In vitro Effects of Probiotics on Clostridium Difficile Toxin Production and Sporulation

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Abstract

Introduction: With increased knowledge of the health benefits of the intestinal microbiome, probiotics are being used to prevent post-antibiotic diarrhea and Clostridium difficile infection (CDI). This study was designed to determine anti-C. difficile activity of five of the top selling probiotics in the U.S. and Canada.

Methods: Two strains of anaerobic bacteria were also selected for study as potential probiotics from patients with recurrent CDI treatment successfully treated with fecal microbiota transplantation, Clostridium clostridioforme (052) and of Bifidobacterium (055). Co-culture studies were performed looking at anti-toxin and anti-sporulation effects of the probiotics using two strains of C. difficile, 43255 (ribotype 087) and BAA 1805 (ribotype 027).

Results: Strains 052 and 055 showed the greatest toxin-neutralization activity for C. difficile 43255, while neutralization against toxin of strain 027 was seen only by BS, 055 and CVS. Of interest, the least active probiotic, PC, contained 10 different probiotic strains, demonstrating that containing more probiotic strains may not confer more activity. All probiotics showed anti-sporulation effects against C. difficile strain 087 while the most active inhibitors of sporulation for C. difficile 027 were BK, BS and PC with lower levels of inhibition seen for by CVS and 052.

Discussion/Conclusion: Overall BS was the most active anti-C. difficile probiotic combination tested in this study.

Keywords
Probiotics, C. difficile toxins, Sporulation

Abbreviations
CDI: C. difficile Infection; BK: Bio K Plus; BS: Bio Schwartz; WAL: Walgreens Ultra Strength Probiotic; CVS: CVS Health Maximum Strength; PC: Physician’s Choice

Introduction
Numerous studies have shown that Clostridium difficile infection (CDI) occurs secondary to loss of intestinal colonization resistance due to depletion of microbiome diversity from antibiotics, diet or aging. This has led to an interest in the use of probiotics to prevent [1] or in combination with antibiotics to treat CDI [2].

Objective
We designed this study to look for anti-C. difficile effects of commonly used bacterial probiotic prepara-
tions, focusing both on inhibition of toxin production which is related to disease pathogenesis, and inhibition of sporulation, important in disease recurrences.

**Methods**

**Study design**

Five of the top selling commercial bacterial probiotics in the United States and Canada were used in the study: Bio K Plus (BK), Bio Schwartz (BS), Walgreens Ultra Strength Probiotic (WAL), CVS Health Maximum Strength (CVS), and Physician’s Choice (PC). In addition, two anaerobic bacterial strains, identified as *Clostridium clostridioforme* (052) and *Bifidobacterium* (055), were isolated from stools of patients with recurrent CDI who responded to fecal microbiota transplantation (FMT), were included in the study. An Enterobacteriaceae strain (*E. coli* HS) was used as a probiotic-negative control in the toxin-inhibition studies. The bacterial composition of the probiotics used is summarized in Table 1.

Two toxigenic *C. difficile* strains were obtained from American Type Culture Collection (ATCC, Manassas, VA) and used in the neutralization experiments, ATCC 43255 and ATCC BAA 1805. Strain ATCC 43255 belongs to ribotype 087 and toxinotype 0 and is positive for *tcdA* and *tcdB* genes by PCR. Strain ATCC BAA 1805 is a ribotype 027, toxinotype III, NAP1, binary toxin and *tcdA* and *cdtB* positive.

**Co-culturing of probiotics and bacterial strains with *C. difficile* [3]**

*C. difficile* ATCC 43255 and ATCC BAA 1805 strains were grown overnight on the plates with *Clostridium difficile* agar (Remel, Lenexa, KS), at 37 °C under anaerobic conditions and tested for toxin production by ELISA and sporulation as described below. The individual probiotics, BK, BS, WAL, CVS, PC and bacterial strains 052 and 055 from FMT-treated patients with recurrent CDI were dissolved in 0.9% saline and justified to 0.5 McFarland turbidity standard (10⁸ CFU/mL). Each probiotic: *C. difficile* solution (P:CD ratio) were mixed at 1:10³, 1:10⁴, 1:10⁵, 1:10⁶, 1:10⁷ and 1:10⁹ ratios and incubated at 37 °C for 24 hours under anaerobic conditions before plated onto blood agar plates to culture for probiotic strains. The highest ratio for which two consecutive probiotic dilutions showed toxin inhibition was considered the point of toxin neutralization.

