Nutritional and management strategies to improve beef and pork production efficiency

by

Payton Lane Dahmer

B.S., Kansas State University, 2019
M.S., Kansas State University, 2020

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

The weaning transition is a crucial time in swine production, as piglets experience physiological and nutritional changes as well as exposure to potential pathogens such as enterotoxigenic *Escherichia coli* (ETEC). Historically, nutritional interventions such as supplementation of pharmacological zinc oxide (ZnO) have been used to manage post-weaning diarrhea caused by ETEC. However, there is consumer and regulatory pressure to limit these practices, thus, alternative management or nutritional strategies are being investigated.

Two experiments were conducted to evaluate dietary acidifiers and microencapsulated zinc oxide (ZnO) on weanling pig health and performance. In the first experiment, a total of 350 pigs (DNA 400 × 200; initially, 5.7 ± 0.06 kg BW) were used in a 42-d study with 5 pigs per pen and 14 replicate pens per treatment. At weaning, pigs were allotted to pens in a completely randomized design and pens of pigs were randomly assigned to one of five dietary treatments consisting of a negative and positive control (150 vs. 3,000 ppm Zn from ZnO) or three different types of dietary acidifiers. Overall, pigs fed a 1.0% blend of formic acid and glycerol monolaurate had reduced ($P < 0.0001$) ADG compared to those fed control diets. Fecal DM was evaluated from d 7 to d 28 and there was a treatment × day interaction ($P = 0.04$). In the second experiment, a total of 300 pigs (DNA 200 × 400; initially 6.0 ± 0.08 kg BW) were used in a 42-d study with 5 pigs per pen and 12 pens per treatment. At weaning, pigs were randomly allocated to pens and pens randomly allotted to dietary treatments. Dietary treatments consisted of a negative control (110 ppm Zn from ZnO) or two levels of ZnO (400 vs. 3,000 ppm Zn from ZnO) or two levels of microencapsulated ZnO (400 vs. 3,000 ppm Zn from microencapsulated ZnO). There was no evidence of differences in ADG, ADFI, or G:F for the entire treatment period (d 0 to d 28; $P > 0.05$). During the common phase 3 (d 28 to 42) pigs fed the negative

control, High-MZnO, or Low-MZnO had improved ($P < 0.0001$) ADG compared to pigs fed High- or Low-ZnO. A significant treatment × day interaction ($P = 0.04$) was observed for fecal Zn concentrations, where the level of Zn excreted in the feces was dependent on the sampling day in pigs fed a low level of ZnO or low level of microencapsulated ZnO, while excretion of Zn did not differ between d 10 and 28 in pigs fed a negative control, high level of ZnO, or high level of microencapsulated ZnO.

In a third experiment, 80 crossbred, high-risk heifers (initially 250 ± 4.2 kg BW), were transported from an Oklahoma City, Oklahoma sale barn to the Kansas State University Beef Cattle Research Center. Cattle were unloaded and randomly placed into one of four receiving pens. Each pen was randomly assigned to one of four rest-times before processing ranging from 0 to 48 hours. Processing time did not impact ($P > 0.05$) heifer BW or ADG. A significant processing time × day interaction ($P < 0.0001$) was observed for the prevalence of fecal parasites, where the percentage of positive samples was significantly lower 14-d after anthelmintic treatment, regardless of the processing time.

While animal-based research is critical for us to make science-based decisions about animal management, we also must make science-based decisions about student education. Our undergraduate research (UGR) program has grown in recent years, but it was necessary to evaluate the learning outcomes of students engaged in these experiences. To do this,167 undergraduate students in the Department of Animal Sciences and Industry at Kansas State University completed an anonymous, retrospective post-then-pre-test to assess their perceptions of how the UGR experience impacted the development of professional skills and research competence. Students participating in either an independent (student paired with faculty member in 1:1 ratio) or course based (approximately 20 students paired with 1 faculty member) UGR

experience completed the assessment at the end of the academic semester following conclusion of the UGR project. A comparison group of students not completing any form of UGR were also surveyed. Students completing a course-based UGR experience reported significant increases ($P < 0.02$) in professional skill development compared to students not completing UGR, while those who participated in an independent UGR experience were intermediate. Students participating in course based UGR reported increased ($P < 0.0001$) gains across all skill areas related to research methods compared to those not completing UGR. Based on student responses, completion of an UGR experience has positive implications for professional skill development and comprehension of how science is practiced.

