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Quantitative real time PCR detection of *Clostridium difficile* growth inhibition by probiotic organisms

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Abstract

Background: Probiotic microorganisms are potential treatments for *Clostridium difficile* diarrheal disease (CDD) but better methods are needed to determine the relative potency of probiotic microorganisms against pathogenic organisms in mixed cultures. **Aim:** Quantify *C. difficile* in the presence of putative probiotic organisms using molecular methods to determine relative probiotic potency. **Materials and Methods:** *C. difficile* strains were cultivated anaerobically. Serial dilutions of *Lactobacillus* cultures or microbial mixtures from kefir were co-cultured with *C. difficile* for 48 hours. Bacterial DNA was extracted and qPCR was used to measure *C. difficile* toxin A gene, on the basis of cycle threshold (Ct) number. **Results:** Strains of *Lactobacillus* (human and ATCC derived), and mixed cultures from commercial kefir were co-cultured with *C. difficile*. *Lactobacillus* and the microbial mixture from kefir were ranked in order of their potency in *C. difficile* growth inhibition. **Conclusions:** PCR allows facile quantification of *C. difficle* in the presence of other. The technique measures relative potency of over-the-counter probiotics and may predict human strains meriting probiotic status.

Keywords: Probiotic organisms, *Clostridium difficile*, in vitro testing, qPCR, probotic potency, antibiotic-associated diarrhea, *Lactobacillus sp.*

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Introduction

Increasing attention by both the scientific community and lay public is being directed at the use of probiotic organisms for various indications, some of which are supported by clinical research. Probiotics have been suggested as having benefit in decreasing vaginal infections [1, 2] and in regulating the gut flora to decrease the risk of diarrheal disease [3-5]. Significant concern to clinicians is *Clostridium difficile* diarrheal (CDD) disease. Probiotics and functional foods have been used as one approach to prevention or treatment of symptoms but reviews have indicated a beneficial effect [6] or lack of significant beneficial effect of the probiotic approach [7]. Inconsistency among observed clinical effects of probiotics may be in part related to the variation in actual probiotic preparations. This, coupled with lack of in vitro measures of potency, undermines the ability to aggregate findings from multiple clinical trials [8] and limits the ability to rationally select a probiotic for study prior to costly, time-consuming clinical studies.

Probiotics remain one option for medical treatment or prophylaxis, or possibly as an adjunct to other therapies that have a stronger evidence base [9]. Over-the-counter products purporting to have probiotic activity enjoy brisk and unregulated sales. Hence, the medical profession and consumers alike could benefit from development of methods to ascertain probiotic potential.

In contrast to antibiotic susceptibility tests with pure cultures, in vitro evaluation of probiotic activity requires

some form of co-culture of the probiotic organism (or mixture of probiotic organisms) with a target organisms (or mixture of organisms) that are intended to be controlled by the probiotic. Determining the population dynamics of individual organisms in co-culture is methodologically challenging. In this study we have investigated a technique that pairs a probiotic with cultures of *C. difficile*. This pathogenic target organism has clinical importance because of the persistent and increasing problems with *C. difficile* diarrhea (CDD) which typically occurs after antibiotic therapy. While vancomycin and metronidazole are typically employed in cases of CDD, these disorders are often recrudescent after therapy [10] and treatment might be augmented by alternative non-antibiotic strategies.

Our hypothesis is that quantitative PCR may be used to enumerate *C. difficle* in the presence of putative probiotic organisms to determine the relative probiotic potency of these non-clostridial microorganisms.

To support this hypothesis, we report here the development of a specific real time PCR method for *C. difficile* quantitation and demonstrated its ability to provide information on the relative growth inhibiting effects of putative probiotic organisms on *C. difficile* strains of human origin.

