ONTARIO PORK

Ontario Pork Research Final Report (insert project #) Executive Summary

(Use the headings below to create an executive summary of the project with a maximum of 1 page double-spaced)

Project Title: Optimizing an infection model for *E. coli* diarrhea in newly weaned pigs

Project Lead: Vahab Farzan; afarzan@uoguelph.ca

Reporting Date: April 30, 2023

Introduction: The most obvious impacts of enterotoxigenic *E. coli* post weaning diarrhea (ETEC-PWD) are the economic losses, significant reduction in animal welfare, and indirect increase in use of antimicrobials. A precise infection model is required to evaluate the potential impact of alternative methods such probiotics and vaccines on controlling enterotoxigenic *E. coli* (ETEC) diarrhea in pigs. One of the issues in the *E. coli* challenge model is a considerable variation in piglet responses to ETEC which can mainly explained by the fact that the pigs' response to ETEC infection may be dependent on several factors including pig sources, pig genetics, breed, age, diet, gut microbiome, dose of infection, stomach acidity, and ETEC ability to adhere the intestine epithelial cells.

Objectives: (1) Evaluate the response to enterotoxigenic *E. coli* (ETEC) F4 among susceptible and resistant pigs (based on *MUC4* polymorphism) challenged with different dosages and at different ages.

(2) Compare the response to ETEC challenge in susceptible pigs weaned at different ages.

(3) Determine the impact of infection dosage on post-weaning diarrhea in susceptible pigs challenged with ETEC.

(4) Determine the pig susceptibility to *E. coli* (ETEC) F4 infection based on *CHCF1* polymorphism **(Additional objective)**.

Materials and Methods: Nine trials were done. For each trial 60-80 piglets were tested for susceptibility to ETEC-F4 infection based on *MUC4*. In addition, for 6 trials, pigs were tested for susceptibility based on the *CHCF1* gene. For each trial 18 pigs in 3 pens were challenged by ETEC with different infectious dosage, while 6 pigs in one pen assigned as control. Pigs were monitored daily and scored for their general appearance, diarrhea, dehydration, and appetite. Fecal swabs were collected and examined for the presence of ETEC. Pigs were euthanized one week post challenge and tissue samples were taken from intestine and examined for histopathology.

Results and Discussion: Overall, 57.9% of 601 pigs and 40.3% of 144 pigs tested for inclusion in the trial were susceptible to ETEC-F4 infection based on *MUC4* and *CHCF1* gene, respectively. The susceptible pigs (based on *MUC4*) were more likely to have diarrhea than resistant pigs while there was no association between the *CHCF1* pig susceptibility and diarrhea. However, both *MUC4* and *CHCF1* susceptible pigs shed higher levels of ETEC in feces and had more bacterial colonization in their ileum than resistant pigs. Pigs challenged with a higher infectious dosage of ETEC were more likely to demonstrate diarrhea than control pigs. Further, diarrhea was higher among pigs born to primiparous sows compared to those pigs born to multiparous sows. Weaning age was not associated with diarrhea and bacterial shedding.

Conclusions: The findings indicate that *MUC4* gene alone may not be sufficient to identify the susceptible pigs to ETEC, and additional genetic markers such as the polymorphism in the *CHCF1* should be considered to examine the pigs for genetic susceptibility to ETEC. The challenge dose used in this study, could increase the rate of diarrhea and bacterial shedding without causing mortality. Altogether, the parameters examined in this study could be implemented to evaluate the efficacy of methods for controlling post-weaning diarrhea in pigs before utilizing on swine farms.

