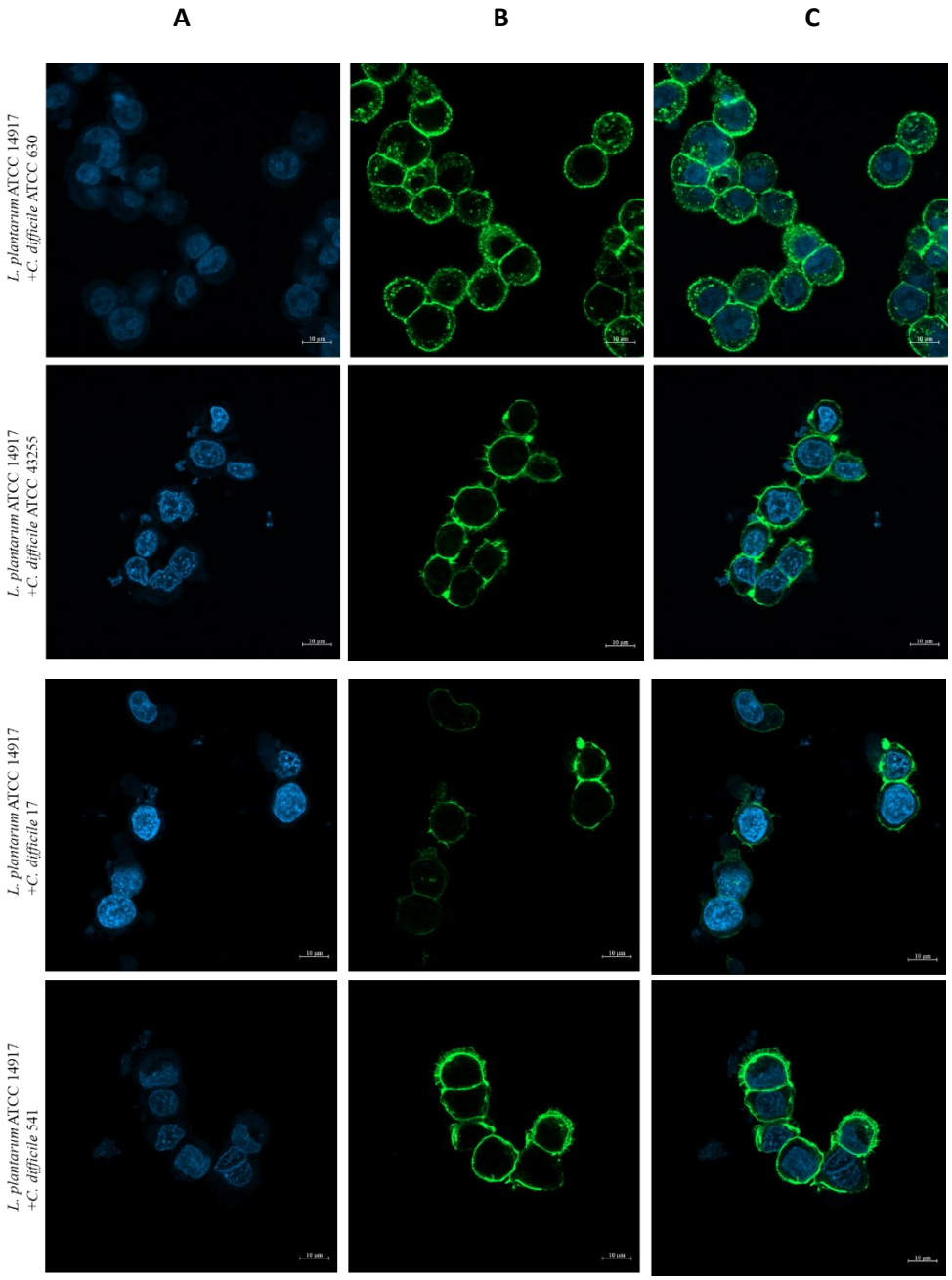


Figure 19. Immunofluorescence images obtained by confocal scanning laser microscopy of HT-29 cells after 24 h incubation with cell-free supernatant of individual *C. difficile*, and probiotic, and with cell-free supernatant obtained after incubation of probiotic with *C. difficile.* A; DAPI-stained nucleus (blue, excited at 405 nm), B; *F-actin* stained with Phalloidin-Alexa-Fluor-488 probe (green, excited at 490 nm), C; combination of nucleus and *F-actin* stained (cont.)

3.6 Evaluate activity of probiotic against *C. difficile* spore

Nine *E. faecalis* strains and *L. plantarum* ATCC 14917 were screened for their activity of probiotic against *C. difficile* spore by inhibitory germination test. These result showed that these 9 *E. faecalis* strains (PK1003, PK1201, PK1202, PK1301, PK1302, PK1802, PK2003, PK2004 and PK2502) and *L. plantarum* ATCC 14917 could utterly inhibit the spore germination of toxigenic *C. difficile*. The percentage of spore germination of toxigenic *C. difficile* are 0 – 6.34±0.06% (Table 15). All of the nine *E. faecalis* strains showed the effective efficiency to inhibited toxigenic *C. difficile* spore germination.