**ELISA detection of *Clostridium difficile* toxins A and B from co-cultured growth**

*C. difficile* toxin A and B were determined by using ProSpectT *C. difficile* Toxin A/B (TechLab© Blacksburg, VA 24060) according to the manufacture instructions.

**Inhibition of *C. difficile* spore production**

*C. difficile* isolates were grown on plates with *Clostridium difficile* agar (Remel, Lenexa, KS) overnight at 37 °C and diluted with 0.9% saline, justified to 0.5 McFarland turbidity standard (10⁶ CFU/mL). The resulting suspension was added to each of the five commercial probiotics, strains 052 and 055 in a ratio of 1:1 to 1:10⁻³. 100 μL of the resulting solution was then plated on the

**Table 1: Commercial Probiotics used in the study are listed along with their Bacterial Composition.**

| Strain                        | Probiotics | Bacterial Strains from FMT Treated Patients with CDI |
|------------------------------|------------|------------------------------------------------------|
|                              | BK         | BS         | WAL        | CVS        | PC         | 052 | 055 |
| Bifidobacterium breve       | -          | -          | -          | +          | +          | -   | -   |
| Bifidobacterium brevis      | -          | -          | +          | -          | -          | -   | -   |
| Bifidobacterium bifidum     | -          | -          | +          | -          | +          | -   | -   |
| Bifidobacteriumbulgaricus   | -          | -          | -          | +          | -          | -   | -   |
| Bifidobacteriumlactis       | -          | +          | +          | -          | +          | -   | -   |
| Bifidobacteriumlongum       | -          | -          | -          | +          | +          | -   | -   |
| Lactobacillus acidophilus    | +          | +          | +          | +          | -          | -   | -   |
| Lactobacillus casei          | +          | -          | +          | -          | +          | -   | -   |
| Lactobacillus paracasei      | -          | +          | +          | -          | -          | -   | -   |
| Lactobacillus plantarum      | -          | +          | +          | -          | +          | -   | -   |
| Lactobacillus rhamnosus      | +          | -          | +          | +          | -          | -   | -   |
| Lactobacillus salivarius     | -          | -          | +          | +          | -          | -   | -   |
| *Clostridium clostridioforme*| -          | -          | -          | -          | +          | -   | -   |
| Bifidobacterium spp.         | -          | -          | -          | -          | -          | +   | -   |
| Bacteroidesovatus/theta iotaomicron | -          | -          | -          | -          | -          | -   | -   |

**Notes:**

- BK: Bio K Plus; BS: Bio Schwartz; PC: Physician’s Choice; WAL: Walgreens Ultra Strength Probiotic; CVS: CVS Health Maximum Strength Probiotic and Bacterial Stains Obtained from Patients with Recurrent *C. difficile* Infection Treated with Fecal Microbiota Transplantation (FMT).
specific primers (PG48) [4]. PCR was run on strain/probiotic plates that had more than 1 spore growth. If a plate had 8 or fewer spores on it, each spore was tested individually. If any plate had more than 8 spores, then a swab of the whole plate was taken and used as a sample.

Results

The results of toxin neutralization for probiotics are provided in Table 2. Two probiotics inhibited toxin of *C. difficile* strain 43255 at 1:10⁹ P:CD ratio, 052 and 055. Three commercial probiotics inhibited toxin at P:CD ratio of 1:10⁷, BS, WAL and CVS, while one probiotic neutralized toxin at P:CD ratio of 10⁶, BK. PC inhibited toxin at a P:CD ratio of 10⁵.