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Table of Contents

List of Figures

List of Tables

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over the years, and the continued guidance you will provide as I attempt to fill your shoes. To the 2019 – 2023 teams, I've learned more from each of you than I could have ever taught you. Thank you for the memories traveling thousands of miles in a 15-passenger van – I know that our friendships will be lifelong.

Most importantly, I'd like to thank my family for their unwavering support. Mom and dad – you have always pushed me to be better than I was the day before and afforded me every opportunity to chase my dreams. Thank you for being a constant source of encouragement and support through the highs and lows. To Paxton and Payge – I'm not sure how I got lucky enough to end up with my two best friends as siblings, but I appreciate everything that each of you have done for me over the years and can't wait to continue watching you both crush your goals. Whitney – you probably deserve an award for the countless hours you spent listening to me vent when I was stressed or handling things so that I could either be on the road or have a late night in the office. You've been a constant supporter of mine and have an ability to help me see the silver lining of every situation. I'm so proud of where we are today and can't wait to see what's in store for us next! Finally, this experience would have never been possible without one person being my biggest cheerleader and encouraging me to keep going. To my grandmother, Peggy, I say thank you for always being in my corner – I miss you beyond words.

Dedication

To my parents, Cory and Amy. I hope to someday be half the person that each of you are.

Preface

This dissertation is original work completed by the author, P.L. Dahmer. Chapters 2 (doi: 10.1019/tas/txac145) and 4 (doi: 10.1093/tas/txac085) were published in Translational Animal Science. Each of the chapters was formatted according to the required standards of the corresponding journal.

Chapter 1 - Quantitative assessment of the variability among enterotoxigenic *Escherichia coli* (ETEC) challenge experiments in weanling pigs[1]

Abstract

Post-weaning diarrhea in pigs is often caused by the F4 or F18 strains of enterotoxigenic *Escherichia coli* (ETEC). To evaluate interventions for ETEC, experimental infection via a challenge model is critical. Others have reviewed ETEC challenge studies, but there is a lack of explanation for the variability in responses observed. Our objective was to quantitatively summarize the responses and variability among ETEC challenge studies and develop a tool for sample size calculation. The most widely evaluated response criteria across ETEC challenge studies consist of growth performance, fecal consistency, immunoglobulins, pro-inflammatory cytokines, and small intestinal morphology. However, there is variation in the responses seen following ETEC infection as well as the variability within each response criteria. Contributing factors include the type of ETEC studied, dose and timing of inoculation, and the number of replications. Generally, a reduction in average daily gain (ADG) and average daily feed intake (ADFI) are seen following ETEC challenge as well as a rapid increase in diarrhea.

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[1]This work has been submitted for publication in *Translational Animal Science:* Dahmer, P.L., J.M. DeRouchey, J.T. Gebhardt, C.K. Jones, and C.B. Paulk. 2023. Quantitative assessment of the variability among enterotoxigenic *Escherichia coli* (ETEC) challenge experiments in weanling pigs. Submitted 03/01/23. TAS-2020-1396.

The magnitude of response in growth performance varies, and methodologies used to characterize fecal consistency are not standardized. Likewise, fecal bacterial shedding is a common indicator of ETEC infection, but the responses seen across the literature are not consistent due to differences in bacterial enumeration procedures. Emphasis should also be placed on the piglet's immune response to ETEC, which is commonly assessed by quantifying levels of immunoglobulins and pro-inflammatory cytokines. Again, there is variability in these responses across published work due to differences in the timing of sample collection, dose of ETEC pigs are challenged with, and laboratory practices. Small intestinal morphology is drastically altered following infection with ETEC and appears to be a less variable response criterion to evaluate. For each of these outcome variables, we have provided quantitative estimates of the responses seen across the literature as well as the variability within them. Using this information, a Microsoft Excel-based tool was created to calculate sample sizes for future studies utilizing the summary of information compiled in this review. While there is a large degree of variability across ETEC challenge experiments, we have provided a quantitative summary of these studies and generated a tool that can aid researchers in designing future work.