Table 15. Relative percentage of germination of toxigenic *C difficile* when treated with potential probiotic

Strains	*C. difficile* ATCC 630	*C. difficile* ATCC 43255	*C. difficile* 17	*C. difficile* 541
E. faecalis PK1003	0.01±0.02	0.01±0.02	0.13±0.13	0.17±0.24
E. faecalis PK1201	0.01±0.01	0.01±0.02	0.05±0.05	1.67±2.36
E. faecalis PK1202	0.01±0.02	0.01±0.02	0.13±0.13	0.17±0.24
E. faecalis PK1301	0.00±0.00	0.01±0.02	0.00±0.00	0.32±0.02
E. faecalis PK1302	0.00±0.00	0.00±0.01	0.00±0.00	0.01±0.01
E. faecalis PK1801	0.01±0.02	0.13±0.18	0.14±0.14	1.80±2.17
E. faecalis PK2003	0.01±0.02	0.01±0.01	0.13±0.13	0.08±0.12
E. faecalis PK2004	0.06±0.09	0.04±0.05	6.34±6.34	0.05±0.07
E. faecalis PK2502	0.01±0.02	0.03±0.04	1.25±1.25	0.03±0.05
L. plantarum ATCC14917	0.00±0.00	0.01±0.02	0.03±0.03	0.05±0.02

Nine *E. faecalis* strains and *L. plantarum* ATCC 14917 possessing activity against *C. difficile* sporulation were test by inhibitory sporulation test. These result showed that (Figure 20) the percentage of spores in each *C. difficile* without treated with probiotics were 44.20±15.97 - 78.07±7.30. The percentage of spores in each *C. difficile* treated with nine *E. faecalis* and *L. plantarum* ATCC 14917 were significantly decreasing, based on each *C. difficile* and *E. faecalis* strains. For example, in *C. difficile* ATCC 630 treated with *E. faecalis* PK 1302. Spore formation was lower (6.73±2.20%) when compared with *C. difficile* ATCC 630 (44.20±15.97). Some *E. faecalis* strains

showed strongly reduced percentage of spore (<2%) such as *E. faecalis* PK1301, and PK2003.

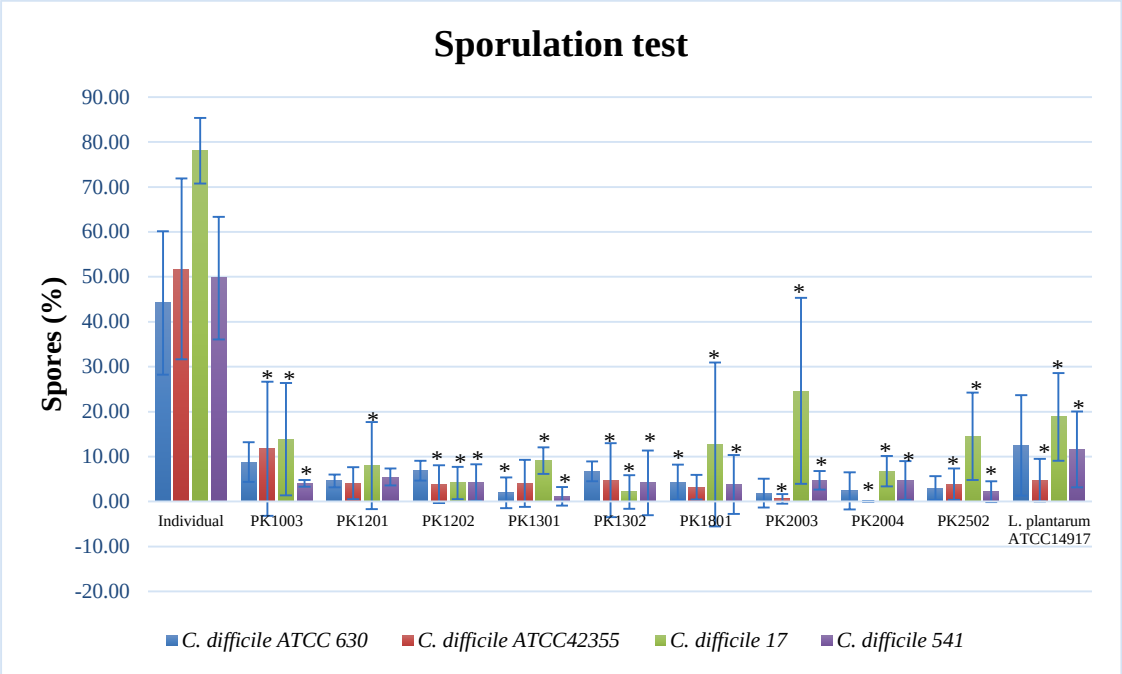


Figure 20. Percentage of *C. difficile* spore with or without treated with each *E. faecalis* strains and *L. plantarum* ATCC 14917. *; significant (p-value <0.05)

3.7 To develop and evaluate formula of potential probiotic

3.7.1 optimization of diary probiotic for *E. faecalis*

In this study we choose 4 skim milk including Dumex, Anlene, Diary, and Skin Milk Replacer to optimize diary probiotic. These result showed that, *E. faecalis* PK1202 and PK1302 suspended in fermented milk can slightly decreased pH. They showed hemolysis on blood agar and none of isolated strains exhibited antimicrobial activity. So this diary probiotic was not suitable for *E. faecalis.*

3.7.2 Optimization of cryoprotectants for *E. faecalis*

Many cryoprotectants have been proved to help the enhancement of survival ability of bacteria such as skim milk, glycerol, mannitol, sorbitol, trehalose, sucrose, maltose, lactose, fructose, glucose, betaine, and amino acids (135). In this study we choose skim milk, sucrose, glucose, and distilled water (DI) to optimize freeze-drying condition. These results showed that, the survival rate after freeze-drying of *E. faecalis* PK1202 suspended in skim milk (96.5-61.92%) were more than glucose, sucrose, fructose, and DI (42.00, 5.71, 9.23, and 15.38%, respectively). Moreover, the survival rate after freeze-drying of *E. faecalis* PK1302 was similar to *E. faecalis* PK1202, except *E. faecalis* PK1302 suspended in 10% fructose (Figure 21). These results indicate that, some cryoprotectants can protect and increase survival rate of *E. faecalis*. So, skim milk were selected for further experiments.

