ADDENDUM
COLUMN E EXPLANATION (2 Protocols)

1. **Registration Number:** 74-R-0075

2. **Number of animals used in this study:** Protocol #1 = 21; Protocol #2 = 14

3. **Species (common name) of animals used in Protocol #1 and #2:** Hamsters

4. **Explain the procedure producing pain and/or distress:**

   a. **Protocol #1:**

Overview: The protocol is designed to evaluate the effectiveness of new antibiotic molecules for the treatment of *Clostridium difficile* infections (CDI, toxic megacolon). The primary basis for determining an effective compound is that it will lengthen the survival time of hamsters when compared to the control group (described below).

Control Animal Group: In these experiments, the Control group is defined as subjects receiving the infectious dose of *C. difficile* and is not given the test drugs or analgesics. The control group is critical to the interpretation of the test data i.e. the statistical evaluation of the test data relies solely on what happens in the control group. To ensure the validity of the data, both the test groups and control groups are treated in exactly the same manner, except for the use of antibiotic test drug in the test group. Therefore, since analgesics are not given to the test group, analgesics are also not given to the control group; this ensures that both the test group and control group are evaluated under the same conditions with the only variable being the test antibiotic used in test group. Because of this approach in standardizing the control group to the test group, (1) control group animals are only included when parameters change (instead of each time samples are tested) and (2) the overall numbers of animals in the control group is drastically reduced.

Procedures Producing Pain: In this model, pain results from the establishment of a gastrointestinal infection in the hamster, following administering an infectious dose to the animal.

   b. **Protocol #2:**

Overview: The protocol is designed to evaluate the effects of mutations in key enzymes in *Clostridium difficile* on how this organism causes disease i.e. its pathogenesis in causing diarrhea. The primary basis for determining that the mutation affects the pathogenesis of the bacterium is as follows: Animals infected with a mutant that is less pathogenic will survive for a significantly longer period than animals infected with the wild type (un-mutated virulent bacterium).

Control and Test Animal Groups: In these experiments, the groups receive either mutant bacteria or wild-type bacteria (control group). After infecting the hamsters with either test or control bacteria, the survival of the hamster is measured over a period of days. To ensure the validity of the data, both the test groups and control groups are treated in exactly the same manner and the results

analyzed for statistical difference. Analgesics are not given to either group as this may alter the course of infection and yield false results. Since this is a short term project, we do not anticipate having to repeat experiments with the control group, which ensures that the overall number of animals used are lowered. Secondly, we only put into animals unique mutants and ensure we do not reproduce data for the same phenotype. Only when parameters change would we do further tests on either group. At present this is not the case as these experiments are still being established, but our goal is to reduce animal numbers.

Procedures Producing Pain: In this model, pain results from the establishment of a gastrointestinal infection in the hamster, following administering an infectious dose to the animal.

5. **Provide scientific justification why pain and/or distress could not be relieved. State methods or means used to determine that pain and/or distress relief would interfere with test results.**

    a. **Protocol #1:**

Justification for not administering analgesics:

Administration of analgesics may alter the course of infection leading to an increase in survival of hamsters in the control group, or conversely cause a decrease in survival; which would severely affect the statistical interpretation of test results, since the quality of control group data is critical to evaluating the results of the test group as mentioned above (*1-5*). The effect of analgesics may occur through a potential delay of gastric clearance, which could retain and increase the levels of toxin load in gut, thereby increasing disease severity and likely a faster time to death (*6-10*). According to White et al. (*6*) pain management is challenging in patients with toxic megacolon because non-steroidal anti-inflammatory drugs may exacerbate bleeding; further, opioids may also adversely affect bowel peristalsis causing an increased risk of colonic perforation. Therefore, the use of analgesics to relieve pain may adversely affect these experiments. Although, we have not found there to be use of analgesics in the hamster model of CDI, from the literature (Jan 11th 2012; Ref. 1-5 as examples), the main reasons for not using analgesics in our experiments are described above. Should analgesics be used in the control group, it would also be scientifically required that they also be used in the test group to mitigate differences between the two groups. However, since different drug compounds are evaluated in the study, there is the potential for analgesics to affect their activity or pharmacokinetic (PK) properties, which affects the outcome of the experiment and generate false data. Further, the use of analgesics would place a greater demand for number of animals, that the control group must be run each time new antibiotic molecules are tested so as to encompass possible PK disruptions, which is unlike the current practice stated above (i.e. our approach above reduces numbers, whilst the inclusion of analgesics may increase the number requirement over time).