Introduction

During the weaning transition, piglets undergo rapid changes environmentally, nutritionally, and immunologically (Zheng et al., 2021). These factors in combination with exposure to gastrointestinal pathogens can result in post-weaning diarrhea (PWD), which is one of the most economically important issues for the swine industry due to increased mortality, suppressed growth rates, and costs associated with treatment (Bonetti et al., 2021). The main etiological agent prompting PWD is enterotoxigenic *Escherichia coli* (ETEC), which are

categorized according to virulence factors known as adhesins and enterotoxins (Smith and Linggood, 1971; Berberov et al., 2004; Zhang et al., 2007). Adhesins located on the bacterial fimbriae regulate the binding of the pathogens to epithelial cells and subsequent colonization within the intestine. The most common fimbriae associated with ETEC infection in post-weaning pigs are F4 (K88) or F18. There are other fimbriae types such as F5 (K99), F6 (987P), F17, and F41, but they are commonly associated with neonatal diarrhea prior to weaning (Jin and Zhao, 2000). Upon fimbriae attachment and ETEC colonization, enterotoxins are produced which induce a rapid loss of electrolytes and secretion of fluid into the intestinal lumen, leading to the clinical observation of diarrhea (Nagy and Fekete, 2005). Heat labile (LT) and heat stable [STa, STb, and enteroaggregative heat-stable toxin 1 (EAST1)] are the two classes of enterotoxins produced by ETEC. Strains of ETEC with F4 (K88) fimbriae typically produce LT and STb (on some occasions STa may or may not be produced) enterotoxins upon bacterial adhesion and colonization, while strains exhibiting the F18 fimbriae will produce STa and STb toxins (Duan et al., 2012).

Also referred to as enteric colibacillosis, PWD caused by ETEC typically occurs within the first two weeks post-weaning and results in watery, profuse diarrhea that can last anywhere from 1 to 5 days after infection (Sun and Kim, 2017). Subsequently, dehydration, reduced growth, and in some cases eventual mortality can occur (Verdonck et al., 2007). Prior to the establishment of the Veterinary Feed Directive in 2017, prevention or control of PWD caused by ETEC included therapeutic treatment with antibiotics, but today the addition of pharmacological levels of zinc oxide (ZnO) to the nursery diet is perhaps more common (Bonetti et al., 2021; Juhász et al., 2022). Due to concerns with antimicrobial resistance and excess zinc excretion in feces, there is strict consumer and regulatory pressure to limit this practice in both Europe and

Canada (Commission, 2016). The demand for alternative strategies to combat PWD is growing in need and has become a focal point for many in the swine industry worldwide. Many proposed solutions include altering diet composition through lower crude protein (Bikker et al., 2006; Wellock et al., 2008), different fiber combinations (Htoo et al., 2007), and the inclusion of feed additives like diet acidifiers (Tsiloyiannis et al., 2001), pre- and pro-biotics (Hayakawa et al., 2016), nucleotides (Martinez-Puig et al., 2007), or essential oils (Tian and Piao, 2019). However, to effectively evaluate any intervention mechanism for PWD caused by ETEC, it is important to appropriately replicate ETEC infection by purposefully inoculating pigs with the pathogen to induce an experimental infection, known as an *in vivo* challenge model (Adewole et al., 2015).

