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...
1991 and 2002 (Kelly and LaMont, 2008). In children, the number of severe cases of CDI has been reported to be continuously 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 BI/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 norther 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](image1)
Adapted from Clements et al., 2010
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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 *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

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

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

**Fig. 4.** Pathogenicity locus of *C. difficile*
Adapted from Carter et al., 2012
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 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 gramicidins. 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                                                | *afuA*                                                      |
| *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) | type-III secretion, Shiga toxin, flagella, motility, cell division                          | *LEE operon, stx, ptsN, sulA, flhD, fla, fliC, motA, qseA, qseBC 404 genes (microarray) |
| *Escherichia coli* EHEC (O127:H6) | Motility (flagellin expression)                                                              |                                                             |
| *Neisseria meningitides* | Bacteremic infection                                                                         |                                                             |
| *Photorhabdus luminescens* | Carbapenem biosynthesis                                                                       | *cm*                                                       |
| *Porphyromonas gingivalis* | Pro tease, hemagglutinin activities, hemin acquisition                                       |                                                             |
| *Salmonella Typhi*       | Virulence factors:                                                                          |                                                             |
| *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:                                                                            | *tcpP*, *tcpA*, *ctxAB*-70, *virulence genes (microarray)* |
| *Vibrio harveyi*         | Light production, colony morphology, siderophore product                                      | *luxCDABE*                                                 |

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