Project Report: **Inhibition of *C. difficile***
A study of the effects of probiotics and various antibody-rich sources

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**Background, Purpose and Hypotheses:**

*Clostridium difficile* is an anaerobic, Gram positive, spore forming, commensal bacterium found in the gut. However, antibiotic therapy may alter the balance of the gut's natural microflora, creating conditions where *C. difficile* can flourish and release toxins, causing potentially serious diseases such as diarrhea or pseudo-membranous colitis. *Clostridium difficile* Associated Disease (CDAD) is normally treated by the discontinuation of antibiotic therapy when possible, or the alternative use of vancomycin or metronidazole. We investigated several possible prophylactic treatments designed to reduce the occurrence of CDAD, including probiotics (commercially available health food products including live *Lactobacillus acidophilus* cultures - a beneficial bacterium usually found in the gut) and non-specific sources of antibodies, which could potentially bind either the bacterium or its toxins, thus preventing disease.

Our hypotheses were: 1) Fresh bovine colostrum (the first milk of mammals; a non-specific antibody source) would inhibit both bacterial growth and toxins in a dose dependent manner. 2) Commercial colostrum would have similar effects, but not to the same degree as fresh colostrum. 3) Milk and canine serum would show little to no effect on bacterial growth or toxins. 4) Probiotics would inhibit bacterial growth, but would not neutralize toxins. 5) Results for probiotic-related products would be similar to results for probiotics, but of lower magnitude. 6) All positive results would vary in a dose-dependent manner.

**Procedures:**

We began experimentation by determining the actual bacterial content of the probiotics we chose to investigate by culturing on MRS media and incubating them for 24 hours at 36 degrees Celsius in an anaerobic chamber. A toxin A negative/ toxin B negative isolate of *C. difficile* was located and subcultured every 48 hours. Experimentation was conducted using this non-pathogenic isolate.

The sensitivity of *C. difficile* to probiotics and antibodies was tested using the following procedure: Using a standardized method, bacterial lawns were grown on blood agar plates. A 6 mm sterile skin biopsy punch was used to make three evenly spaced wells in each agar plate. The wells were filled with concentrations of 1, 1/10, and 1/100 of each treatment method and the plates were incubated anaerobically for 48 hours at 36 degrees Celsius. Zones of inhibition (i.e. no bacterial growth) were measured in millimeters.

To determine the effect of each treatment method on toxins produced by *C. difficile,* a dose concentration of 1 was incubated with an equal volume of a previously collected pure toxin solution containing toxin A and toxin B. This solution was prepared for us by the laboratory, and was made by growing a toxin A/ toxin B positive isolate and washing the plate with sterile saline. The wash was filtered with a Millipore filter to remove bacteria. The toxin solution was mixed 50:50 with each treatment and incubated for 15 minutes. This mixture was then tested with an enzyme-linked immunosorbent assay (ELISA) for the detection of *C. difficile* toxin A and B. Reactions were scored as either positive or negative. A negative test indicated that toxin A and/or B was no longer

able to react in the ELISA assay. We interpreted this to indicate that the toxin molecule was altered and could no longer bind to cells.

**Results/Observations and Conclusions:**

Commercial probiotics available in health food stores are of variable quality. Two of the four products we tested, which claimed to have over two billion active cells per dose, showed no bacterial growth when cultured. The remaining two of four probiotics appeared to have no significant value in the treatment of *C. difficile*. Although bacterial content in products was verified, products inhibited neither bacterial growth nor toxins *in vitro*.

Contrary to our hypothesis, natural colostrum had no effect on either bacterial growth or toxin levels *in vitro*. As we hypothesized, commercial colostrum had an intermediate effect in treatment. It had no effect on bacterial growth, but proved to have an inhibiting effect on toxins. Both milk and serum, as we hypothesized, had no effect on either bacterial growth or toxin levels.

Contrary to our hypothesis, probiotic related products did have significant positive treatment effects *in vitro*. Dyna-ProTM, which is advertised as a biological equine and canine food additive designed to maximize digestive efficiency, caused bacterial inhibition but had no effect on toxins. Ration PlusTM, a product advertised to restore the digestive system by ensuring a healthy environment for the growth of beneficial microflora, showed the most remarkable results. It induced bacterial inhibition in a dose dependant manner, and neutralized toxins completely. After a control test was performed using an acid of similar pH, we were able to show that low pH levels in these

products were not contributing to the effects of this treatment. These results are significant because they suggest that there are potential alternatives to minimize the risk of *C. difficile* colonization and disease.

Ration Plus TM should be studied further due to its inhibiting effects on both bacterial growth and toxins. In addition, Ration Plus TM had positive outcomes at concentrations of lower than 1 on bacterial inhibition, and therefore would be a more likely candidate for further studies and possible clinical use as prophylactic treatment.

Although commercial colostrum showed more effect than the fresh/frozen colostrums we used during experimentation, we are skeptical that these results are entirely valid. The commercial colostrum we used is made from a large pool of colostrum, taken from many cows. This means that the level of different antibodies it contains is much more diverse than the level of different antibodies contained in the colostrum collected from a single cow. If the colostrum we used in our experiment came from a cow that had not been exposed to *C. difficile*, the cow would have not made antibodies against it, and it's colostrum would therefore provide no protection. We feel that results concerning fresh colostrum could vary greatly should we repeat the experimentation using fresh colostrum from different cows.

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