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Probiotics and Inhibition of *Clostridium difficile* Toxin

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Abstract

The definitions of international authorized probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The scientific basis for the prevention and treatment of bowel disease among the functions of probiotics has only recently been established. Probiotics, which are actively studied, are lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species. The *C. difficile* toxin is controlled by quorum sensing, which causes intestinal disease and other gastrointestinal disorders, leading to antibiotic-associated diarrhea. The use of probiotics for prevention and treatment has been discussed in this review.

Keywords

Clostridium difficile, probiotics, quorum sensing, toxin

Toxin of *Clostridium difficile* and *C. difficile*-associated diarrhea

Clostridium difficile (*C. difficile*) was initially named *Bacillus difficilis* by Hall and O'Toole in 1935. It was first isolated from the stool of newborns and called "difficile" because it was difficult to culture (Schroeder, 2005). Later, *C. difficile* was found to be affiliated with the class Clostridia and the binomial name was changed to "*Clostridium difficile*." It was initially considered non-pathogenic bacterium. However, in 1978, it was reported to caused antibiotic-associated pseudomembranous colitis (Bartlett *et al.*, 1978; Tedesco *et al.*, 1974).

C. difficile is an exotoxin-producing (toxin A and B), gram-positive, rod-shaped bacterium. It has the ability to grow under an anaerobic conditions, while the spores can only survive under aerobic conditions. Spore formation increases the probability of *C. difficile* infection (CDI). *C. difficile* is transmitted via the fecal-oral route in the form of spores or whole cells and hands of the healthcare providers between patients in hospitals (Shaughnessy *et al.*, 2011). The number of CDI has been rising steadily worldwide along with increasing morbidity and mortality over time. In 2003, CDI was found to be most prevalent disease in Canada, leading to the worldwide recognition of the threat of CDI. In Quebec, the number of severe cases of CDI quadrupled in 2003 compared to that in the period between

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1991 and 2002 (Kelly and LaMont, 2008). In children, the number of severe cases of CDI has been reported to be continuously to increasing, from 3,565 cases in 1997 to 7,779 cases in 2006 (Zilberberg *et al.*, 2010). The incidence of CDI is at an annual rate of approximately 3 million cases in the United States (Schroeder, 2005). From 2003 to 2006, the number of CDI occurrences and death In Austria increased significantly from 977 to 2,192 and from 80 to 150, respectively (Kuijper *et al.*, 2008). The first outbreaks of *C. difficile* 027 occurred in South Korea, Hong Kong, and Costa Rica between 2008 and 2010 (Quesada-Gomez *et al.*, 2010; Tae *et al.*, 2009; Cheng *et al.*, 2009). Increase in the incidence and severity of CDI can be attributed to a newly discovered strain of *C. difficile* strain BI/NAP1/027 (designated restriction endonuclease analysis type BI, North American pulsed-field gel electrophoresis type 1 (NAP1), polymerase chain reaction (PCR) ribotype 027) (McDonald *et al.*, 2005). CDI due to the new strain B1/NAP1/027 rapidly increased after 2000. The expression of *tcdA* and *tcdB* that encoded toxin A and toxin B was higher in *C. difficile* 027 than in other *C. difficile* strains. Therefore, *C. difficile* 027 causes more severe colitis and mortality than those caused by other strains (Fig. 1) (Clements *et al.*, 2010). *C. difficile* BI/NAP1/027 has several characteristics such as *tcdC* (a negative regulator of *C. difficile* toxin production) down-regulation and higher fluoroquinolone resistance (Clements *et al.*, 2010). Therefore, *C. difficile* 027 expresses higher levels of *tcdA* (16-fold) and *tcdB* (23-fold) than that by the nother toxin type O strain, and causes more severe colitis and higher mortality than those caused by other strains (Warny *et al.*, 2005).