Utilizing an ETEC challenge model in post-weaning pigs has been demonstrated on numerous accounts; however, results from these studies are often extremely variable. A review by Luise et al. (2019b) summarized ETEC F4 and F18 infection models and focused on factors to consider when planning the challenge, such as genetic susceptibility to ETEC, preconditioning practices (i.e., administration of antibiotics or fasting prior to inoculation), and the timing and dose of ETEC inoculation. These authors also outlined methods to determine the effectiveness of an ETEC challenge based on diarrhea, rectal temperature, fecal bacterial shedding, immunoglobulin A, and intestinal mucosa ETEC receptor expression. Luise et al. (2019b) provided a great summary of features to consider when planning F4 or F18 challenge experiments in effort to decrease the variability in the challenge response. However, to our knowledge, there has been no attempt to provide quantitative assessment of this variability that can be useful to continue refining and standardizing ETEC infection studies. Since sample size calculations are often conducted using information extracted from published studies, the immense variability across the body of literature has perhaps resulted in subsequent sample sizes

not truly reflective of what is required to generate a statistically significant outcome. Therefore, our objective was to review recent ETEC challenge experiments and provide quantitative measurements of the responses and variability among the most widely evaluated response criteria. Using this information, a second aim of this work was to generate a sample size calculation tool that accounts for this variability and can assist researchers with designing future ETEC challenge studies.

Materials and Methods

The literature search was conducted utilizing PubMed (www.pubmed.ncbi.nlm.nih.gov), Web of Science (www.webofscience.com), and Scopus (www.scopus.com). The aim was to identify studies that challenged weanling pigs with ETEC F4 or F18. Search terms included a combination of the following: weanling pig OR nursery pig AND Escherichia coli OR ETEC F4 OR ETEC F18 OR ETEC K88 OR challenge. To narrow the search so that response criteria could be identified, a subset of search terms were included: growth OR immune OR fecal. Bibliographies from papers generated from the search were also scanned to identify relevant literature. Articles were restricted to peer-reviewed, in vivo studies, with English as the primary language and published between the years of 2010 and 2022. This time frame was selected in effort to account for the vast changes in genetic composition, nutrition, health, and management seen in the swine industry over recent years. Additionally, these parameters allowed for enough data to be extracted without overlapping previously written reviews. Studies utilizing a lipopolysaccharide (LPS) challenge were not included. Once relevant papers were located, data were organized to identify the most frequently evaluated response criteria. Next, the means and measure of variability were extracted for each response criterion where quantitative values were

provided (i.e., data presented as figures or charts with no numeric measure were not utilized) and summarized in a series of tables. For each response criteria, the mean and standard deviation (SD) were presented by the type of ETEC utilized for the challenge, then chronologically according to the publishing date for each reference. Additionally, for each response the percent change between the control and treatment groups was calculated to provide an estimated magnitude of effect. The data from these tables were then organized in a literature database within a Microsoft Excel spreadsheet. This database was utilized to develop two calculators located within the Excel spreadsheet that use the mean and SD from the literature to determine the sample size needed to detect a significant difference between two treatment groups (calculator 1) or given a desired magnitude of difference (calculator 2). More detailed information regarding the sample size calculators is provided at the end of this review.

Results and Discussion

Considerations of the Challenge Model

The multi-dimensional nature of published ETEC infection models makes it difficult to directly compare studies. Several factors including the use of experimental controls, the age and genetic background of experimental animals, the timing and dosage of inoculation with ETEC, study length, response criteria measured and methodology to evaluate them, and the number of replicates is vastly different across the body of literature. The importance of experimental controls has long been understood (Boring, 1954), however, as it relates to ETEC challenge studies, the research question ultimately dictates the type of controls that will be used. If a researcher is interested in investigating how ETEC infection impacts the pig, then including a non-challenged control group is critical. However, this adds another layer of complexity when

designing the study, as strict biosecurity measures are needed to prevent the cross-contamination between treatments to keep the non-challenged pigs healthy. Often times, this requires additional personnel or enhanced biosecurity level 2 (BSL-2) facilities with separate housing environments, which could limit the feasibility of the study. Conversely, if the researcher's objective is to study the efficacy of an intervention strategy against ETEC infection, then perhaps a non-challenged group of pigs may not be required. In this case, all pigs may be challenged with ETEC, but the use of a strong negative or positive control intervention would accomplish this objective. Among studies included in this review, 67% included a non-challenged control group, and each of these papers provided statistical comparisons of non-challenged pigs to pigs challenged with ETEC.

