

BIOAG PROJECT FINAL REPORT

TITLE: Harnessing microcins for control of scours in neonatal calves

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ABSTRACT:

Calf diarrhea (scours) is a major animal health challenge for cattle producers because it causes dehydration, depression, mortality, and long-term reduction in vitality of infected animals. Enterotoxigenic *E. coli* (ETEC; K99) and enterohemorrhagic *E. coli* (EHEC; e.g., serovars O157:H7, O26 and O111) can cause enteric colibacillosis in calves, and EHEC strains are also significant food safety pathogens. We have discovered a novel microcin that is capable of killing both ETEC and EHEC *E. coli*. In this project, which directly addresses the BIOAg “Livestock and Animal Health” priority for FY12, we employed a neonatal calf model to determine if inoculation with microcin producing *E. coli* can inhibit colonization by susceptible strains of *E. coli* including *E. coli* O157:H7. Twenty-four to 72 hr old calves were given oral doses of a microcin-producing strain (E-25) simultaneously with a susceptible strain. The latter included a microcin knockout strain (E-25 background), an antibiotic susceptible commensal strain of *E. coli*, or a strain of *E. coli* O157:H7. A competition index was calculated at six days post-inoculation to compare the number of microcin-producing bacteria with the number of susceptible bacteria in the feces. The microcin-producing strain had a significantly higher competition index ($ci = 0.04$; $n = 8$ calves) compared with the susceptible strains ($ci = -0.22$; $n = 5$ calves) ($P=0.039$). Analysis at time of necropsy demonstrated that E-25 strain colonizes the rectal-anal junction of the calves, which means that it is likely to be in close proximity of EHEC bacteria like O157:H7. Out of 29 purchased calves, the majority could not be used because of health problems or because they harbored inherently high backgrounds of antibiotic resistant *E. coli*. This latter challenge was partially circumvented when we generated a new strain with an alternative antibiotic resistance marker. We also discovered that calves need to be inoculated within 24-48 hrs of birth or the inoculated strains could not compete with established flora. Our results demonstrate an *in vivo* selective advantage for the microcin-producing strain, although we have concluded that a simple oral inoculation delivery model is likely to be insufficient to control pathogenic bacteria. In the spring of 2013 we developed a method to screen the genome of a susceptible strain and identified a putative membrane bound receptor that we hypothesize is required for the microcin to gain entry to the periplasm. Work continues towards development of microcin-based product to prevent or treat ETEC-based disease in livestock.

PROJECT DESCRIPTION

Calf scours (diarrheal disease) causes significant financial losses to dairy and cow-calf producers. Between 1991-2000 beef and dairy producers reported that 19.8% to 26% of calf losses were attributable to scours and other digestive system disorders and 52.2% of pre-weaned heifer calf deaths have been attributed to scours (as perceived by herd managers). The etiology of scours ranges from viruses to bacteria and protozoa. *E. coli* is the most important cause of bacterial scours in young calves with enterotoxigenic *E. coli* (ETEC; F5 or K99) contributing to spontaneous diarrheal disease during the first week and enterohemorrhagic *E. coli* (EHEC; including O-serotypes O157, O26, and O111) potentially contributing to disease in the first two months of life. The EHEC strains also have zoonotic potential.

Regardless of the etiology, treatment of scours involves rehydration therapy with oral or intravenous administration of electrolytes. In severe cases sepsis can ensue and requires treatment using antibiotics. Consequently, prevention of the *E. coli* scours would offer significant savings in terms of effort required for care of sick animals and the cost of mortalities. If this can be accomplished using non-chemotherapeutic practices, it would be the ideal economic and animal welfare solution that would simultaneously help reduce antibiotic use on farms while improving agricultural sustainability.

Probiotics offer one alternative means to control diarrheal disease including a study that showed some promise of probiotic activity against EHEC O111 and O157 (but not O26). The mechanisms responsible for probiotic activity include competitive exclusion, production of antibiotics or other toxins, and immunomodulation. We discovered a commensal *E. coli* strain (SSuT-25; also called E-25) that inhibited *E. coli* O157:H7 and *E. coli* K99 by a “proximity-dependent inhibition” (PDI) mechanism. Using flow cytometry we recently demonstrated that E-25 inhibits *E. coli* O157:H7 by a killing mechanism and we discovered that E-25 produces a toxin (called a microcin) that is responsible for this killing phenotype (ms in prep). The microcin that we discovered is plasmid-borne and in a recent experiment it was effective against every tested strain of O157:H7 (n=17), O26 (n=4), O111 (n=2) and ETEC K99 (n=2) with the mean reduction in cfu ranging from 3.9 to 9.2 log cfu. Importantly, we know from previous studies that administration of the cattle derived E-25 strain has no negative consequences for neonatal calves. Consequently, this novel microcin appears to be an ideal candidate for limiting EHEC and ETEC infections in calves.