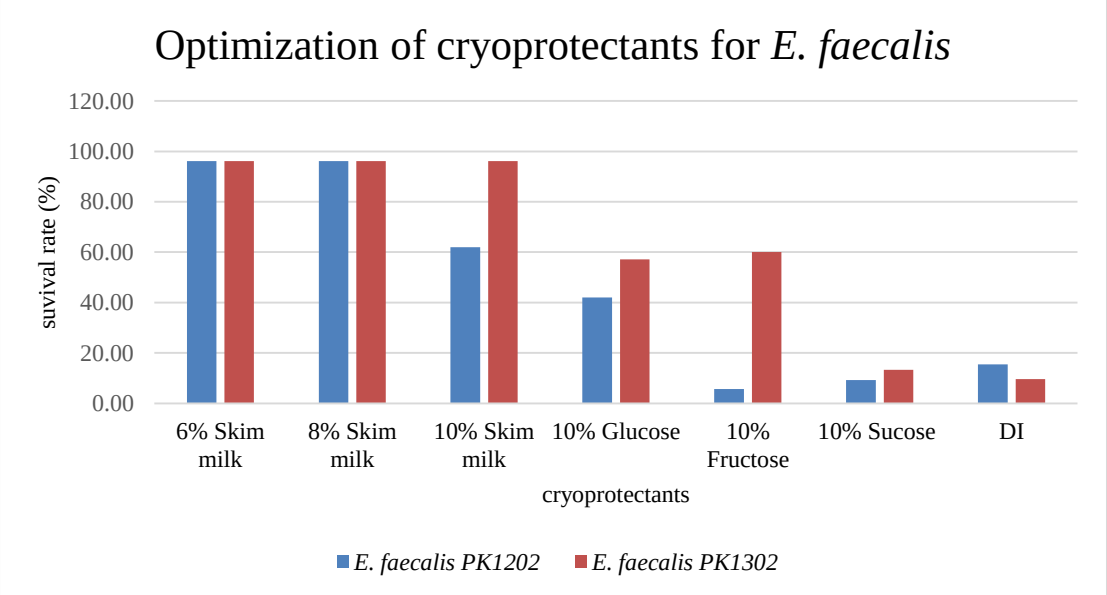


Figure 21. Survival rate (%) of *E. faecalis* PK1202 and PK1302 suspended in each cryoprotectants after freeze drying.

3.7.3 Survival of freeze dried probiotic strains

To evaluated the survival rate of *E. faecalis* after freeze-drying and storage, these result summarized in the table 16. All of nine *E. faecalis* strains suspended in 6%, 8%, and 10% skim milk. After freeze-drying, viability cell of nine *E. faecalis* strains significantly similar to pre freeze-drying. *E. faecalis* PK 1202 showed the highest viability cell after freeze-drying and storage at 4 °C for 30 days (pre freeze-drying= 14.18 ± 0.29, cell after freeze-drying= 14.16 ± 0.04, days 30= 14.05± 0.16 log CFU/mL). While, after storage 4 °C for 30 days the survival rate of nine *E. faecalis* strains were slightly decrease; however, no significance was observed. The best storage condition depend on bacterial strains, time of storage, and crycoprotectants. So, these result indicate that 6%, 8%, and 10% skim milk as cryoprotectants are suitable for nine *E. faecalis*

Table 16. Effect of storage on viability of *E. faecalis* after freeze-drying and storage at 4 °C for 30 days