The dose and timing of inoculation with ETEC are both crucial parameters that are extremely variable across the studies presented in this review. For the sake of simplicity, we have displayed the inoculum dosages across this review as the total colony forming units (CFUs) administered to the animal, since many studies report the dosage of inoculum in mL and concentration on a per mL basis. The range of total CFUs administered to pigs to elicit a challenge is just one of many factors that makes comparison of challenge models difficult, and future work focusing on standardizing this dose should be done. Across the studies included in this paper, the range in dosage of ETEC F4 was from 1.5×10^5 to 1.0×10^{12} CFUs, while F18 dosages varied between 2.0×10^9 and 9.0×10^{10} CFU. It has been demonstrated that ETEC infection can occur with a single dose of approximately 10^9 CFUs (Berberov et al., 2004; Wellock et al., 2008; Boeckman et al., 2022). However, it appears as though the type and dosage of ETEC play a role in the response observed, depending on the outcome variable being evaluated. This is discussed further in subsequent sections.

The timing of inoculation with ETEC is also important to consider and varies among studies. Based on our review of the literature and information presented in Luise et al. (2019b), inoculation appears to be most effective when it coincides with the expression of ETEC receptors in the piglets small intestine, which are impacted by age. According to Fairbrother et al. (2005), F4 fimbriae receptors within the piglet's intestine are expressed from birth until maturity. Conversely, others have suggested that F18 receptors may not be fully expressed until closer to 18-20 days of age (Nagy et al., 1992; Nadeau et al., 2017). This indicates that administration of the inoculum during ETEC challenge studies should coincide with the time at which the small intestine has adequate receptivity for bacterial attachment. The weaning age of piglets within the included papers varied from 19 to 28 days of age, therefore we would expect to see expression of both ETEC F4 and F18 receptors by all pigs. The F4 and F18 strains of ETEC are commonly associated with PWD; therefore, it is important that ETEC challenge studies simulate the multiple stressors pigs undergo during the weaning transition which coincide with the incidence of PWD. Thus, across the tables in this review, the timing of inoculation is reported as the number of days post weaning (dpw), rather than the age of pigs at time of inoculation. The amount of time that passes between the weaning transition and ETEC challenge may impact the response observed, making dpw a more suitable characteristic to compare studies.

Sample size is one of the most variable features of ETEC challenge studies. Determining the number of replicates needed per experimental group is a crucial component of designing a study (Wittes, 2002). Generally, sample sizes are calculated from previously published studies using the mean and measure of variability. Thus, the wide range of sample sizes utilized across ETEC studies is not surprising, given the extreme variation in responses that are reported across the literature. In the subsequent sections of this review, we summarize the types of responses and

variability seen among several of the most widely evaluated response criteria in ETEC F4 and F18 challenge studies. Using data extracted from these papers, we have generated tables to display quantitative values for these responses that can assist researchers with sample size calculations for future studies.

Growth Performance

Maximizing growth performance in the post-weaning period is of great importance yet is challenging due to low feed intake the first 7 – 10 days after weaning. Proper development of the GI tract during this time is critical, where the rate and efficiency of gain can be heavily impacted by GI health and function (Becker et al., 2020). Evaluating average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (G:F) are commonplace in swine nutrition research. However, when considering challenge studies, the nature of the research question ultimately dictates whether growth performance is a valuable response criterion to examine. For example, those investigating a nutritional intervention to ETEC infection may place greater emphasis on growth performance than researchers studying the effect of a vaccination program on immunological response. In the most practical sense, quantifying changes in growth performance following ETEC challenge can provide useful information to the swine industry, and collection of these data is inexpensive and rather simple compared to responses discussed through the remainder of this review. However, our findings indicate that there is a high degree of variability surrounding growth performance responses to both F4 and F18 ETEC.