BS, 055 and CVS inhibited *C. difficile* strain BAA 1805

| Table 2: Neutralization of *C. difficile* Toxin by Probiotics after Combination of Probiotics to *C. difficile* in ratios of 1:10³ to 1:10⁹. |
|---------------------------------------------------------------|
| **Probiotic Mixed with Increase *C. difficile* Strains** | **Ratio (Probiotic / *C. difficile*)** | 1:10³ | 1:10⁴ | 1:10⁵ | 1:10⁶ | 1:10⁷ | 1:10⁹ |
|---------------------------------------------------------------|
| **Studies with *C. difficile* strain 43255** | | | | | | | |
| BK | NTD | NTD | NTD | NTD | TD | TD |
| BS | NTD | TD | NTD | NTD | NTD | TD |
| WAL | NTD | NTD | NTD | NTD | NTD | TD |
| CVS | NTD | NTD | NTD | NTD | NTD | TD |
| PC | NTD | NTD | NTD | TD | TD | TD |
| 052 | NTD | NTD | NTD | NTD | NTD | NTD |
| 055 | NTD | TD | NTD | NTD | NTD | TD |
| *E. coli* (strain HS) Negative Control | TD | TD | TD | TD | TD | TD |
| **Studies with *C. difficile* strain BAA 1805** | | | | | | | |
| BK | TD | TD | TD | TD | NTD | TD |
| BS | TD | TD | NTD | NTD | NTD | NTD |
| WAL | NTD | TD | TD | TD | TD | TD |
| CVS | TD | TD | NTD | NTD | TD | TD |
| PC | TD | NTD | TD | TD | TD | TD |
| 052 | NTD | TD | TD | TD | TD | TD |
| 055 | NTD | NTD | NTD | NTD | NTD | TD |
| *E. coli* (strain HS) Negative Control | TD | TD | TD | TD | TD | TD |

NTD: no toxin detected; TD: Toxin detected.

| Table 3: Spore counts (x 10³) before and after co-incubation of probiotics and bacterial strains with *C. difficile*. |
|---------------------------------------------------------------|
| **Category** | **C. difficile ATCC 43255** | **C. difficile ATCC BAA 1805** |
| Spore Counts | | |
| **C. difficile strains alone** | 86 | TNTC |
| **Ratio Probiotics / *C. difficile*** | BK | BS | WAL | CVS | PC | 052 | 055 | BK | BS | WAL | CVS | PC | 052 | 055 |
| 1:1 | 10 | 1 | 1 | 0 | 10 | 28 | 0 | 1 | 316 | 680 | 18 | 664 | TNTC |
| 1:10⁻¹ | 0 | 0 | 0 | 0 | 0 | 11 | 3 | 0 | 0 | 176 | 7 | 128 | TNTC |
| 1:10⁻² | 0 | 0 | 0 | 3 | 0 | 3 | 5 | 1 | 2 | 1 | 0 | 1 | 14 | 808 |
| 1:10⁻³ | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 64 | 0 | 1 | 0 | 248 | |

TNTC: too numerous to count.
Discussion

In the present study we examined the inhibitory effects on *C. difficile* toxins and spore formation for commonly used probiotics. Also, the inhibitory effects of probiotics on both toxin production and sporulation of *C. difficile* strains differed by probiotics. The strength of the study is using whole licensed probiotic products (whole licensed probiotic products) to determine overall anti-*C. difficile* activity. A number of studies have looked at activity against *C. difficile* using purified bacterial strains looking for effects on inhibition of the organism and its biologic characteristics [5-8]. Mixing multiple strains together as is done for commercial probiotics, may produce additive bioactivity intended but may also have inhibitory effects [9]. We wanted to study the anti-*C. difficile* effects of the probiotic mixtures currently being used by patients.

The number of probiotic strains included in various preparations used in this study varied between three and ten. Having a larger number of probiotic strains in a preparation is no guarantee of improved activity [9]. In the present study, PC (contained 10 different probiotic strains) inhibited toxin production by *C. difficile* strain 43255 less well than two probiotics containing three bacterial strains, BK and BS. Probiotic PC failed to neutralize the toxin of *C. difficile* strain BAA 1805.

Commercial probiotics commonly employ strains within the bacteria genera of *Bifidobacterium*, phylum Firmicutes, and phylum Actinobacteria or *Lactobacillus*. Strains within these two classes of anaerobe strains have been shown to effect *C. difficile* colonization [6], toxigenicity [10], inflammation [11] germination [12] and growth [13,14]. Biologic activity for probiotics has been shown to be related to individual strain, not family or class [15].

We included in this study two anaerobic strains isolated from patients with successful FMT treatment of recurrent CDI, a strain of *Clostridium difficile* and a strain of *Bifidobacterium* possessed the highest level of toxin-neutralization for one of the *C. difficile* strains.