CDI occurs mainly in patients who have disturbances in the intestinal microbiota because of antibiotics. Infection occurs primarily in hospitals. *C. difficile* attaches to the mucus layer and enterocytes of patients with the aid of proteases and causes colonization. *C. difficile* secretes toxins to damage the colonic mucosa, and when the patient cannot produce antibodies to the toxin, clinical manifestations appear (Fig. 2) (Deneve *et al.*, 2009). Alteration of intestinal microflora by antibiotics enables the overgrowth and dominance of pathogenic *C. difficile* in the gastro-intestinal environment and triggers CDI via the production of toxins A and B to destroy intestinal cells (Johnson and Gerding, 1998). CDI also has several risk factors, and the most important ones are advanced age

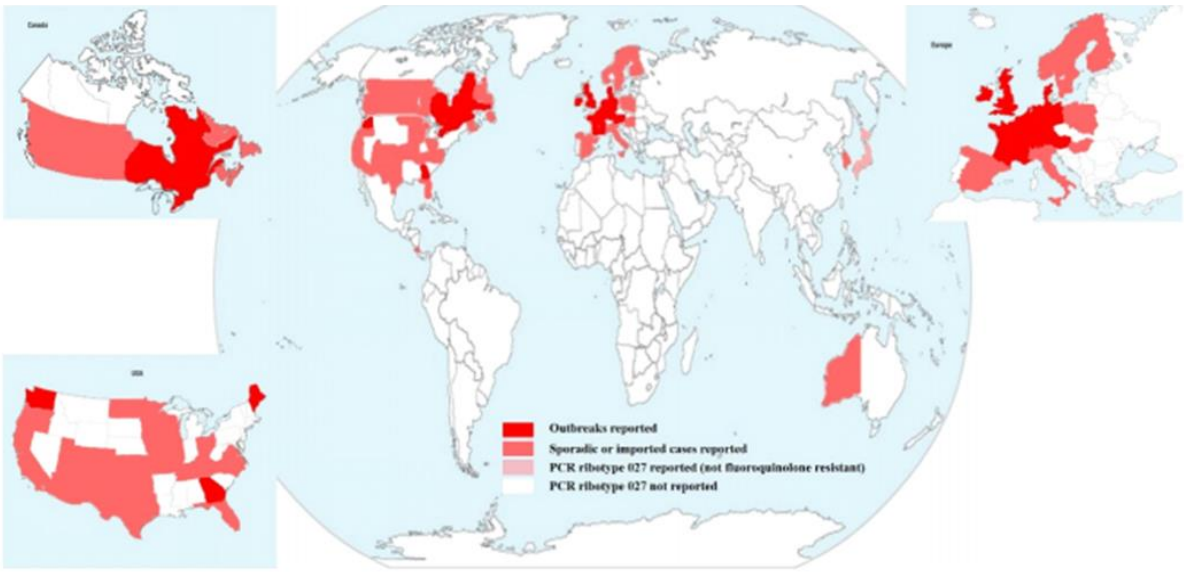

Fig. 1. Worldwide CDI occurrence of *C. difficile* PCR ribotype 027
Adapted from Clements *et al.*, 2010

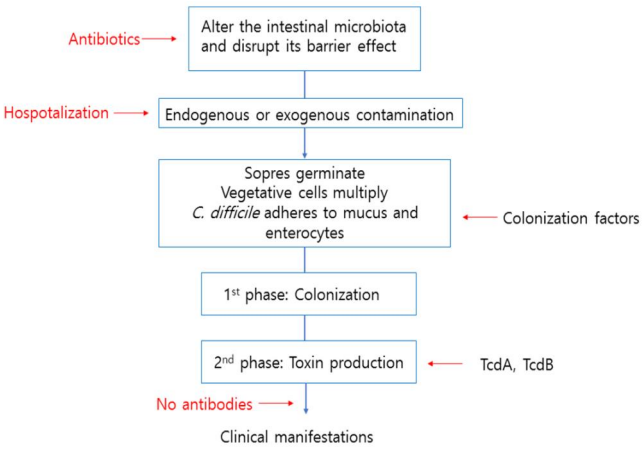

Fig. 2. Infection process of *C. difficile*
Adapted from Deneve *et al.*, 2009

and excessive use of antibiotics. Among patients infected with *C. difficile*, those who are 65 years of age or older and exposed to many antibiotics display the following progression of symptoms: inflammatory lesions, formation of pseudomembranous in the colon, toxic-megacolon or bowel perforation, sepsis, shock, and death (Fig. 3) (Rupnik *et al.*, 2009).