Materials and Methods

Microbial strains: *C. difficile* positive specimens from the Mercy Medical Center (Des Moines, IA) clinical microbiology laboratory were identified by EIA toxin testing (Meridian Diagnostics, Cincinnati, OH). The organisms were purified by propagation in thioglycollate broth (Oxoid, Basingstoke, Hampshire, UK) for 72 hours at 37° C followed by heat shock at 75 ° C for 15 minutes. Purity of the culture was checked by growing heat-shocked cultures on CCFA (cycloserine-cefoxitin-fructose agar) medium supplemented with horse blood (Remel, Lenexa KS). Cultures were maintained throughout the study by subculture in chopped meat glucose medium (Difco/Becton-Dickenson, Sparks, MD). In addition to clinical isolates of *C. difficile*, ATCC strain 9689 was used as a reference and maintained the culture as for the clinical isolates.

Potential probiotic organisms included two human strains of *Lactobacillus* which were provided by Dr. Lin Tao, University of Illinois Chicago College of Dentistry. These were unspeciated and were designated as strains "A" and "C". Two known strains of *Lactobacillus* were obtained from ATCC (*L. rhamnosis* ATCC 53103 and *L. reuteri* ATCC 53609). Lactobacilli were maintained on MRS agar (Difco, Sparks, MD) and were cultivated anaerobically in MRS broth prior to use in challenge experiments. In addition, organisms were subcultured from commercial kefir (Lifeway, Morton Grove, IL) which lists 10 live cultures among its ingredients. These include 5 species of *Lactobacillus* (*casei, lactis, acidophilus, plantarum*, and *rhamnosus*),2 Saccharomyces species (*diaceylactis* and *florentinus*), 2 *Bifidobacterium* species (*longum* and *breve*) and *Leuconostoc cremoris*. Chopped meat glucose medium was used to cultivate kefir organisms, and these were recovered the liquid phase of this medium by centrifugation at 12,000 x g for 3 minutes and re-suspended in MRS broth prior to use in challenge experiments.

Challenge experiments: Lactobacillus overnight cultures were diluted 10, 100 or 1000- fold in fresh MRS broth and 900 µl placed in a sterile tube and combined with 100 µl of a fresh *C. difficile* culture (diluted 1:100) to create co-cultures of various combinations of *C. difficile* and *Lactobacillus*. These co-cultures were incubated anaerobically (GasPak Anaerobic System, Becton-Dickenson, Sparks, MD) at 37 ° C for 48 hours. An aliquot of the *C. difficile* inoculum was saved for determination of baseline qPCR signal from the inoculum for comparison to results from the experimental cultures.

DNA preparation: At the conclusion of the challenge experiment, a 100 µl aliquot from each co-culture was removed, and bacteria were recovered by centrifugation at 12,000 x g followed by a distilled water wash and centrifugation. After removal of the supernatant fluid 20 µl of Lyse-and-Go® reagent (Pierce, Chicago Ill) was added and the mixture heated according to manufacturers directions to release DNA. The mixture was centrifuged and 1 µl of the supernatant fluid was used as target DNA for PCR analysis.

PCR detection of C. difficile: Primers were synthesized for both Toxins A and B (IDT DNA, Coralville, IA) and were used to develop a detection system for the pathogen. Toxin-specific primer sequences were obtained from the literature [11] and are listed in Table 1. 1 µl each of the forward and reverse primers (each at 50 picomoles/µl) and the target DNA directly from the Lyse-And-Go reaction were combined with 12.5µl TaKaRa xTaq version (Premix Taq, Takara Bio Inc., Otsushiga, Japan) and sufficient nuclease free water to produce a 25 µl reaction mixture. PCR was carried out according to the following program: 4 min 95 ° C, followed by 35 cycles of 95 ° C for 30 seconds, 48 ° C for 30 seconds (for Toxin A) or 45° C (for Toxin B), 72 ° C for 1 minute with a final 4 min extension step at 72 ° C. Products from conventional PCR were detected on 1% agarose gels stained with ethidium bromide.