	Storage time (days)	Viability cell (log CFU/mL)									
		PK1003	**PK1201**	**PK1202**	**PK1301**	**PK1302**	**PK1801**	**PK2003**	**PK2004**	**PK2502**	***L. plantarum* ATCC14917**
6% Skim milk	Pre freeze-drying	14.74 ± 0.04	14.18± 0.21	14.18 ± 0.29	14.32 ± 0.28	14.32 ± 0.29	14.49 ± .04	14.46 ± 0.02	14.34 ± 0.05	14.50 ± 0.17	14.94 ± 0.02
	0	14.14 ± 0.06	14.17 ± 0.01	14.16 ± 0.04	14.17 ± 0.02	14.16 ± 0.04	14.17 ± 0.18	14.17 ± 0.02	14.15 ± 0.05	14.14 ± 0.04	14.18 ± 0.06
	30	14.11 ± 0.01	13.93 ± 0.06	14.05± 0.16	14.10± 0.43	14.14 ± 0.06	14.01± 0.13	14.45± 0.10	14.03 ± 0.06	13.39 ± 0.03	13.94 ± 0.00
8% Skim milk	Pre freeze-drying	14.75 ± 0.02	13.96 ± 0.28	13.69 ± 0.29	14.28 ± 0.35	14.32 ± 0.29	14.46 ± .15	14.45 ± 0.05	14.32 ± 0.09	14.53 ± 0.21	15.05 ± 0.05
	0	14.17 ± 0.17	14.16 ± 0.01	14.13 ± 0.01	14.10 ± 0.05	14.18 ± 0.02	14.15 ± 0.02	14.18 ± 0.02	14.19 ± 0.01	14.15 ± 0.02	14.18 ± 0.00
	30	14.11 ± 0.09	13.99 ± 0.11	14.09± 0.01	14.12± 0.10	14.17 ± 0.02	14.12± 0.04	14.10± 0.01	14.06 ± 0.06	14.08 ± 0.04	13.94 ± 0.05
10% Skim milk	Pre freeze-drying	14.64 ± 0.21	14.53± 0.49	14.05 ± 0.21	14.75 ± 0.23	14.47 ± 0.01	14.58 ± 0.25	14.42 ± 0.09	14.50 ± 0.25	14.53 ± 0.21	14.98 ± 0.11
	0	14.15 ± 0.05	14.15 ± 0.04	14.14 ± 0.05	14.12 ± 0.05	14.17 ± 0.01	14.15 ± 0.05	14.11 ± 0.11	14.01 ± 0.29	14.08 ± 0.17	14.16 ± 0.04
	30	14.08 ± 0.02	14.03 ± 0.07	14.10± 0.00	14.12± 0.05	14.18 ± 0.01	13.95± 0.15	13.96± 0.12	14.10 ± 0.00	13.95 ± 0.15	14.20 ± 0.04
	Storage time (days)	Survival rate (%)									
		PK1003	**PK1201**	**PK1202**	**PK1301**	**PK1302**	**PK1801**	**PK2003**	**PK2004**	**PK2502**	***L. plantarum* ATCC14917**
6% Skim milk	0	95.96 ± 0.20	99.91 ± 1.55	103.42±1.96	98.96 ±1.99	98.85 ±2.23	97.79 ±0.75	97.94 ±0.23	98.67 ±0.39	97.51 ±0.93	94.87 ±0.40
	30	95.70 ± 0.35	98.22 ± 1.02	102.63±1.10	98.49 ±1.74	98.75 ±2.18	96.66 ±0.11	97.07 ±0.75	97.85 ±0.60	96.45 ±1.27	93.27 ±0.49
8% Skim milk	0	96.07 ±0.13	101.56±2.13	103.28±2.25	99.15±2.12	98.95±2.10	97.97±1.05	98.03±0.44	99.06±1.28	97.48±1.28	94.18±0.31
	30	95.62 ±0.56	100.26±1.62	102.95±2.12	98.93±2.87	98.95±2.05	97.64±1.03	97.61±0.41	98.25±0.35	96.85±1.14	93.27±0.64
10% Skim milk	0	96.67 ±1.59	97.49 ±3.07	100.65±2.74	95.79 ±1.85	97.94 ±0.56	97.06 ±1.79	97.89 ±1.28	96.70 ±3.59	96.86 ±2.08	94.48 ±0.51
	30	96.23 ±1.47	96.67 ±3.79	100.38±2.80	95.79 ±1.43	98.03 ±0.52	95.68 ±0.72	96.86 ±2.82	97.27 ±1.67	96.00 ±2.24	94.77 ±0.93

3.7.4 Antimicrobial activity

Antimicrobial activity was evaluated after freeze-drying (Day 0) and 30 days after freeze-drying against toxigenic *C. difficile* including *C. difficile* ATCC 630, *C. difficile* ATCC 43255, *C. difficile* 17, and *C. difficile* 544. These result showed that, at day 0 and 30, nine *E. faecalis* strains suspended in 6%, 8%, and 10% skim milk were able to inhibit five toxigenic *C. difficile* (Table 17-18). Moreover, *E. faecalis* PK1302 suspended in 10% skim milk showed strongly inhibit *C. difficile* ATCC 43255, *C. difficile* 17, and *C. difficile* 544. So, this result indicate that antimicrobial properties of nine *E. faecalis* (PK1003, PK1201, PK1202, PK1301, PK1302, PK1802, PK2003, PK2004 and PK2502) were remained after freeze-drying and storage at 4 °C for 30 days.

3.7.5 Hemolysis activity

None of nine *E. faecalis* strains showed hemolysis activity on 5% blood agar and was confirmed by hemolysis tube test. The result showed that these strains cannot destroys red blood cell.

3.7.6 Survival test (*G. mellonella)*

The effect of *G. mellonella* infection with each nine freeze-dried *E. faecalis* strains and *L. plantarum* ATCC 14917 were evaluated with *E. faecalis* ATCC 4736 as positive control and PBS as negative control. The result showed that *E. faecalis* ATCC 4736 induced 85 % death of *G. mellonella* larvae. While, control of PBS and no treated did not affect the five-day survival of *G. mellonella* larvae (survival rate 100% and 90% respectively). Nine *E. faecalis* strains and *L. plantarum* ATCC 14917 showed survival rate between 70%-100% (Table 19). Thus, there suggested that nine are safe.

Table 17. Effect of after freeze-drying on antimicrobial activity of *E. faecalis*

Bacteria		PK1003	PK1201	PK1202	PK1301	PK1302	PK1801	PK2003	PK2004	PK2502	*L. plantarum* ATCC14917
6% Skim milk	*C. difficile* ATCC 630	++	++	+++	++	++	+	++	++	++	+
	C. difficile ATCC 43255	++	++	++	++	+++	++	++	++	++	-
	C. difficile 17	++	++	++	++	++	++	++	++	++	-
	C. difficile 541	++	++	++	++	++	++	++	++	++	-
8% Skim milk	*C. difficile* ATCC 630	++	++	++	++	++	++	++	++	++	+
	C. difficile ATCC 43255	++	++	++	++	++	++	++	++	++	-
	C. difficile 17	++	++	++	++	++	++	++	++	++	-
	C. difficile 541	++	++	++	++	++	++	++	++	++	-
10% Skim milk	*C. difficile* ATCC 630	++	++	++	++	++	++	+++	++	++	+
	C. difficile ATCC 43255	++	++	+++	++	+++	++	++	++	++	-
	C. difficile 17	++	++	++	++	+++	++	+++	++	++	-
	C. difficile 541	++	++	++	++	+++	++	++	++	++	-

-; not determined, +; 5-10 mm, ++; 10-15 mm, +++; 15-20 mm.