The pathogenicity locus (PaLoc) of *C. difficile* consists of

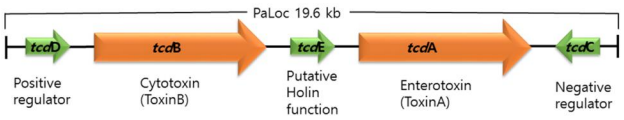

Fig. 4. Pathogenicity locus of *C. difficile*
Adapted from Carter *et al.*, 2012

tcdA, and *tcdB* and accessory genes *tcdC, tcdD,* and *tcdE* (Fig. 4) (O'Connor *et al.*, 2009). *TcdA* and *tcdB* encode toxins A and B, which are the major toxins produced by *C. difficile* (Braun *et al.*, 1996). *C. difficile* overgrowth occurs in the intestine after destruction of the normal intestinal flora, and it produces toxin A (308 kDa) and toxin B (270 kDa). Toxin A enters the cells by endocytosis with the help of toxin B. Toxin B binds preferentially to the cell membrane and causes cytoskeletal changes, resulting in disruption of tight junctions and loosening of the epithelial barrier. In addition, toxins A and B inactivate the Rho protein in the cytosol. Rho proteins functions by regulating the intestinal epithelial barrier, cell movements, intercellular junctions, immune cell migrations, and standard cellular functions (Jank *et al.*, 2007). Rho proteins are modified by the glucosyltransferases of

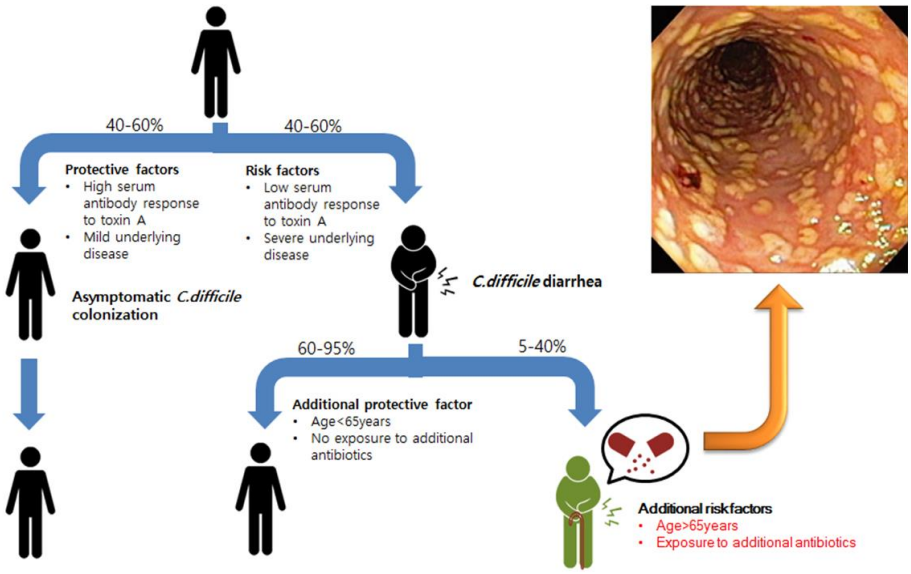

Fig. 3. Protective and risk factors of *C. difficile*-associated diarrhea
Adapted from Poutanen and Simor, 2004

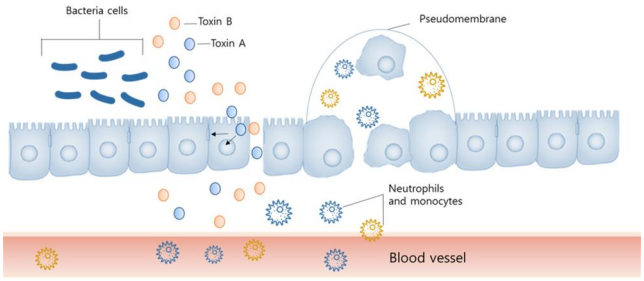

Fig. 5. Pathogenesis mechanism of *C. difficile*
Adapted from Maja Rupnik *et al.*, 2009