ONTARIO PORK

Ontario Pork Research Final Report (insert project #)

(maximum of 6 pages double-spaced)

Date: April 30, 2023

Introduction

Post-weaning diarrhea is a significant problem for the pig industry. The most common cause of diarrhea in newly weaned pigs is enterotoxigenic *E. coli* (ETEC). Pathogenic *E. coli* tend to possess certain virulence factors, including fimbriae and enterotoxins (Gyles, 1994). Enterotoxins are secreted by *E. coli* and then act on intestinal epithelial cells causing them to secrete large volumes of fluids and electrolytes, resulting in diarrhea. Unfortunately control of post-weaning *E. coli* diarrhea is difficult. Economic losses are attributed to the death of individual animals, reduced growth rate, the cost of medication and other preventive measures as well as increased labour.

Antibiotics have been a common solution to controlling post-weaning *E. coli* diarrhea, but there is pressure from the public to reduce the use of antibiotics in food animal agriculture. In addition, ETEC strains have developed antimicrobial resistance making it more difficult to control this disease in the long term with routine inclusion of antibiotics in starter feeds and creating public health concerns because of the fear of resistance transfer (Jahanbakhsh et al., 2016). Water medication with antibiotics including neomycin and apramycin are commonly used to treat outbreaks, along with individual pig injections. Zinc oxide is also widely used in feed to control post-weaning diarrhea. However, using zinc oxide may damage the environment caused by depositing a heavy metal onto land and creating a concern that surface run-off might contaminate watercourses and rivers (Wood, 1991). Alternative methods such as probiotics, organic acids, and vaccination are thus considered to replace the zinc oxide to prevent post weaning *E. coli* diarrhea in pigs. The effectiveness of those alternatives needs to be evaluated under experimental challenge condition before utilizing on swine farm. The use of proper animal models is critical in the success of developing and studying novel therapies. However, making a reproducible experimental challenge model for PWD can be difficult (Roubos-van den Hil et al., 2017; Spitzer et al., 2014; Jensen et al., 2006), especially if the pigs used in the study have been previously exposed to ETEC infection (Jensen et al., 2006).

There have been inconsistent findings among different studies to induce post weaning *E. coli* diarrhea in newly weaned pigs. A scoping review of published studies between 1986 and 2020 found that diarrheal response was not developed in many challenge studies while bacterial shedding was frequently reported (Goodman, 2021). Similarly, histological changes observed in post-mortem examination and *E. coli* colonization was uncommon and inconsistent among different studies. The variations among studies can mainly explained by the fact that the pigs' response to ETEC infection may be dependent on several factors including pig sources, pig genetics, breed, age, diet, gut microbiome, dose of infection, stomach acidity, and ETEC ability to adhere the intestine epithelial cells. Hence, it is necessary to examine aspects of existing experimental challenge models to optimize a more precise infection model.

Objectives

1. Evaluate the response to enterotoxigenic *E. coli* (ETEC) F4 among susceptible and resistant pigs (based on *MUC4* polymorphism) challenged with different dosages and at different ages.
2. Compare the response to ETEC challenge in susceptible pigs weaned at different ages.
3. Determine the impact of infection dosage on development of post-weaning diarrhea in susceptible pigs challenged with ETEC.
4. Determine the pig susceptibility to *E. coli* (ETEC) F4 infection based on *CHCF1* polymorphism **(Additional objective)**.

Materials and Methods

Pig genetic susceptibility to enterotoxigenic *E. coli* F4. For each trial, tail dockings were collected from 60-80 piglets housed at the Arkell Swine Research Facility a few days after birth. Pig DNA extracted from tail tissue samples was used to determine pig susceptibility to ETEC-F4 infection based on the presence of a polymorphism in the Mucin 4 (MUC4) *gene* (Jørgensen et al., 2004; Jensen et al., 2006). For six of the nine trials (trials 4-9), pig susceptibility genotype was also determined by assessing pigs for the presence polymorphism in the *CHCF1 gene (*Rampoldi, 2013).