Four of the probiotics used in this study were effective in inhibiting sporulation by *C. difficile* strain 43255, BK, BS, WAL, CVS and PC. For *C. difficile* strain BAA 1805, sporulation was inhibited by two of the commercially available probiotics, BK and BS. The probiotics with greatest levels of inhibition of sporulation of the *C. difficile* strain 43255 were BK, BS, WAL, PC, and CVS with inhibition of a lower level seen for the two purified anaerobic bacteria. For *C. difficile* strain BAA 1805, BK and BS appeared to have the greatest effect. 055 had no obvious anti-sporulation effects. The probiotic mixture with greatest anti-*C. difficile* activity in the study was BS.

We believe that in the future it will be possible to identify strains of intestinal anaerobic bacteria with strong potential for gut engraftment and with important biologic properties that will be harnessed as advanced probiotics for health benefits. The commercially available probiotics currently in use were developed before understanding the composition of the intestinal microbiome [16] and may not be ideal strains to reverse reduced microbiome diversity.

This study demonstrated that commonly employed probiotics in the U.S. and Canada differ in their inhibition of *C. difficile* virulence factors. Also, the anti-toxin effects of probiotics were shown to be dependent upon strain of *C. difficile*. Toxigenicity and sporulation for the so called hypervirulent ribotype 027 strain were more resistant to the effects of study probiotics.

Limitations

One limitation in our study is that we used only two toxigenic strains of *C. difficile* to test the activity of probiotics on the inhibition of *C. difficile* toxin production and sporulation. In the sporulation assays, we only performed the study once and were unable to perform statistical comparisons. We do not know the importance of inhibition of these virulence factors in CDI as predictors of protection in the natural disease. Effects of probiotics on intestinal adhesion and cytoprotection, antimicrobial and anti-inflammatory properties, and engraftment potential may also be important and were not the focus of this investigation. Clinical studies are needed to determine if this in vitro observation allows prediction of success in treating CDI.

With the recognition that *C. difficile* infects people with reduced colonic microbiota diversity, renewed interest in probiotics as a way to prevent infection has emerged [17]. Studies of probiotics in the hospital setting have resulted in mixed results. In one small placebo-controlled study, the use of a probiotic mixture in the treatment of patients with CDI also treated with antibiotics, provided evidence the diarrhea was shortened by the probiotic [18]. In three systematic review of
published studies, probiotics appeared to provide some protection from CDI when given to patients receiving antibiotics [1,19,20]. In a multicenter, double-blind placebo and randomized controlled trial a fungal probiotic, Saccharomyces boulardii did not prevent CDI in hospitalized patients receiving antibiotics [21]. In a retrospective cohort hospital-based study patients receiving intravenous antibiotics who also received a single probiotic (Bio-K+), there was no difference in the hospital associated case rate of CDI [22].

More work is needed to determine the value of probiotics in the treatment and prevention of CDI in high-risk patients. With newer studies of the intestinal microbiome, engraftment of strains, and biologic activity of pure strains we may see the development of newer, more potent probiotic strains and combinations emerge in the future.

Conclusion

Commercial probiotic products exhibited variable degrees of anti- C. difficile activity. In vivo studies are needed to determine the significance of these findings.

Highlights

• Commonly employed probiotics in the U.S. and Canada differ in their inhibition of C. difficile virulence factors.
• Toxigenicity and sporulation for the so called hypervirulent ribotype O27 strain, were more resistant to the effects of study probiotics.
• Activity of single strains of anaerobic Clostridium and Bifidobacterium neutralized C. difficile toxin and are candidates for probiotic development

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Statement of Ethics

UTHHealth Committee for the Protection of Human Subjects HSC-SPH-14-0020.

Disclosure Statement

Authors, Valeria De Las Casas, Sam Miller, Zhi-Dong Jiang and Herbert DuPont have no conflict of interest to report.

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Author Contributions

Valeria De Las Casas: performed assays, manuscript preparation Sam Miller: performed assays, manuscript preparation; Herbert DuPont: study design, data interpretation, manuscript preparation; Zhi-Dong Jiang: study design, data interpretation, manuscript preparation.

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