RESEARCH REPORT
SWINE HEALTH

Title - Age related changes in the intestinal microbiome protect neonatal piglets from *Clostridium difficile* infection **NPB# 12-190**

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Industry Summary

Clostridium difficile has been recognized as a significant cause of illness in neonatal pigs, but is not encountered in piglets older than 1 week of age. We hypothesized that this age related resistance is due to the increase in bacterial diversity (species and numbers of bacteria) in the intestinal tract that occurs as piglets grow and are exposed to more of their environment. Piglets were challenged with *C. difficile* at 2- 14 days of age and the microbial diversity of the cecal contents was determined. Half of the piglets that were challenged with *C. difficile* at 2 and 4 days of age showed signs of disease. The incidence of disease decreased with piglet age and none of the piglets challenged at ≥10 days of age showed any signs of disease. The bacterial populations of piglets also clustered by age so that the populations from piglets that were 2-4 days of age were more closely related to one another than to the populations in older piglets. This relatedness occurred across litters from 4 different sows and supports our hypothesis that the resistance to *C. difficile* disease in piglets greater than 1 week of age may be directly related to the diversity and complexity of the microbial community in intestinal tract.

Key words - *Clostridium difficile*, neonatal diarrhea**,** intestinal microbiome, bacterial diversity, succession

Scientific abstract

Clostridium difficile has been recognized as a significant cause of morbidity in neonatal pigs, but is not encountered in piglets older than 1 week of age. We hypothesized that this age related resistance is due to the natural microbial succession of the intestinal tract. Piglets were challenged with *C. difficile* at 2- 14 days of age and the microbial diversity of the cecal microbiome was determined. Half of the piglets that were challenged with *C. difficile* at 2 and 4 days of age showed signs of disease. The incidence of disease decreased with piglet age and none of the piglets challenged at ≥10 days of age showed any signs of disease. The cecal microbiomes of piglets also clustered by age with those that were 2-4 days of age more closely related with one another than to those of older piglets. This clustering occurred across litters from 4 different sows and supports our hypothesis that the resistance to *C. difficile* disease in piglets greater than 1 week of age may be directly related to the diversity and complexity of the intestinal microbiome.

Objective - The objective of the project was to determine if: Alteration in the intestinal microbiome due to naturally occurring bacterial succession protects piglets from infection by *C. difficile*

Introduction

Clostridium difficile- associated diarrhea (CDAD) is a significant cause of gastroenteritis in neonatal piglets and a wide variety of other mammals, including humans (3). Since the organism can be cultured from healthy animals, as well as those with diarrhea, the diagnosis of CDAD requires demonstration of the toxins, TcdA and/or TcdB, and the presence of intestinal lesions in the cecum and/or spiral colon. While the majority of very young piglets are culture positive for the organism, the intestinal population of *C. difficile* appears to decline over the first 2 months of life (8). Significant disease in piglets is confined to the neonatal period, generally 2-5 days after birth (6, 7).

Natural microbial succession of the intestinal microbiome occurs as neonatal animals are exposed to the maternal microbiota and to the diversity of microbes present in their environment (4). This increase in microbial diversity and complexity of the intestinal microbiome changes rapidly at the time of weaning as the young animals diet shifts to solid foods containing complex carbohydrates. We hypothesize that the acquisition of a more complex intestinal microbiome protects animals from overgrowth of *C. difficile* and that this protection may be able to be extended to neonatal animals through the use of probiotic cultures that resemble the microbiota of older animals.

Materials and Methods

Animals. Pregnant sows were obtained from a commercial source and farrowed in BSL-2 facilities. After birth piglets were allowed to nurse ad libitum. On days 2, 4, 6, 8, 10, 12 or 14 days of age a pair of piglets were inoculated with 5×10^4 - 10^6 spores of *C. difficile* using a gastric tube (1, 5). Challenged piglets were housed in clean plastic tubs and fed milk replacer. At 72 hr. pi, each pair of *C. difficile*-challenged piglets plus one control piglet was necropsied. During the first experiment the control piglets were kept with their respective sows until necropsy. During the second experiment control piglets were removed from the sow at the same time as the challenged piglets and fed milk replacer.

Necropsy. At necropsy gross lesions were noted and content from the cecum and spiral colon was collected for bacterial culture, toxin analysis by ELISA or Vero cell assay. Cecal samples for metagenomic analysis were frozen at -80 until processing. In addition, tissue sections from cecum and spiral colon were fixed in formalin and processed for standard H & E evaluation for intestinal lesions

DNA isolation and library preparation. Total DNA from cecal content was extracted using a MoBiol Power Soil DNA isolation kit (9). PCR was used employing a high fidelity Taq polymerase (HotStar High Fidelity Taq, Qiagen) to amplify the V4 regions of the 16S rRNA genes. Sequencing was performed using the Illumnia MiSeq platform and the data was analyzed using the QIIME v1.4.0 program(2).