The objectives of the current study were to:

(1) Determine if inoculation with microcin producing *E. coli* can inhibit colonization of neonatal dairy calves by *E. coli* O157:H7. Animals were euthanized at the end of each trial so we can map the distribution of the different *E. coli* strains throughout the gastrointestinal tract. This objective was the focus of the current grant and we were successful. Twenty-four to 72 hr old calves were given oral doses of a microcin-producing strain (E-25) simultaneously with a susceptible strain. The latter included a microcin knockout strain (E-25 background), an antibiotic susceptible commensal strain of *E. coli*, or a strain of *E. coli* O157:H7. A competition index was calculated at six days post-inoculation to compare the number of microcin-producing bacteria with the number of susceptible bacteria in the feces. The microcin-producing strain had a significantly higher competition index (ci = 0.04; n = 8 calves) compared with the susceptible strains (ci = -0.22; n = 5 calves) ($P=0.039$). Analysis at time of necropsy demonstrated that E-25 strain colonizes the rectal-anal junction of the calves, which means that it is likely to be in close proximity of EHEC bacteria like O157:H7. Out of 29 purchased calves, the majority could not be used because of health problems or because they harbored inherently high backgrounds of antibiotic resistant *E. coli*. We did not anticipate that the prevalence of kanamycin resistant *E. coli* would be so high for dairy calves, but this challenge was partially circumvented by generating new strains with alternative antibiotic resistance markers. We also discovered that calves need to be inoculated within 24-48 hrs of birth or the inoculated strains could not compete with established flora. Our results demonstrate an *in vivo* selective advantage for the microcin-producing strain, although we have concluded that a simple oral inoculation delivery model is likely to be insufficient to control pathogenic bacteria. The important point from this work is that (a) the microcin is physiologically functional under “natural” conditions, and (b) the next challenge is to identify a more efficient means to deliver the microcin so it can be employed effectively. This will be the basis for a subsequent grant application.

(2) Identify the receptor that is required for susceptibility to the microcin. Classic protein-protein interaction methods were used as originally proposed, but we finally employed a whole-genome knockout library to identify potential genes of interest. In this case, we determined that a commercially available lab strain of *E. coli* is susceptible to the microcin and there is a “library” of all possible open

reading frame knockouts for this strain. We purchased the library completed screening >3,500 mutants by the end of December 2012. Confirmatory screening reduced the list of putative hits to six genes of which one encodes a highly conserved membrane bound porin protein. This protein is highly conserved in *E. coli* suggesting that the microcin will be effective to a diverse collection of *E. coli* unless the strain in question also has the ability to encode an immunity system specific to the microcin (described earlier by our team).

One potential unexpected outcome from this work is a collaboration with another research team from the Department of Veterinary Microbiology and Pathology. We determined at an earlier date that our microcin producing strain will inhibit a very important cattle disease organism called shipping fever that is caused by *Mannheimia haemolytica*. This pathogen is responsible for about 1 billion dollars in annual losses for the cattle industry. If our microcin is responsible for the inhibition of *M. haemolytica*, then there is a significant opportunity to co-join these projects to develop a multi-target anti-bacterial agent that is independent of traditional antibiotics. This work is underway at present and independent of BioAg funding.

OUTPUTS

- Work Completed: Calf trials have been completed; library screening completed.
- Publications, Handouts, Other Text & Web Products: A manuscript describing the calf experiments will be submitted by the end of May 2013. A manuscript describing the putative receptor will be submitted after we confirm the results using independent gene-knockout experiments.
- Outreach & Education Activities: A poster was presented at the CSANR symposium, December 6th, 2012. Our results will be submitted for peer-review and publication in *Applied and Environmental Microbiology* or an equivalent journal.

IMPACTS

- Short-Term: Lauren Eberhart, the PhD student for this project, has successfully defended her PhD work and graduated in May 2013.
- Intermediate-Term: discussions are currently underway with two commercial companies regarding expansion of funding and licensing.
- Long-Term: if successful, we expect to develop a very effective probiotic or similar product that could be used to prevent or control both enteric disease and pulmonary disease in cattle and other ruminants. We are also investigating applications in other food animals.

ADDITIONAL FUNDING APPLIED FOR / SECURED

Current work is supported by a USDA-AFRI grant for which D. Call is a co-investigator. Our success with the current work led to redirection of project resources to support two additional years of work on the microcin project. We are continuing discussions with two companies who have expressed interest in further developing the microcin for use in food animals and potentially as a food additive; these discussions are being facilitated by the Washington State University Office of Intellectual Property. Both companies have indicated that their interest will be enhanced considerably when we have identified the mechanism by which the microcin kills susceptible strains and if we can demonstrate that a recombinant microcin protein is biologically active. We are currently investigating these questions. If the commercial entities do not choose to invest in this technology, we will pursue funding through the USDA-NIFA-AFRI program and/or through a USDA SIBR funding mechanism.

GRADUATE STUDENTS FUNDED

This project provided summer stipend support (2012) for a Ph.D. candidate, Ms. Lauren Eberhart, in the Immunology and Infectious Disease Ph.D. program. She has completed the manuscript describing the calf work that we will submit by the end of May. An additional manuscript describing the receptor component of this system will be submitted once we have confirmed the results from our library screen.

RECOMMENDATIONS FOR FUTURE RESEARCH

The current work has demonstrated that the microcin will function in vivo. Once a functional microcin can be produced and isolated we can investigate alternative delivery systems (e.g., delivery as a high-dose suppository) and determine the physiological conditions under which the protein remains functional. This latter work will allow us to determine if the microcin can be used as a food additive to kill or limit the growth of pathogenic bacteria in foodstuffs and in animal drinking water. A third avenue of investigation is to develop heterologous expression systems that would produce a high concentration of the microcin as a probiotic and thereby circumvent the limitations of commensal strains such as E-25.