Quantitative PCR: 1 µl of target DNA was added to 12.5 µl of Sybr Green master mix (iQ SYBR Green Supermix, BioRad Laboratories, Hercules, CA) and 50 picomoles each of forward and reverse primers added along with a sufficient volume of nuclease free water to make a 25 µl reaction mix. Quantitative PCR employed the BioRad Chromo4 instrument and proceeded with a 94 ° C melt step for 4 minutes, and then 44 cycles of 94 ° C for 20 seconds, 45 ° C for 20 seconds, 72 ° C for 30 seconds with green fluorescence read at the end of each cycle. A melt curve was performed at the end of each PCR run.

Table 1 PCR Primers for *C. difficile* toxin genes [11]

Gene	Forward Primer Sequence	Reverse Primer Sequence	Product Size
Toxin A	5' TCT ACC ACT GAA GCA TTA C 3'	5' TAG GTA CTG TAG GTT TAT TG 3'	158 bp
Toxin B	5' ATA TCA GAC ACT GAT GAG 3'	5' TAG CAT ATT CAG AGA ATA TTG T 3'	108 bp

Evaluation: The relative abundance of *C. difficile* toxin A gene copies was related to the cycle threshold (Ct) which is the number of PCR cycles needed to obtain a detectable fluorescent signal above background and typically was the point on the amplification curve where the curve became linear. Ct was experimentally confirmed to be related to copy number of *C. difficile* by amplifying a series of serial 10-fold dilutions of organism DNA. Specificity was established by showing *Lactobacillus* DNA did not amplify or interfere with the qPCR estimation of *C. difficile*.

For comparison of the relative concentration of *C. difficile* in various co-cultures, the Ct value of the experimental culture was subtracted from the Ct of the control culture to establish a Ct relative to the control. A Ct $\geq$ 2 was considered to be indicative of inhibition as replicate co-cultures typically had standard deviations for Ct values less than 2 cycles.

Statistical evaluation: Most experiments were repeated at least twice and allowing Ct values to be averaged and standard deviations calculated. These data were compared by t-test. Guided by results, we tabulated the number of *C. difficile* strains inhibited by each *Lactobacillus* strain and made comparisons of the relative potency in terms of the number of strains inhibited based on Ct. The number of C. difficile strains inhibited by each *Lactobacillus* was compared by Chi-square test.

Results

Validation of Technique

The initial goal of this research was to establish the details of a molecular approach to quantitative detection of *C. difficile* in the presence of other organisms. A total of 9 *C. difficile* isolates (8 of which were clinical isolates) were tested with conventional PCR for the presence of Toxin A and Toxin B genes. This evaluation demonstrated that Toxin A was reliably amplified in all organisms which suggested this would be an appropriate gene target for estimating the relative abundance of *C difficile* in a culture.

To be a valid method for co-culture experiments, it was necessary to prove that the presence of DNA from microorganisms other than *C. difficile* did not interfere with its detection. As shown in Figure 1, *C. difficile* in the presence of *Lactobacillus* successfully amplified, but no product with *Lactobacillus* alone. The significance of this finding was confirmation of the specificity of the amplification.

The utility of the Toxin A primer set with organisms to be used in this study were adapted for real time quantitative PCR. When *C. difficile* DNA was amplified using toxin A and SYBR green detection, a sigmoid-shaped amplification curve with a single sharp the melt curve peak at 74.5^{o} C was obtained. In contrast, the same test performed with *Lactobacillus* DNA showed no amplification product (data not shown). This finding confirmed the potential utility of qPCR for co-culture experiments.

The final validation experiment involved demonstrating the linearity of quantitation of *C. difficile* DNA. ATCC *C. difficile* strain 9689 was grown in thioglycollate broth to stationary phase and DNA preparations from serial ten-fold dilutions through $1x10^{-6}$ were amplified with Toxin A primers and the results plotted with Ct versus dilution. The undiluted culture had a Ct=14.79 cycles (average of 2 runs) and the highest dilution had a Ct of 29.77 (average of 2 runs) cycles with an r^2 value for the standard curve of 0.994.