Trial design. Nine trials were done. For seven trials (trials 1-7), 12 resistant and 12 susceptible pigs, and for trials 8 and 9, 24 susceptible pigs were selected. Pigs were transported to the Level 2 Animal Isolation Facility at the University of Guelph (Day 0) and housed in 4 pens (Day 1) **(Table 1)**. One day after arrival (D1), pigs in 3 pens were challenged by ETEC isolates (O149:LT:STa:STb:EAST1:F4ac) while pigs in the control groups received sterile placebo.

Clinical observations. Pigs were monitored daily and scored for their general appearance, diarrhea, dehydration, and appetite. Diarrhea was scored as 0 (normal), 1 (mild: pasty, or loose stool), 2 (moderate: stool was quite liquid but coloured), and 3 (severe: watery, clear diarrhea). Additionally, the number of days pigs displayed diarrhea was also assessed as a percentage of diarrhetic days out of total trial days for each group (pig/day/diarrhea).

Sample collection. Fecal swabs were collected daily and cultured for the presence of hemolytic E. coli (trials 1-9; yes/no) and *for* CFU/g (trials 3-9) of the fecal sample. The isolates were tested by PCR for five fimbrial (F4, F5, F6, F18, and F41) and toxin (LT, Sta, and STb) genes (Jensen et al., 2006).

Post-mortem observations and histopathology. All pigs were euthanized on Day 8. Intestinal content was scored during necropsy as 0 (normal), 1 (pasty), 2 (presence of more solid particles than liquid), and 3 (presence of more liquid than solid particles or totally liquid). Tissue samples were taken from the jejunum, ileum, and colon and submitted to the Animal Health Laboratory at the University of Guelph for histological examination.

Data analysis. Multilevel mixed-effects logistic regression models with trial, sow, and pig as random effects were used to assess any associations between the outcome variables and independent variables of interest.

Results and Discussion

Genetic susceptibility to E. coli F4 based on MUC4 and CHCF1 gene polymorphism. In total, 601 pigs (122 female and 94 male) were genotyped using the *MUC4* polymorphism, of which 42.1% and 57.9% of pigs were resistant and susceptible to ETEC-F4 infection, respectively **(Figure 1)**. Pigs from six of the nine trials were further tested for the *CHCF1* SNP after the challenge trials had been completed. Of the 144 pigs tested for the *CHCF1* SNP, 59.7% (86/144) were the resistant while 40.3% (58/144) were susceptible **(Figure 2)**. Based on a Cohen's kappa test, there was a slight agreement ($\kappa = 0.21$) between the MUC4 and CHCF1 to identify the susceptible/resistant pigs ($p=0.003$).

Sow parity, weaning age, and diarrhea. Age of pig was not a significant variable for any outcome tested in this study. The weaning age for the selected pigs ranged from 12-23 days with the majority of pigs falling around 14-15 days (28.2%) or 18-19 days (48.6%). It is possible that the passive immunity acquired from sows may interfere with pigs' response if pigs are challenged at younger age (Roubos-van den Hil et al., 2017). On the other hand, pigs might have already developed active immunity and resist to infection if they are challenged at older age (Roubos-van den Hil et al., 2017). Pigs born to primiparous sows were also more likely to have diarrhea than pigs born to multiparous sows ($p = 0.010$). It is likely that multiparous sows have had increased opportunity for exposure to ETEC on-farm and were able to pass on that immunity to their piglets. Gilts may not yet have been exposed to ETEC or other intestinal pathogens, which may explain the increased incidence of diarrhea observed in their piglets. However, sow parity was not significant with fecal shedding or intestinal colonization of ETEC in the piglets. It is possible that the immunity gained from the sow was only sufficient to reduce the severity of disease symptoms and not provide complete protection against a direct ETEC challenge dose.