Table 18. Effect of after storage at 4 °C for 30 days on antimicrobial activity of *E. faecalis*

Bacteria		PK1003	PK1201	PK1202	PK1301	PK1302	PK1801	PK2003	PK2004	PK2502	*L. plantarum* ATCC14917
6% Skim milk	*C. difficile* ATCC 630	++	++	+++	++	++	+	++	++	++	+
	C. difficile ATCC 43255	++	++	++	++	+++	++	++	++	++	-
	C. difficile 17	++	++	++	++	++	++	++	++	++	-
	C. difficile 541	++	++	++	++	++	++	++	++	++	-
8% Skim milk	*C. difficile* ATCC 630	++	++	++	++	++	++	++	++	++	+
	C. difficile ATCC 43255	++	++	++	++	++	++	++	++	++	-
	C. difficile 17	++	++	++	++	++	++	++	++	++	-
	C. difficile 541	++	++	++	++	++	++	++	++	++	-
10% Skim milk	*C. difficile* ATCC 630	++	++	++	++	++	++	++	++	++	+
	C. difficile ATCC 43255	++	++	+++	++	+++	++	++	++	++	-
	C. difficile 17	++	++	++	++	+++	++	++	++	++	-
	C. difficile 541	++	++	++	++	+++	++	++	++	++	-

-; not determined, +; 5-10 mm, ++; 10-15 mm, +++; 15-20 mm.

Table 19. Survival rate (%) of G. mellonella infected with each nine freeze-dried *E. faecalis* and *L. plantarum* ATCC 14917

Strains	**6% skim milk**	**8% skim milk**	**10% skim milk**
E. faecalis PK1003	80	80	80
E. faecalis PK1201	80	80	70
E. faecalis PK1202	80	70	90
E. faecalis PK1301	80	70	90
E. faecalis PK1302	80	80	90
E. faecalis PK1801	80	70	70
E. faecalis PK2003	70	70	100
E. faecalis PK2004	70	70	90
E. faecalis PK2502	80	70	80
L. plantarum ATCC 14917	90	80	100
E. faecalis ATCC 4736	15		
PBS	100		
No treated	100		

CHAPTER 4

DISCUSSION

In this study, we isolated, identified, characterized properties and evaluated mechanism of the new probiotic strains that isolated from breast-fed infants with capability of inhibition *C. difficile* in vitro test. All isolated strains from breast-fed infant samples are *Enterococcus faecalis*; PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 confirmed by MALDI-TOF MS and 16s rDNA sequencing. These isolated strains can inhibit toxigenic *C. difficile.* Therefore, we chose all these strains for characterized probiotic properties and evaluated mechanism of new probiotic. Major sources of microbial colonization of the GIT are maternal microbiota (during vagina delivery, the infant's diet, and environment during birth, gestational age, hygiene measures and antibiotic treatment) (136, 137). These sources of isolation for probiotics are generally recognized as safe (GRAS) for human consumption and probiotics. The beneficial effect of isolated strains from the stool of breast-fed infant beyond strains isolated from diary food were the high ability to survive during passage through the GIT condition, compete nutrient from pathogen bacteria and colonize in GIT (138). Moreover, some factors in maternal milk are immunoglobulin, lactoferrinn, oligosaccharides, and prebiotic substances that can against infections (112). In addition, these factors may stimulate the growth of a limited number of beneficial bacteria in the GIT of infants (101, 138). Thus, several researches have been screened new probiotic from breast-fed infant stool since it dominate with bifidobacteria, lactobacilli, enterococci, and other LAB (112, 139-143). In previous study, Rodríguez E, et al. (2012) were isolated *L. paracasei* INIA P272, *L. rhamnosus* INIA P344, *L. rhamnosus* INIA P426, *L. mucosae* INIA P459, *B. pseudolongum* INIA P2, *B. breve* INIA P18, *E. faecium* INIA P445 and *E. faecalis* from breast-fed infants and characterized as new probiotics (112). Another study, LAB also was isolated as probiotic from the feces of breast-fed infants (140). Probiotic bacteria get interesting in

recent years to control the CDI and AAD that major cause of serious health problem in the world. According to the WHO, FAO, and EFSA (the European Food Safety Authority) (67, 68), probiotics have to safety and functionality criteria, moreover those related to their technological advantage. The bacterial safety is clarified by its origin, the lack of association with the pathogen, and the antibiotic resistance pattern. It must survive passage through the GI tract condition. The functional requirements of probiotics include tolerance to human gastric juice and bile, adherence to epithelial surfaces, persistence in the human GI tract, and antimicrobial activity (69, 70). The resistance of antibiotic is the most important factor for evaluation of safety of probiotic. In case of enterococcus, vancomycin resistance is significant concerned for enterococcus safety because it can transfer to other strains by horizontal gene transferring (122, 144). Surprisingly, nine *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 isolated in this study were sensitive to vancomycin. In addition, all of nine *E. faecalis* strains were not carried *Van-A* and *Van-B* genes detected by PCR. However, all nine strains were resistant to gentamicin, and some strains were resistant to erythromycin and tetracycline, which are similar to previous study that strains isolated from fecal samples were resistant to erythromycin and tetracycline because *E. faecalis* carried genes resistant to those antibiotics as intrinsic genes. So, after host being exposed to antibiotics, these bacteria can survive in GIT (91). In addition, enterococcus is part of the normal GI flora in humans. Moreover, enterococci are used as probiotic organisms because of their good growth, adhesion ability, lactic acid production and the stability of their bacteriocin, an antimicrobial peptide that can inhibit other related strains of bacteria. Although, some enterococcus species carry virulence gene. It has been a part of food production and probiotics for many centuries since it contribute to the typical taste and flavor of fermented foods (91, 115, 120, 145, 146). Some enterococci probiotics products in the market include Symbioflor 1 (SymbioPharm, Herborn, Germany), ECOFLOR (Watlters Health Care, DenHaag, The Netherlands), and Cylactin® (Hoffmann — LaRoche, Basel, Switzerland) (147). Furthermore, nine *E. faecalis* strains were tested the enterococcal virulence gene. The nine isolates strains showed $efaA_{fs}$ gene that are involved in the adhesion process. *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, and PK1801 were negative for *cyl*A, cylB and cylM, genes that are considered