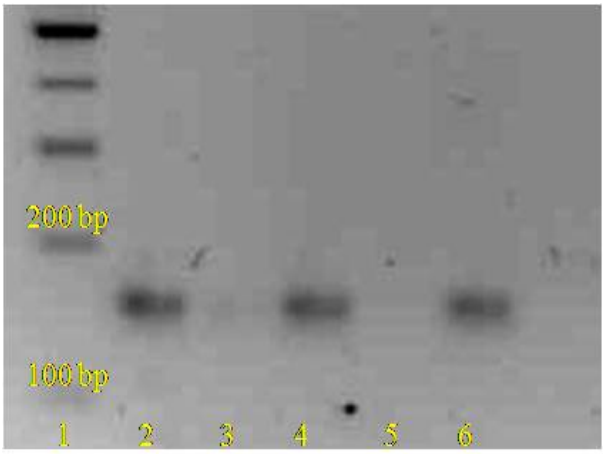

Fig. 1 Electrophoresis of conventional PCR amplification products using Toxin A primer set and genomic target DNA from *C. difficile* ATCC 9689 alone (lane 2), *Lactobacillus reuteri* ATCC 53609 alone (lane 3), *C. difficile* plus *L. reuteri* (lane 4), *L. rhamnosus* ATCC 53103 alone (lane 5) and *C. difficile* plus *L rhamnosus* (lane 6). Molecular weight standards are in lane 1 and the correct Toxin A product appears at 158 bp. The presence of a second organism in the presence of *C. difficile* did not inhibit the PCR reaction.

Ct Values

The relative abundance of *C. difficile* toxin A gene copies, used as a surrogate marker for the number of *C. difficile* genomes in the sample, was related to the cycle threshold (Ct). Ct is the number of PCR cycles needed to obtain a detectable fluorescent signal above background. A preliminary co-culture study combined a $1x10^{-4}$ (final) dilution of *C. difficile* (ATCC 9689) with a 1:10, 1:100 and 1:1000 fold dilutions of *Lactobacillus* strain A. The Ct values resulting from 48 hours of co-culture are shown in Figure 2. Growth of *C. difficile* in the control culture was reflected by the difference between the Ct for the inoculum (Ct=17) and the same culture at the end of the incubation period (Ct=8). The cultures containing *Lactobacillus* had Ct values that indicated inhibition and a gradation of effect with changing *Lactobacillus*

concentrations. These observations indicated that the co-culture experiments were biologically plausible and were able to detect inhibition of *C. difficile*. In this experiment we found that *C. difficile* from inoculation to study end was significantly different ($p=2x10^{-6}$ by two tailed t-test) and *Lactobacillus* at 1:10 and 1:100 dilutions co-cultured with *C. difficile* provided significantly higher Ct values than *C. difficile* alone ($p=0.027$ and $p= 0.0025$ respectively by two tailed t-test) which was interpreted as inhibition of *C. difficile* by the inhibitory probiotic organism. The significance of this finding was that the method proved satisfactory in demonstrating probiotic effects and could also detect gradations in the potency of the effect.

