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ORIGINAL ARTICLE

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 successful 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. We were encouraged by the finding that single strains of bacteria had important anti-CDI activity in vitro. In conclusion, commercial probiotic products exhibited variable degrees of anti-*C. difficile* activity. *In vivo* studies are needed to determine the significance of these findings.

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-

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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 *C. 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 (10^8 CFU/mL). Each probiotic: *C. difficile* solution (P:CD ratio) were mixed at 1:10^3, 1:10^4, 1:10^5, 1:10^6, 1:10^7 and 1:10^9 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 (10^8 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	-	-	+	-	-	-	-
Bifidobacteriumbifidum	-	-	+	-	+	-	-
Bifidobacteriumbulgaricus	-	-	+	-	+	-	-
Bifidobacteriumlactis	-	+	+	-	+	-	-
Bifidobacteriumlongum	-	-	-	+	+	-	-
Lactobacillus acidophilus	+	+	+	+	+	-	-
Lactobacillus casei	+	-	+	-	+	-	-
Lactobacillus paracasei	-	+	+	-	+	-	-
Lactobacillus plantarum	-	+	+	-	+	-	-
Lactobacillus rhamnosus	+	-	+	+	-	-	-
Lactobacillus salivarius	-	-	+	-	+	-	-
Clostridium clostridioforme	-	-	-	-	-	+	-
Bifidobacterium spp.	-	-	-	-	-	-	+
Bacteroidesovatus/ thetaiotaomicron	-	-	-	-	-	-	-

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).

C. difficile plates and incubated at 37 °C under anaerobic conditions for 1 week. After this incubation, bacterial colonies were collected and diluted to 0.5 mL of 0.01 M sterile PBS (7.2 pH) and centrifuged at 5000 g for 10 minutes to wash the sample, this was done twice. Afterwards, the pellet was resuspended in 4 mL of 0.9% saline and heated in a 70 °C water bath for 10 minutes to inactivate vegetative cells. The solution was then diluted from1:1 to $1:10^{-3}$, from which 100 µL was plated on a BHI medium containing D-cylcoserine (250 µg/ml), cefoxitin (8 µg/ml), and 0.1% taurocholate (Fisher Scientific, Federal Way, WA) for 48 hours in the 37 °C incubator. After incubation, the spores were counted and recorded for each plate. The assay was performed once.

Spores identified after co-incubation were confirmed by amplification of the 16S rRNA gene using *C. difficile* 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 $1:10^9$ P:CD ratio, 052 and 055. Three commercial probiotics inhibited toxin at P:CD ratio of $1:10^7$, BS, WAL and CVS, while one probiotic neutralized toxin at P:CD ratio of 10^6, BK. PC inhibited toxin at a P:CD ratio of 10^5.

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 $1:10^3$ to $1:10^9$.

Probiotic Mixed with Increase *C. difficile* Strains	**Ratio (Probiotic / *C. difficile*)**					
	$1:10^3$	**$1:10^4$**	**$1:10^5$**	**$1:10^6$**	**$1:10^7$**	**$1:10^9$**
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	TD	NTD	**NTD**
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 10^3) before and after co-incubation of probiotics and bacterialstrains with *C. difficile*.

Category	***C. difficile* ATCC 43255**							***C. difficile* ATCC BAA 1805**						
***C. difficile* strains alone Spore Counts**	**86**							**TNTC**						
Ratio Probiotics / *C. difficile*	**BK**	**BS**	**WAL**	**CVS**	**PC**	**052**	**055**	**BK**	**BS**	**WAL**	**CVS**	**PC**	**052**	**055**
1:1	1	0	0	1	1	10	28	0	1	316	680	18	664	TNTC
$1:10^{-1}$	0	0	0	0	0	11	3	0	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	0	64	0	1	0	248

TNTC: too numerous to count.

toxin at P:CD ratios of $1:10^9$, $1:10^7$ and $1:10^6$, respectively. The remaining probiotics did not show neutralization at any P:CD ratios. The *E. coli* HS control strain was negative for toxin neutralization for both strains of *C. difficile*.

The results of the sporulation experiment are summarized in Table 3. For *C. difficile* strain 43255, obvious inhibition of sporulation was seen for BK, BS, WAL, CVS and PC. Moderate reduction in spore counts were seen for 052 and 055. For *C. difficile* strain BAA 1805, BK and BS were effective than other probiotics in inhibiting sporulation. PC had moderate anti-sporulation effects and WAL and CVS and 052 showed minimal anti-sporulation effects. 055 had no effects on sporulation for this strain of *C. difficile*.

Identified spores in each study group were confirmed as *C. difficile*-specific by 16S rRNA gene amplification using primer PG48.

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 *C. clostridioforme* 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 43225 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

UTHealth 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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