C. difficile toxins that inhibit GTPases activation by GEF. Inhibition of GTPases, which regulate intracellular actin dynamics, induces the weakening of cell junctions. Both toxins are cytotoxic and induce the release of various immunomodulatory mediators from epithelial cells, phagocytes, and mastocytes and cause the inflammation and the accumulation of neutrophilic leukocytes (Fig. 5) (Herrmann *et al.*, 1998; Sehr *et al.*, 1998; Jank *et al.*, 2007, Martin-Verstraete *et al.*, 2016). *C. difficile* toxin production decreases in the exponential phase and increases in the stationary phase because *tcdC* (negative regulator of *C. difficile* toxins) is up-regulated and *tcdD* (positive regulator of *C. difficile* toxins) is down-regulated in exponential phase. Therefore, *tcdC* and *tcdD* regulate *C. difficile* toxin production (Hundsberger *et al.*, 1997; Dupuy and Sonenshein, 1998).

Toxin production and quorum sensing of *C. difficile*

Quorum sensing (QS) is a bacterial cell-to-cell communication process, which regulates gene expression in response to cell number fluctuations (Fuqua *et al.*, 1994). QS is involved in the production and detection of extracellular signaling molecules, known as autoinducers. Autoinducers regulate cell responses such as cell division and production of virulence factors (Xavier and Bassler, 2003). Secreted autoinducers regulate the expression of specific genes in accordance to the cell density. Gram-positive and gram-negative bacteria regulate various activities using QS, such as

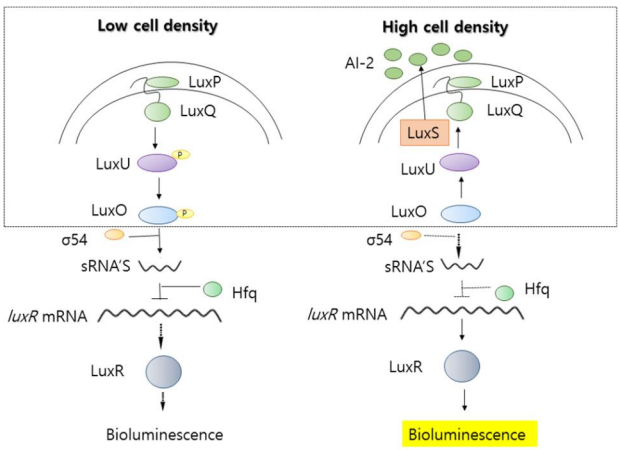

Fig. 6. Quorum sensing mechanism of *V. harveyi*.
Solid line allow, active path; Dotted line allow, inactive path.
Adapted from Banik *et al.*, 2009

symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller and Bassler, 2001; Waters and Bassler, 2005). QS was first observed in *Vibrio fischeri* and *Vibrio harveyi* (Nealson and Hastings, 1979). *V. harveyi* produces an acyl-homoserine lactones (acyl-HSL) autoinducer-1 (AI-1), which associates with the grampositive two-component phosphorelay system and produces and detects the autoinducer-2 (AI-2). Various gram-positive and gram-negative bacteria have a *luxS* quorum sensing system that produces and detects AI-2 (Xavier and Bassler, 2003). *LuxS* QS in *V. harveyi* consists of the following mechanisms. At low cell densities, *luxO* is phosphorylated to control the production of multiple small RNA that inhibits post-transcriptional translation of the QS master regulatory protein (*luxR*). In contrast, *luxS* activated at high cell densities produces AI-2, and decreased production of small RNA activates *luxR*. This activation increases expression of the light-emitting gene of *V. harveyi* (Fig. 6) (Banik *et al.*, 2009). Unlike HSL and other oligopeptide autoinducers, all AI-2-producing bacteria have identical a biosynthetic pathways, chemical intermediates, and AI-2 molecules (Miller and Bassler, 2001). These findings suggest that AI-2 is a universal signal that enables intercellular communication. AI-2 regulates the virulence factors of *C. perfringens*, motility of Escherichia coli EHEC (O127:H6), and biofilms of *Salmonella* Typhi (Table 1).