the main pathogenic factor. *cylA* is cytolysin production, *cylB* is involved on the transport of cytolysin, and *cylM* plays a role in posttranslational modification of the cytolysin (121). Actually, nine strains share the virulence genotype with many of the *E. faecalis* isolates used as a commercial starter or were found in raw or fermented milk products. Although many virulence genes present in the nine isolated strains, they are not necessary associated with pathogenesis and expression of some genes may allow the bacteria to colonize in GI epithelial cell and show probiotic functionality (147). Isolated *E. faecalis* strains are saved because phenotypic assays showed none gelatinase activity, mucin degradation, and hemolysin production except *E. faecalis* PK2003, PK2004, and PK2502. Our findings are similar to those of a previous study, that *E. faecalis* NM815 being positive for *agg, ccf,* and *efaAfs* genes (91). In 2005, Reviriego C et al, to screen virulence determinants in *E. faecium* and *E. faecalis* isolated from breast milk, the result showed that all the strains were clear of the majority of potential virulence determinants including *esp, cpd, cob, ccf, cad, gelE, cylM, cylB*, and *cylA*. None of the strains showed gelatinase activity, hemolysin production, or aggregation phenotype, and none carried the *vanA* or *vanB* genes (120). In 2001, Eaton TJ and Gasson MJ, screen enterococcus virulence determinants and potential for genetic exchange between food and medical isolates (148). Moreover, the effect of virulence factors of *E. faecalis* were tested by animal model (*G. mellonella*) that have characteristics useful for the study of human pathogens. The innate immune system of *G. mellonella* larvae including structural and functional are similar to vertebrate. In this study, the pathogenisis of nine *E. faecalis* strains were determined by *G. mellonella* killing assay. The nine isolated *E. faecalis* strains and *L. plantarum* ATCC 14917 showed survival rate between 80%-100%. This result similar to control groups. In 2009, Gaspar F et al, to evaluate *fsrB* and *gelE* in virulence of *E. faecalis* dairy strains by *G. mellonella* model. *E. faecalis* dairy strains were able to kill larvae. Fortunately, all of nine isolate strains did not kill larvae. These data suggested that *E. faecalis* PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 are safe.

We then characterized properties of probiotic. Probiotics must survive the passage through the GIT, tolerance to low pH (pH 1.5-3), tolerance to simulated gastric (pH 2.0, 3 mg/mL pepsin, 3 h) and pancreatic digestion (pH 8.0, 1 mg/mL pancreatin, 4h), tolerance to bile salts (0.3%, w/v bile salts, 4h) (73, 112, 117). Nine *E. faecalis*

strains survive in the pH 2. The pH of the gastric juice is regarded among the major factors affecting the survival of bacteria upon passage through the stomach to the intestine (117). Similar studies have shown high survival of *E. faecalis* NM815, *E. faecalis* NM915, and *E. faecium* NM1015 in all the tested pH conditions (91) . In another study, showed the number of *E. faecalis* INIA 127 decreased less than 2.89 log CFU after incubation at pH 2 for 3 h (112). For tolerance to pepsin, pancreatin, and bile salts, the nine *E. faecalis* strains show higher levels of viability in tolerance to pepsin and bile salts than *L. plantarum* ATCC 14917; however, viability of *L. plantarum* ATCC 14917 in bile salts medium were similar to all of the *E. faecalis* strains. Our findings are like those of a previous study that three enterococcus strains (NM815, NM915, and NM1015) showed good survival in the presence of pepsin, pancreatin, and bile salts (91). Nine *E. faecalis* (PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502) can survive in the gastrointestinal tract. Adhesion of probiotic bacteria on human epithelium cells has been suggested as an important mechanism for preventing colonization by pathogens. Nine *E. faecalis* strains showed high adhesion to HT-29 cells. Isolated strain PK1801 showed the highest adhesion ability (78.83 ± 4.16%). This result similar to a previous study, that *E. faecium* NM1015, *E. faecalis* NM815, *E. faecalis* NM915 showed the adhesion ability are 58%, 49% and 44%, respectively (91). In 2012, Rodrı´guez E et al. tested adhesion of probiotic bacteria on human epithelium cells. *L. paracasei* INIA P272 and *L. rhamnosus* INIAP344 were the most adhesive strains, with counts of adherent bacteria higher than the positive control *L. rhamnosus* GG (112). And another result from Monteagudo-Mera, A., et al. (2012), *L. lactis* 660 and *L. rhamnosus* ATCC 53103 showed strong adhesion ability (16% and 9.24%, respectively). Thus, nine isolated strains may adhere to human epithelium cells more than another probiotic depend on type of cell lines. Moreover, the hydrophobicity of *E. faecalis* strains from breast-fed infant that varied from 47.51 ± 3.02% to 85.00 ± 2.93%. In a previous study, the hydrophobicity of tested *L. brevis* KU15006 isolated from kimchi was 48% (149). Therefore, these result suggest that, nine *E. faecalis* strains from breast-fed infant have safety and probiotic properties that are assessment approved them as potential probiotic strains.