Diarrhea in unchallenged pigs. Overall, 52.1% (25/48) of control pigs displayed diarrhea at least once compared to 67.3% (113/168) of challenge pigs. As weaning is a time of high stress and change, it is possible that diarrhea in the control pigs and some challenged resistant pigs may have been caused by dietary changes or other microbial agents such as rotavirus (Fairbrother et al., 2005)

Diarrhea and genetic susceptibility to ETEC F4. Overall, 52.1% (25/48) of control pigs displayed diarrhea at least once compared to 67.3% (113/168) of challenge pigs **(Table 2)**. Diarrhea was observed in 71.0% (93/131) of susceptible pigs, compared to 52.9% (45/85) in resistant pigs. The susceptible pigs (based on *MUC4)* were more likely to have diarrhea ($p = 0.001$) than resistant pigs. It is possible that the F4 receptor has been expressed even within resistant pig populations (Rasschaert et al., 2007). However, The *CHCF1* SNP was not significantly associated with diarrhea in pigs. Overall, a little is known about the *CHCF1* SNP, it is possible that despite not having a significant association with the likelihood of diarrhea this marker may still be valuable as a marker for other aspects of ETEC-F4 infection in pigs. Further, as this SNP was tested for after trial completion, the abnormal distribution of genotypes and complete lack of susceptible pigs in the population tested may have influenced this significance of this result.

Diarrhea and infectious dosage of ETEC. The incidence of diarrhea was highest among the high dose group pigs (75.0%) and among pigs challenged three times (75.0%). Additionally, pigs that received one and two challenge dose(s) were 3.13 times ($p < 0.001$) and 1.98 times ($p = 0.005$) more likely to display diarrhea than control pigs during the trial, respectively. Higher rates of diarrhea (pig/day/diarrhea) were seen as the ETEC challenge dose CFU/mL increased ($p < 0.001$). Only 4 pigs (2 susceptible and 2 resistant) pigs in high dose group were euthanized due to severe diarrhea.

ETEC fecal shedding. In total, 89.4% (193/216) pigs shed the ETEC challenge strain in feces at least at one sampling time point after challenge, of which 38.3% (74/193) were resistant while 61.6% (119/193) were susceptible based on *MUC4.* Fecal shedding of ETEC-F4 peaked on Day 3 (2 dpi) in both resistant and susceptible pigs **(Figure 3)**. Pigs susceptible to E. coli infection based on the CHCF1 gene were 6.96 times more likely to shed ETEC in feces than resistant pigs ($p < 0.001$).

The number of ETEC shed by pigs. The number of ETEC (CFU/g) in fecal samples varied between trials and treatment groups **(Figure 4)**. Pigs with the *MUC4* susceptible genotype had a higher mean log CFU/g of ETEC in feces compared to resistant pigs at each sampling time point. Additionally, the bacterial load of ETEC detected in feces **(Figure 4)** increased as the challenge inoculum dose (CFU/mL) increased, with pigs in the high dose group having the highest number of ETEC (Log CFU/g = 4.17), and control pigs having the lowest (0.85). Pigs that received a higher dose CFU/mL ($p < 0.008$) and pigs that had diarrhea at least once during the trial ($p = 0.002$) had higher levels of ETEC recovered from fecal samples.

Additionally, susceptible pigs (based on *CHCF1*) shed higher levels of ETEC in feces than resistant pigs ($p = 0.020$) indicating that the *CHCF1* SNP may be a promising candidate marker for future studies in pig populations. There was also an association between pig *MUC4* susceptibility and higher levels of ETEC shedding in feces, but it was approaching significance level ($p = 0.095$).

ETEC colonization in ileum tissue. Overall, pigs that were susceptible to ETEC based on the *CHCF1* SNP were 11.62 more likely to have E. coli colonization of ileal tissue ($p < 0.001$). Pigs that had diarrhea at least once during the challenge trial were also 6.14 times more likely to have *E. coli* colonization of ileal tissue ($p < 0.001$). Additionally, pigs in the highest dose group were more likely to have ETEC in ileum tissues than any other dose groups. Pigs susceptible to E. coli based on MUC4 gene ($p = 0.006$) and susceptible pigs based on the *CHCF1* SNP ($p < 0.001$) were both more likely to have higher amounts of E. coli in ileum tissues. Additionally, pigs that had higher CFU/g of E. coli in feces ($p = 0.009$) and pigs that received a higher dose CFU/mL ($p < 0.001$) showed higher levels of E. coli in the ileum.