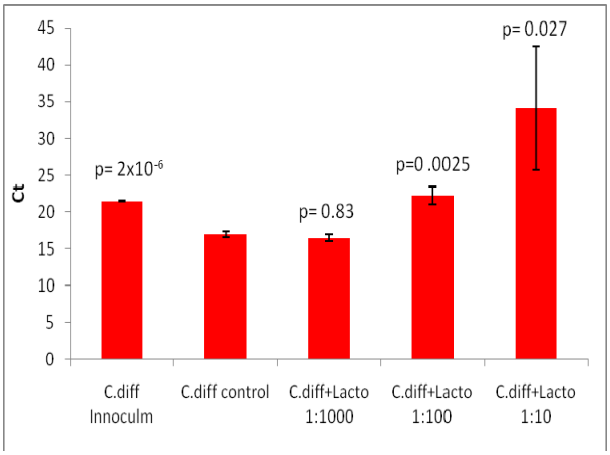

Fig. 2 The ability of *Lactobacillus* strain A to exert a probiotic effect on *C. difficile* (ATCC 9689) by means of qPCR is illustrated. Bars indicate the average Ct for duplicate PCR reactions and error bars represent the standard deviation. Shorter bars indicate lower Ct values and represent larger amounts of specific bacterial DNA in the reaction mixture. The full growth potential of *C. difficile* is illustrated by the first two bars and the difference proved significant by two-tailed t test. While the amount of *C. difficile* from co-culture with *Lactobacillus* at a 1:1000 dilution was identical to the *C. difficile* cultured alone (suggesting no inhibition), the other two *Lactobacillus* dilutions were significantly inhibited (t-test versus *C. difficile* alone).

Relative potency of probiotic organisms vs. C. difficile

The relative probiotic potency of the various *Lactobacillus* strains available for co-culture experiments was undertaken with proportions of each organism suggested by the initial experiment. Thus, *Lactobacillus* was used at a dilution of $1x10^{-2}$ versus *C. difficile* at a dilution of $1x10^{-4}$. Results compared the Ct of the co-cultures and Ct of the uninhibited control and several iterations allowed the reporting of average Ct values for replicate experiments with a CT which reflecting the degree of inhibition.

Figure 3 shows the CT for each of the *C. difficile* isolates in relationship to 5 probiotic strains or probiotic products. If we considered CT values ≥ 2 which would represent a four-fold difference in *C. difficile* DNA as indicative of inhibition, the percent of *C. difficile* strains inhibited is ranged from a low of 44% for *L. rhamnosus* to 89% for the kefir organisms. The importance of this finding is in its ability to help in the prediction of which probiotic organisms or combination might be best for selection in future clinical trials.

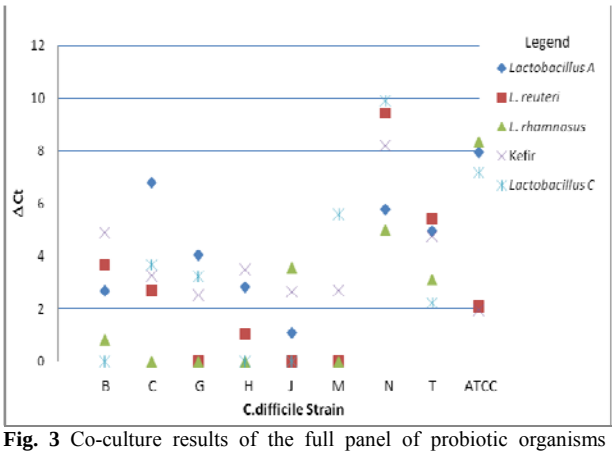

Fig. 3 Co-culture results of the full panel of probiotic organisms or mixtures of organisms (from kefir) indicated by different symbols, against the full panel of 9 strains of *C. difficile* indicated by labels on the x axis. In this graph, inhibition is illustrated by Ct (y axis) which means the difference between the Ct value for qPCR of *C. difficile* alone versus *C. difficile* cultivated in the presence of the probiotic organism(s). Ct values appearing on the baseline indicate that *C. difficile* alone showed equal or lower Ct than that obtained from *C. difficile* co-cultured with the indicated probiotic organism(s) and as Ct values above 2 were considered indicative of a probiotic effect, virtually all potential probiotic organisms showed some activity against *C. difficile.*

Discussion

The literature portrays CDD as a persistent problem and physicians working primarily in hospitals and nursing facilities are well acquainted with the both the disease and its tendency for recrudescence. In addition, the spectrum of disease has expanded to more community acquired cases [12, 13]. The scope of disease and potential for disastrous outcomes has kindled interest in therapies beyond antibiotic treatments. Among the alternative therapies that have received significant attention is use of probiotic microorganisms, functional foods containing probiotic organisms or prebiotics which support colonization by a favorable flora. Well-controlled clinical studies are limited and have failed to provide support for the probiotic approach to CDD [7] but interest in probiotic treatments remains and further clinical studies can be anticipated, especially in parts of the world where medical resources are limiting factors.