Then, we evaluated mechanism of nine *E. faecalis* as a probiotic. Some compounds produced by probiotic bacteria, such as metabolites, organic acids, and bacteriocins that may contribute to the antimicrobial activity against foodborne pathogens (134). For the previous study, some probiotic such as *Bifidobacteria* can produce antimicrobials and acids that inhibit *C. difficile* growth and adhesion to intestinal epithelial cells (85). *L. lactis* produce lacticin 3147 that inhibits *C. difficile. B. thuringiensis* capable of producing a Thuricin CD which was shown to be as effective as metronidazole at inhibiting *C. difficile*. Probiotics can produce BSH enzymes that transformation of bile acids form conjugated bile acid to unconjugated bile acid that can inhibit spore germination of *C. difficile* (85, 86). Enterococci probiotics were used to test their effectiveness in the prevention of AAD (145, 150, 151). *E. faecalis* NM815, *E. faecalis* NM915, and *E. faecium* NM1015 can inhibit *C. difficile* (91). Likewise, our result showed nine *E. faecalis* strains were able to inhibit *C. coli* ATCC 11353, *C. difficile* DMST 16662, *C. difficile* ATCC 630, *C. difficile* ATCC 42355, and two clinical toxigenic *C. difficile.* These *E. faecalis* may produce organic acids, bacteriocins, and/or BSH. Bacteriocins from LAB strains can kill bacteria by several mechanisms, including pore formation in the cell membrane, inhibition of cell wall or protein synthesis, and degradation of cellular DNA (152, 153). Moreover, organic acids were found to be caused mainly by the undissociated form of organic acids. Non-dissociated organic acids can penetrate through lipid membrane of the bacterial cell and once internalize into the neutral pH of the cell cytoplasm dissociate into anions and protons, both of which exert an inhibitory effect on bacteria. Releasing proton ions causes the internal pH to decrease leading to disruption of proton motive force, and inhibiting substrate transport mechanisms (154, 155).

C. difficile colonizes the intestine following disruption of the host gut normal flora. *C. difficile* adheres to the gut mucosa and toxigenic strains secrete toxins that are toxin A and/or toxin B. These toxins disrupt the actin cytoskeleton and tight junctions leading to decrease epithelial barrier function, fluid accumulation, destruction of the intestinal epithelial cells, rounding cell, and cell death (3, 26, 36). Another *C. difficile* toxin is binary toxin, associated with increased severity of the symptom, modifying actin molecules, trigger microtubule protrusion, thereby increasing the adherence of *C. difficile* to gut epithelium (37, 38). Some probiotics can against *C. difficile* toxins such

as *S. boulardii* produce protease protein which hydrolyzed TcdA and TcdB and inhibited their binding to their respective intestinal brush border receptors (85, 86). Moreover, probiotic can upregulate specific anti-TcdA secretory IgA expression in CDI patient (86-88). In our result, nine *E. faecalis* may secreted some molecules such as organic acid, BSH, and/or bacteriocin that reduce or protect cytotoxic effect of toxin on HT-29 cells by inactivated *C. difficile* toxin or kill toxigenic *C difficile*.

Moreover, nine *E. faecalis* can inhibited toxigenic *C. difficile* spore germination and reduce sporulation of *C. difficile*. From the previous study, Enterococci can secreted some molecule that inactivated *C. difficile* toxin and activated immune cell to against *C. difficile* (91, 156, 157). From Valdes VL et al. (2016) (128), they screened of bifidobacteria and lactobacilli that are able to antagonize the cytotoxic effect of *C. difficile* upon intestinal epithelial HT29 monolayer. This result showed that, *B. longum* and *B. breve* to reduce the toxic effect of the pathogen on HT29. This strain prevents HT29 cell rounding, preserving the F-actin microstructure and tight-junctions between adjacent cells. This is similar to our result. So, further work, we will needed to identify the secretion molecules of *E. faecalis* that against *C. difficile.*

Finally, probiotic formulas are divided into two groups including diary probiotic products and non-diary probiotic products; fermented vegetable and fruit, grains, and meat (53). Enterococci occur as starter in a variety of cheeses, fermented milk, and whey starter cultures (158). Enterococci probiotic products in the market are included capsule and micro-encapsulated forms (147). Moreover, physicians tend to recommend tablets and powders. The main technologies were involved in fermentation, encapsulation, drying, rehydration, and storage developed and applied to use in probiotics but there are still many technological challenges in producing and preserving probiotic foods (53, 107). Freeze-dried as lyophilization assay is one of many methods to preserve and storage probiotic starter over a long period of time (111). Several cryoprotectants such as skim milk, glycerol, manmtol, sorbitol, trehalose, sucrose, maltose, lactose, fructose, glucose, betaine, and amino acids have been proved to help the enhancement of survival ability of bacteria (135). Skim milk and sucrose as cryoprotectants have been commonly used. Skim milk can prevent cellular injury of microoganism by stabilizing the cell membrane and providing a protective coating for the cells, whereas sucrose can prevent cell by trapping salts in a highly viscous or glass-