Adherent bacilli. Overall, 26.4% (57/216) of pigs had adherent bacilli detected in intestinal tissues**.** Of those 57 pigs with adherent bacilli, 24.6% were *MUC4* resistant and 75.4% *MUC4* susceptible to ETEC-F4. The highest rate of adherent bacilli (61.1%) was detected in pigs from the moderate dose group. Pigs *MUC4* susceptible to *E. coli* infection ($p < 0.001$) and the *CHCF1* susceptible pigs ($p = 0.001$) were more likely to show adherent bacilli in intestinal tissues post-mortem compared to resistant pigs. This may support the hypothesis that both markers are linked with *the* expression of F4 receptor warranting further investigation.

Conclusions

Post-weaning diarrhea associated with ETEC has a significant impact on the pig industry, both economically and on animal welfare. As such, it's important to further study and understand the factors that may influence *E. coli* infection in pigs. This will help to evaluate the control methods using a more precise infection model. While weaning age has been shown to be associated with pig health and growth performance in other studies, in the present study the weaning age of pigs was not significantly associated with any outcome. It is possible that there were only minor differences observed between the ages of pigs selected for trial inclusion and perhaps weaning age may have been significant if compared against pigs weaned at 28 or more days (Moeser et al., 2007). Pigs born to primiparous sows were more likely to have diarrhea at least once during the trial. Despite this association not being significant in shedding or colonization models, the colostrum from multiparous sows may have provided enough passive immunity or nutrients passed to piglets to reduce the severity of ETEC colonization. Therefore, it is crucial to use pigs raised on ETEC free farm in the challenge studies, though it may be difficult to find such a farm. Dose level was significant in all outcomes with pigs in the high dose groups showing higher rates of diarrhea, fecal shedding, ileum colonization, and adherent bacilli in intestinal tissues. Exposure to a high acute dose of ETEC-F4 is expected to be more than sufficient to ensure adhesion and proliferation of ETEC in the intestines as was seen in the present study. Elucidating the genetic mechanisms behind receptor expression and susceptibility to ETEC-F4 has become an area of keen interest in recent years. Although the causal gene for *F4R* remains unknown, several candidate markers have been proposed that show promise (Goetstouwer et al., 2014; Rampoldi, 2013). The most extensively studied marker, *MUC4*, was significantly associated with diarrhea and adherent bacilli in intestinal tissues. Regardless, the findings herein confirm the usability of *MUC4* as a marker for ETEC-F4 susceptibility, however it is of importance to continue to study other markers such as *CHCF1* which was found to be significantly associated with ETEC fecal shedding, ileum colonization, and adherent bacilli in intestinal tissues. Based on these results, it is clear that further study of pig susceptibility to ETEC-F4 infection is crucial to not only optimizing an infection model also to preventing the impact ETEC has on pig production and animal welfare.

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Knowledge Transfer

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Tables and Figures

Table 1: Trial design for weaned pigs selected for inclusion.

Trial	Genotype[#] (Number of pigs per group (total pigs))		Weaning age (days)	Sex* (Number of pigs per group)		Number of sows [parity]	Number of challenges
	R	S		M	F		
1	3 (12)	3 (12)	19-20	13	11	10 [2,3,4]	1-3
2	3 (12)	3 (12)	18-23	10	14	7 [1,2]	1-2
3	3 (12)	3 (12)	15-18	11	13	8 [1,2]	1-2
4	3 (12)	3 (12)	14	11	13	3 [2,5,7]	1
5	3 (12)	3 (12)	18	8	16	4 [1,2]	1
6	3 (12)	3 (12)	19-20	4	20	3 [5]	1
7	3 (12)	3 (12)	12-14	14	10	5 [1,2]	1
8	0	6 (24)	19	13	11	6 [1,2]	1
9	0	6 (24)	14-15	10	14	4 [1,3,5]	1-2

[#]R/S: Resistant/Susceptible genotype determined by polymorphism in *MUC4* gene.