Table 1. Gene and functions controlled by *luxS* in bacteria

Species	Functions regulated by *luxS*	Genes regulated by *luxS*
Actinobacillus actinomycetemcomitans	Virulence factor: leukotoxin, iron acquisition	*afu*A
Borrelia burgdorferi	Expression of many proteins on two-dimensional-gels ErpA,-1 and –N proteins	
Campylobacter jejuni	Motility	
Clostridium perfringens	Virulence factors: alpha, kappa and theta toxins	*pfo*
Escherichia coli W3110	Cell division, DNA processing, cell shape and morphology	242 genes (microarray)
Escherichia coli EHEC (O157:H7)	Virulence factors: type-Ⅲ secretion, Shiga toxin, flagella, motility, cell division	*LEE operon, stx, ptsN, sulA, flhD, fliA, fliC, motA, qseA, qseBC* 404 genes (microarray)
Escherichia coli EHEC (O127:H6)	Motility (flagellin expression)	
Neisseria meningitides	Bacteremic infection	
Photorhabdus luminescens	Carbapenem biosynthesis	*cpm*
Porphyromonas gingivalis	Virulence factors: protease, hemagglutinin activities, hemin acquisition	*uvrB, hasF*
Salmonella Typhi	Biofilms	
Salmonella Typhimurium	AI-2 ABC transport system	*lsrACDBFGE*
Shigella flexneri	Transcription factors involved in controlling virulence	*virB*
Streptococcus pyogenes	Virulence factors: secreted protease hemolysin	*speB and sagA*
Vibrio cholera	Virulence factors: Cholera toxin, toxin-coregulated pilus	*tcpP, tcpA, ctxAB~70* virulence genes (microarray)
Vibrio harveyi	Light production, colony morphology, siderophore producton	*luxCDABE*

EHEC, enterohemorrhagic E. coli; EPEC, enteropathic E. coli
(Adapted from Xavier and Blassler, 2003)

Probiotics

According to Gismondo *et al.* (1999), probiotics are live microorganisms that provide health benefits to people and animals when consumed. Probiotics are normally found in foods and dairy products. Although there are many probiotic bacteria, *Lactobacillus* and *Bifidobacterium* are mainly used (Kekkonen, 2008). The probiotic mechanism of action is as follows: Probiotics suppress growth and consequent adherence of pathogenic bacteria to the intestinal wall and aid the immune system by improving intestinal wall function (Zareie *et al.*, 2006). Several probiotic strains promote the production of protective cytokines, such as IL-10 and TGF-beta, and inhibit the production of cytokines, such as TNF, that cause inflammatory responses. Probiotics have been reported to relieve allergies and antibiotic-associated diarrhea, inhibit bacterial vaginosis, and reduces blood cholesterol (Cuello-Garcia *et al.*, 2015; McFarland, 2006; Borges *et al.*, 2014; Ooi and Liong, 2010).

Recently, *Lactobacillus* strains and *Saccharomyces boulardii* have been reported to effectively prevent inflammatory bowel diseases and CDI in high-risk patients on antibiotics (Katz, 2006).

The inactivated lactic acid bacterium product has higher safety and stability than the live bacterium product. It can be stored easily because of its high stability, and the product distribution period, therefore, can be extended. Heat-inactivated *Lactobacillus* strains do not grow and,

therefore, differ from live bacteria. The lysate resulting from heat treatment has antibacterial activity and consists of cell components such as the cytoplasm, cell wall, bacteriocins, polysaccharides, and organic acids. In recent years, inactivated *Lactobacillus* strains have been produced industrially to be used as probiotics. They can be used as raw materials for the production of cosmetics, and in fields where live bacteria have been applied (Seo *et al.*, 2010).

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