like phase (159). In present work, we select skim milk to protect *E. faecalis* strains and evaluate the survival rate of nine *E. faecalis* strains after freeze-drying and storage. After freeze-drying and after storage at 4 °C for 30 days, viability cell of nine *E. faecalis* strains significantly similar to pre freeze-drying. It preserve the antimicrobial activity of *E. faecalis.* So, these skim milk are suitable as cryoprotectants for nine *E. faecalis.* Skim milk is inexpensive, easy, and common to use as cryoprotectants. Skim milk are generated porous structure on cell surface that promotes the process of rehydration, meanwhile, protein of which provides a protective coating for cells (160, 161). Our result are similar to previous studies such as Shu G, et al. (2018) (161), that were tested survival of *L. acidophilus* with different composite cryoprotectants (trehalose of 13%, Na_2HPO_4 of 0.33%, lactose of 7.5% and skim milk powder of 21%) during freeze-drying. The survival rate of freeze-dried *L. acidophilus* increased to 93.9 ± 0.12% in comparison to control 36.6 ± 0.08%. Yingjian L, et al. (2017), the most significant factor influencing the resistance of *S. thermophilus* STX2 to freeze-drying was skim milk, followed by sodium glutamate, and then glycerol (135). *L. gasseri* CRL1412 suspended in skim milk showed a significantly higher resistance than when it was suspended in water, but lactose or sucrose did not significantly increase its viability after lyophilization (162). Moreover, Mayur B et al. (2017) were tested the viability of freeze-dried cells suspended with sorbitol, ascorbic acid and skim milk and sustained probiotic properties during storage. Percentage of survivability are 73%–93% for a long period of time (163). In conclusion, 10% skim milk add to the nine *E. faecalis* (PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502) suspensions that can protect and prevent cell damage during freeze-drying process and long term storage. The effects of these process may interfere with the properties of probiotic such as the antimicrobial activity. This property must be taken into evaluation in product development. The nine *E. faecalis* strains as probiotic sustain and retain adequate viability in appropriate storage conditions with probiotic properties.

CHAPTER 5

CONCLUSIONS

We isolate, identify, characterized physiological probiotic properties and evaluated mechanism of nine *E. faecalis* strains including PK1003, PK1201, PK1202, PK1301, PK1302, PK1801, PK2003, PK2004, and PK2502 that isolated from breast-fed infants. *E. faecalis* may produce or secrete some molecules including BSH, bacteriocin, and/or hydrogen peroxide to inhibit *C. difficile* and its spore. These strains are reduce the Clostridial toxic effect upon HT29. It prevents HT-29 cells rounding, preserving the F-actin microstructure and tight-junctions between adjacent cells. Moreover, nine *E. faecalis* strains are safe that were not carry *Van-A, Van-B,* and major virulence gene especially, *cylM, cylB, cylA* gene. None of isolated strains produces mucin degradation, gelatinase activity and hemolysin production except *E. faecalis* PK2003, PK2004, and PK2502. Moreover, none of nine *E. faecalis* strains infected *G. mellonella* indicated that they are safe. Nine *E. faecalis* strains exhibit characteristics of probiotic including all strains display higher levels of viability in the pH 2 as well as tolerance to pepsin and bile salts than *L. plantarum* ATCC 14917. It can survive in pancreatic digestion. All of nine strains can strongly adhere to HT-29 cells. In addition, nine *E. faecalis* suspended with10% skim milk can prevent cell damage during freeze-drying process and long term storage. After freeze-drying process, *E. faecalis* sustain and retain adequate viability in appropriate storage conditions with probiotic properties. *E. faecalis* as probiotic candidates are very attractive for further evaluations in vivo as well as for developing novel foods or pharmaceutical products.

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List of Publications and Proceedings

1. **Chonticha Romyasamit**, Teeranuch Boonpipattanapong, Surasak sangkhathat, Benjamas Thamjarongwong, Kamonnut Singkhamanan. Optimization of direct multiplex PCR for detection of toxigenic *Clostridium difficile*. Oral presentation at the 2 nd Joint Symposium BMS-BME-EU: Post-graduate Health Science and Technology Conference. May 21-22, 2015. Faculty of Medicine, PSU
2. **Chonticha ROMYASAMIT**, Teeranuch BOONPIPATTANAPONG, Surasak SANGKHATHAT, Benjamas THAMJARONGWONG, Kamonnut SINGKHAMANAN. Optimization of multiplex loop-mediated isothermal amplification assay for direct detection of toxigenic *Clostridium difficile*. Poster presentation at 19th National Genetics Conference 2015. July 15-17, 2015. Centara Hotel & Convention Centre Khon Kaen
3. **Chonticha Romyasamit**, Teeranuch boonpipattanapong, Surasak sangkhathat, Benjamas thamjarongwong, Janthima Jaresitthikunchai, Sittiruk Roytrakul, Kamonnut Singkhamanan. Identification of *Clostridium difficile* by MALDI-TOF MS. Poster

presentation at 5thInternational Biochemistry and Molecular Biology Conference 2016. May 26-27, 2016. B.P. Samila Beach hotel, Songkhla, Thailand.

4. Romyasamit C., Boonpipattanapong T., Sangkhathat S., Thamjarungwong B., Singkhamanan K. Sensitive, specific and rapid multiplex direct-PCR for detection of toxigenic *Clostridium difficile* in stool sample **(Submitted)**

5. Chonticha Romyasamit, Teeranuch Boonpipattanapong, Surasak Sangkhathat, Benjamas Thamjarongwong, Janthima Jaresitthikunchai, Sittiruk Roytrakul and Kamonnut Singkhamanan. Identification and Typing of *Clostridium difficile* by MALDI-TOF MS techniques. Oral presentation at the 2th Joint Symposium BMS-BME-EU: Post-graduate Health Science and Technology Conference. May 25-26, 2017. Faculty of Medicine, PSU

6. Chonticha Romyasamit, Anucha Thatrimontrichai, Aratee Aroonkesorn, Kamonnut Singkhamanan. ELIMINATION OF *Clostridium difficile* SPORE GERMINATION BY ENTERIC BACTERIA. Poster presentation at the 6th international conference on biochemistry and molecular biology 20-22 June 2018. Rayong Resort, Rayong, Thailand

7. Chonticha Romyasamit, Natnicha Ingviya, Anucha Thatrimontrichai, Kamonnut Singkhamanan. Identification and evaluation of physiological properties of probiotics isolated from breast-fed infants against *Clostridium difficile.* Poster presentation at 29th European Congress of Clinical Microbiology and Infectious Diseases, 13-16 April 2019, Amsterdam, Netherlands