References:

1. Douce, G. & Goulding, D. Refinement of the hamster model of Clostridium difficile disease. *Methods in molecular biology (Clifton, N.J* **646**, 215-227 (2010).
2. Bartlett, J.G., Onderdonk, A.B., Cisneros, R.L. & Kasper, D.L. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. *J Infect Dis.* **136**, 701-705 (1977).
3. Anton, P.M., *et al.* Rifalazil treats and prevents relapse of clostridium difficile-associated diarrhea in hamsters. *Antimicrob Agents Chemother* **48**, 3975-3979 (2004).
4. McVay, C.S. & Rolfe, R.D. In vitro and in vivo activities of nitazoxanide against Clostridium difficile. *Antimicrob Agents Chemother* **44**, 2254-2258 (2000).
5. Kokkotou, E., *et al.* Comparative efficacies of rifaximin and vancomycin for treatment of Clostridium difficile-associated diarrhea and prevention of disease recurrence in hamsters. *Antimicrob Agents Chemother* **52**, 1121-1126 (2008).
6. White M et al. Pain management in fulminating ulcerative colitis. Paediatr Anaesth. 2006 Nov;16(11):1148-52.
7. Herbert MK et al. Peristalsis in the Guinea pig small intestine in vitro is impaired by acetaminophen but not aspirin and dipyrone. Anesth Analg. 2005 Jan;100(1):120-7.
8. Fekety R. Guidelines for the diagnosis and management of *Clostridium difficile* associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol*. 1997;92:739-50.
9. Walley T, Milson D. Loperamide-related toxic megacolon in *Clostridium difficile* colitis. *Postgrad Med J*. 1990;66:582.
10. Vinagre, AM. Effect of 4-aminoantipyrine on gastric compliance and liquid emptying in rats. Braz J Med Biol Res. 2007 Jul;40(7):903-9.

b. Protocol #2:

Justification for not administering analgesics:

Administration of analgesics may alter the course of infection leading to an increase in survival of hamsters in the control group, or conversely cause a decrease in survival; which would severely affect the statistical interpretation of test results, since the quality of control group data is critical to evaluating the results of the test group as mentioned above (*1-5*). The effect of analgesics may occur through a potential delay of gastric clearance, which could retain and increase the levels of toxin load in gut, thereby increasing disease severity and likely a faster time to death (*6-10*). According to White et al. (*6*) pain management is challenging in patients with toxic megacolon because non-steroidal anti-inflammatory drugs may exacerbate bleeding; further, opioids may also adversely affect bowel peristalsis causing an increased risk of colonic perforation. Therefore, the use of analgesics to relieve pain may adversely affect these experiments. Although, we have not found there to be use of analgesics in the hamster model of CDI, from the literature (Jan 11th 2012; Ref. 1-5 as examples), the main reasons for not using analgesics in our experiments are described above. The effect of analgesics on the microbiology or pathogenesis of *C. difficile* is largely unknown and the potential for obtaining false data due to analgesics is unknown. Further, the use of analgesics would place a greater demand for increasing animal numbers, which is counter to our goal.

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References:

1. Douce, G. & Goulding, D. Refinement of the hamster model of Clostridium difficile disease. *Methods in molecular biology (Clifton, N.J* **646**, 215-227 (2010).
2. Bartlett, J.G., Onderdonk, A.B., Cisneros, R.L. & Kasper, D.L. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. *J Infect Dis.* **136**, 701-705 (1977).
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