The present study was engendered by the recognition that probiotic preparations are variable in their composition and presumably in their relative potency. Apparent probiotic potency may be related to the condition being treated, and even when a probiotic organism is identified by genus and species, different strains of the same organism may differ in potency from one to another and there is no *a priori* means of determining how a probiotic might perform. However, if one scans the internet for products being marketed directly to consumers, it is apparent that the public is faced with strong claims with medical overtones and a dizzying array of products and there is no means of independently addressing an industry largely based on anecdote and testimonials.

We hypothesized that molecular methods could be

exploited in determining the effect of potential probiotic organisms and we successfully devised a real time quantitative PCR method that enumerated the relative number of *C. difficle* genomes even in the presence of an abundance of probiotic organisms. The significance of this finding resides in our ability to do probiotic experiments without having to perform quantitative culture with difficult to handle anaerobic microorganisms.

We applied this technique to a panel of human and one ATCC isolates of *C. difficile* challenged with several *Lactobacillus* and food probiotics. The result was an ability to rank the inhibitory potential of the organisms and at the same time to take into account the diversity not only of the probiotic organisms, but the diversity in the relative susceptibility of the *C. difficle*.

In mixed cultures of a probiotic organism with *C. difficile*, we were able to successfully and specifically amplify the pathogen for the purpose of quantitation, in the presence of the inhibiting microorganism. In addition, it was demonstrated that different clinical isolates of the pathogen of interest displayed varying degrees of susceptibility to a panel of potential probiotics. This emphasizes the importance of experimentally evaluating multiple strains of pathogens as they do not have uniform response to inhibitory microorganisms.

A potential limitation of this technique was focusing our measurement of bacteria in mixed culture *to C. difficile* and not quantitating the probiotic organism. However, we did control the quantity of the *Lactobacillus* or other probiotic mixtures through dilution and this technique was sufficient to indicate the necessity of numerical dominance of the probiotic organism. This finding should emphasize that in clinical studies, efficacy of a probiotic should take into account the dose of the probiotic and whether it is viable or not, and whether it survives in the host. The qPCR method employed here could, with the addition of reactions to quantitate the probiotic organisms, show the relative abundance of all players in the mixed cultures evaluated.

In terms of the apparent probiotic activity of the organisms tested, it was interesting that the organisms present in the kefir fermented milk product appeared most potent, with a *Lactobacillus* of human origin almost as potent. The kefir inoculum was a mixture of bacteria and yeast, whereas the *Lactobacillus* A was a single species. While it is tempting to assume a cocktail of organisms may synergistically inhibit *C. difficile*, the ability of a single species to show similar potency is an important finding, since using a single species of bacterium as a probiotic treatment would be easier to control and monitor than a complex microbial mixture.

In vitro testing of potential probiotic organisms or mixtures of organisms could be helpful in selecting the most potent and most relevant organisms for clinical evaluation of probiotics. In addition, in vitro potency testing could be useful in evaluating products marketed directly to the public, some of which have on occasion proven to have low viability or contain species not listed on the labeling [14]. The biological activity of probiotic candidate organisms or mixtures of organisms has required challenging laboratory techniques such as embedding pathogens in agar-based growth medium and placing potential probiotics on the medium to look for zones of inhibition. Mixed cultures are attractive as they put probiotics and pathogens in immediate contact without inhibitory mediators having to diffuse through semi-solid media, but identifying one organism in the presence of another in such mixed-culture experiments can prove technically challenging. These issues were overcome in the present study by the use of a molecular approach based on specific quantitative PCR of the *C. difficile*.

Conclusion

A new molecular technique for measuring relative potency of over-the-counter probiotics was applied to inhibition of *Clostridium difficle*. The technique is able to rank order putative probiotic organisms in terms of inhibitory efficacy against the *C. difficile* isolates in our panel. We conclude that this method could be a prelude to selecting probiotic products for clinical testing in future clinical trials.

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