Table 2: Number of pigs displaying diarrhea by MUC4 susceptibility and challenge group in 9 trials.

	Number of diarrhetic pigs/number of pigs in group per trial										
Trial	Group 1*		Group 2		Group 3		Group 4		All groups		Total
	R[#]	S	R	S	R	S	R	S	R	S	
1	2/3	3/3	3/3	3/3	3/3	3/3	1/3	2/3	9/12	11/12	20/24
2	1/3	0/3	2/3	2/3	0/3	1/3	0/3	1/3	3/12	4/12	7/24
3	2/3	2/3	0/3	1/3	3/3	3/3	3/3	2/3	8/12	8/12	16/24
4	0/3	0/3	1/3	0/3	0/3	2/3	1/3	2/3	2/12	4/12	6/24
5	1/3	2/3	1/3	0/3	1/3	2/3	1/3	3/3	4/12	7/12	11/24
6	1/3	2/3	3/3	3/3	3/3	2/3	2/3	2/3	9/12	11/12	18/24
7	2/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3	11/12	11/12	22/24
8	-	6/6	-	5/6	-	6/6	-	4/6	-	21/24	21/24
9	3/4	0/2	-	6/6	-	4/6	-	6/6	3/4	16/20	19/24
All	7/16	13/20	8/12	17/24	7/12	18/24	7/12	20/24	29/52	68/92	97/144

*Group 1: Single challenge (trial 1) or control (trials 2-9); Group 2: Single challenge (trials 1-3) or low dose (trials 4-9); Group 3: Multiple challenges (trials 1-3) or medium dose (trials 4-9); Group 4: Multiple challenges (trials 1-3) or high dose (trials 4-9). [#]R/S: Resistant/Susceptible genotype determined by *MUC4-8227* SNP.

Figure 1: Genetic susceptibility to ETEC F4 in 601 pigs based on *MUC4* for inclusion in 9 trials.

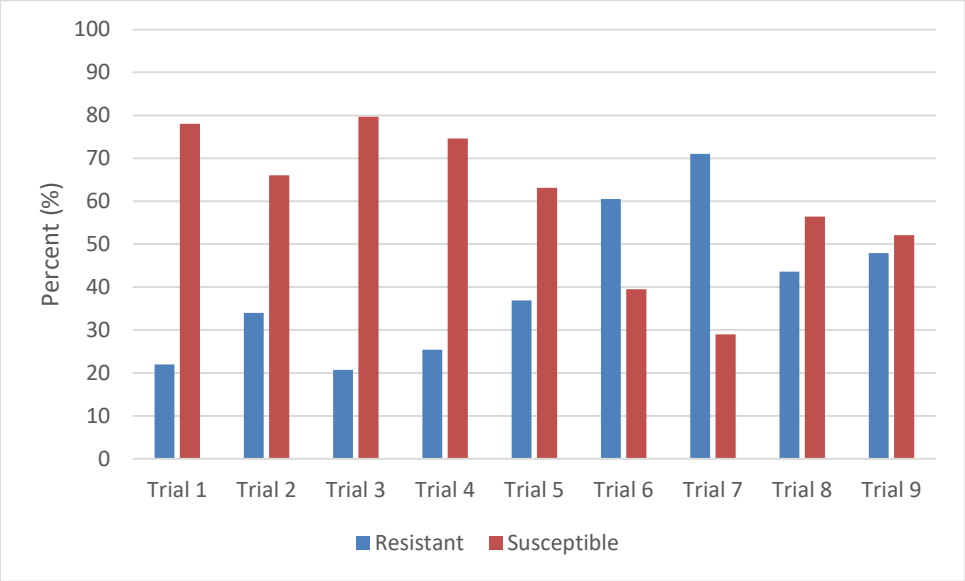

Figure 2: Genetic susceptibility to ETEC F4 based on the *CHCF1* in 144 pigs for inclusion in 6 trials.