Results

Half of the piglets that received the spore challenge at either 2 or 4 days of age displayed evidence of *C. difficile* disease (Table 1) such as the characteristic volcano lesions or toxin was recovered from the cecal contents. Only a minority of piglets challenged at 6 or 8 days of age had evidence of disease (3/16) and none of the piglets challenged at $\geq$ 10 days of age had evidence of disease (0/14). We considered either the presence of toxin or microscopic lesions to be indicators of disease since lesions do not occur without toxin being present at some point in the disease. In addition,

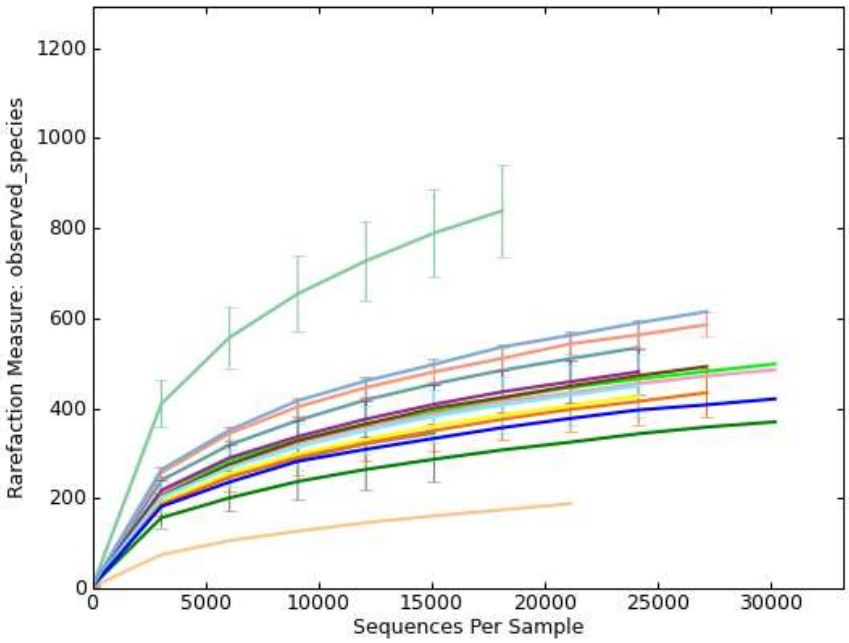
lesions can be segmental and while we did cut in multiple sections of tissue it is likely that some lesions were missed by chance alone.

Table 1. Lesions and TcdA/B detected in piglets challenged with *C. difficile* at various ages.

Day of age	Mesocolonic edema	Toxin positive	Microscopic lesions	Toxin or lesions
2	3/6	3/6	1/6	50%
4	0/6	3/6	0/6	50%
6	1/8	2/8	1/8	25%
8	0/8	1/8	0/8	12.5%
10	0/8	0/8	0/8	0%
12	0/3	0/3	0/3	0%
14	0/3	0/3	0/3	0%
Neg controls	0/8	0/8	0/8	0%

The bacterial diversity of the cecal microbiome increased with the age of the piglets; the youngest animals had the least diversity and the sows had the greatest diversity (Fig 1). There were not any significant differences in microbial diversity or taxon abundances between control piglets that were fed milk replacer and those that remained with the sow. Therefore, the data from both of these groups were combined and analyzed as a single group. There were not any significant differences in bacterial diversity or taxon abundance between piglets that were challenged with *C. difficile* spores and the negative controls. *C. difficile* sequences were recovered from the cecal contents of all of the piglets challenged at 2 and 4 days of age and from 14/31 challenged at a later age. *C. difficile* sequences were also recovered from 4/8 controls.