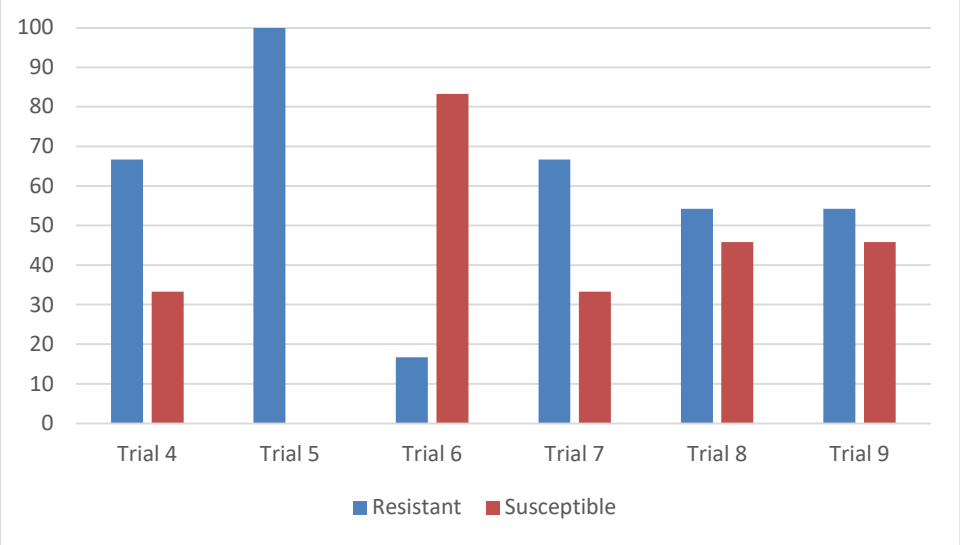

Figure 3: Daily fecal shedding of ETEC in pigs.

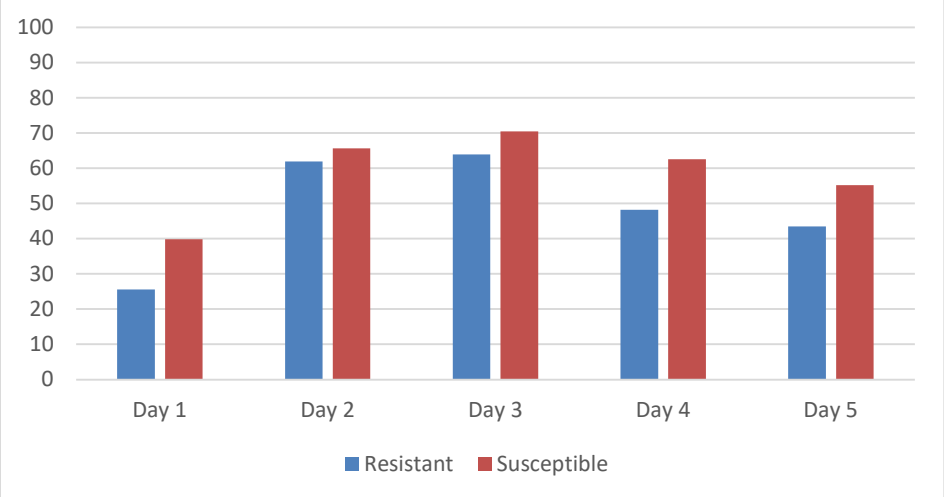

Figure 4: Number of ETEC (presented as the mean log CFU/g) in fecal samples from resistant and susceptible pigs (based on MUC 4 gene)