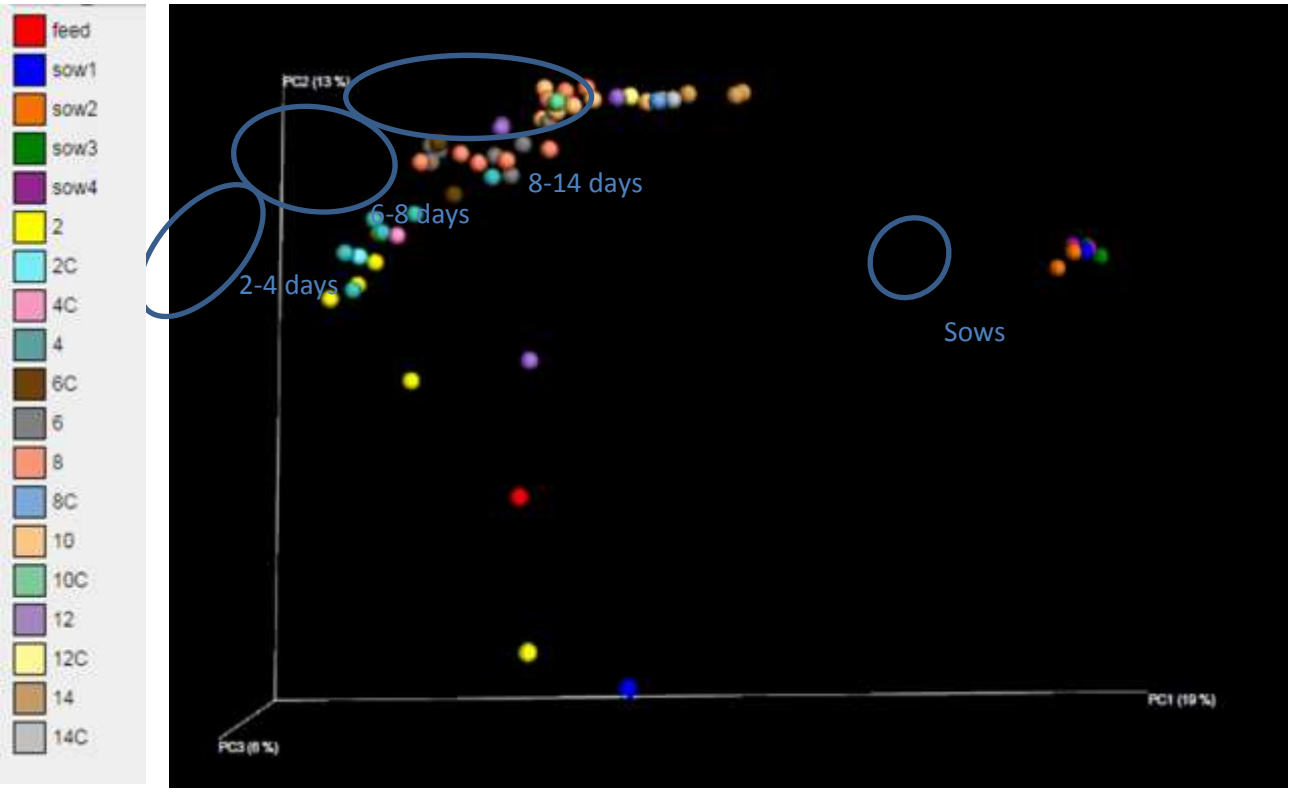
Fig 1. Mean bacterial diversity (Alpha diversity) of the cecal microbiomes of piglets by age. Each line represents all of the piglets challenged at a specific day of age.



	2
	4
	6
	8
	10
	12
	14
	C2
	C4
	C6
	C8
	C10
	C12
	C14
	feed
	sows

When the microbial diversity from the ceca of individual piglets was compared to each other the animals clustered by age (Fig 2). The microbiomes of piglets challenged at 2 or 4 days of age were more closely related to one another than they were to pigs that were challenged when they were older. Piglets that were 8 days of age at challenge clustered with 6 day old piglets and with older piglets suggesting that the microbiome is shifting and becoming distinctly more complex even by 1 week of age. This clustering occurred across 4 different litters of piglets.

Fig. 2. Relative differences between the cecal microbiomes of individual piglets (Beta diversity). Each circle represents an individual animal, colors indicate age of animal at time of challenge with *C. difficile*.



Discussion

Our challenge data confirm that *C. difficile* disease is confined to very young piglets. Half of the animals challenged at 2 or 4 days of age showed evidence of disease but that rate decreased by day 6 and none of the piglets that were challenged at 10 days of age or later showed any signs of illness. This is in agreement with the consensus in the literature and from epidemiological studies. *C. difficile* can cause disease in 10 day old piglets if the animals are only given minimal amounts of colostrum and fed solely on milk replacer (1). Since we wished to more closely replicate production practices all of our piglets remained with the sow until they were challenged (48 hr for the youngest group). In addition to the maternal antibodies the piglets received, continued contact with the sow likely hastened the development of the intestinal microbiota of the piglets.

As expected the microbial diversity of the cecal contents increased with the age of the piglets. The natural succession of the intestinal microbiome of infants has been investigated using molecular methods however, we are not aware of any such studies in piglets. The clustering of the piglets by age supports our hypothesis that the resistance to *C. difficile* disease in piglets greater than 1 week of age may be directly related to the diversity and complexity of the intestinal microbiome. At this point we cannot determine the mechanism for this protection, but possibilities include competitive exclusion for either binding sites and/or nutrients, production of an antagonistic metabolite or some other change in the environment that inhibits the growth of *C. difficile* in older piglets. It would be of interest to determine the specific changes in the microbiome that occur at the genus/species level when the piglets are 8 -10 days old. If consistent changes occur between individuals this could suggest suitable organisms that should be included in a

preventative probiotic. If such a product proved to be efficacious, it would likely have substantial appeal to pork producers.

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