Many factors can impact whether a significant growth performance response is observed and the magnitude of that response, such as the number of replicates, the age of piglets at time of inoculation, the dosage of inoculation, and the degree of change between the control and treatment groups. Based on the studies reviewed, both a larger dose and a greater effect size were

needed to observe a significant difference in ADG when pigs were inoculated with F18 ETEC compared to F4. A final inoculum concentration of 1.95×10^{10} CFU and a difference of approximately 45% were needed to see a significant ADG response following an F18 challenge. Conversely, in F4 challenge studies, a dosage of 5×10^{9} CFU and a difference of 24% yielded a significant change in ADG. It has been shown that expression of F4 receptors in the small intestine is earlier than that of F18 (Sun and Kim, 2017); however, it is difficult to confer whether this directly impacts the magnitude of response seen, given other factors of the challenge model can also play a role. Inoculum dose and the percent change between control and treatment groups did not play as large of a role in piglet responses in ADFI following ETEC challenge, and very few studies observed significant responses in G:F.

A goal of our review was to provide a summary of the variability around these response criteria to assist in sample size calculation for future studies. The importance of appropriate replication does not need reiterated but appears to be a factor that explains the variability in piglet growth performance responses following ETEC challenge. In a study by Koo et al. (2020), the authors challenged pigs with 5×10^{10} ETEC F4 and observed a 33% and 22% reduction in ADG and ADFI, respectively, but these differences were not statistically significant. Along with a relatively small sample size (n = 7 pigs/treatment), pigs in this study were challenged on d 10, but data collection post-challenge only lasted until d 13. Generally, a longer data collection period would be preferred to ensure detection of statistically significant differences in performance, if present. However, since clinical signs of ETEC infection can manifest rather quickly post-challenge, leading to drastic reductions in growth performance, it is difficult to conclude whether the 3-d collection period explains the lack of statistical difference reported by these authors. An ETEC F4 challenge study by Xu et al. (2020) used 1×10^{10} CFU and observed

a 27% decrease in ADG with 6 replications per treatment and a 7-d data collection period, but again, these differences were not statistically significant. The number of replicates used across studies varies considerably. This is likely due to the immense variability in the type of growth performance responses reported as well as the variability within these responses across literature, which has made sample size calculation challenging. Table 1 summarizes the responses seen and variability surrounding ADG, ADFI, and G:F. The means and standard deviations (SD) represent the period post-inoculation from each study. Additionally, a percent change column was included for each response to understand the difference that was observed between the control and treatment groups for each study. It is important to note that not all papers included in this review utilized a non-challenged control group. A greater proportion of studies reported a significant response in ADG and ADFI when compared to G:F. However, due to the relatively small sample size used in most ETEC challenge studies, interpretation of growth responses should be done carefully. Regardless, the importance of quantifying changes in growth performance due to ETEC challenge is apparent, especially when considering nutritional intervention strategies for PWD caused by ETEC. However, from our review of the literature, the concentration of inoculum and number of replications per treatment group appear to heavily impact the response seen.

Fecal Parameters

Fecal Consistency

One of the most notable and easily detected clinical signs of ETEC infection is diarrhea, which can be defined as an increased frequency of defecation accompanied by feces which contain reduced dry matter content (Pedersen et al., 2011a). Diarrhea can last anywhere from 1 to 5 days following ETEC infection (Sun and Kim, 2017). In research settings, evaluation of fecal

consistency as an indicator of diarrhea is a widely used tool to determine the effectiveness of the challenge model (Luise et al., 2019b). Generally, visual fecal scoring or analysis of fecal dry matter (DM) are utilized to assess fecal consistency; however, there is substantial variability in the methodologies used to evaluate these response criteria. Fecal scoring requires the subjective evaluation of the consistency of feces, usually per experimental unit, according to a Likert-type scale. Various fecal scoring scales have been implemented within the literature, but most studies included in this review utilized one of two scoring systems: a 1 to 5 scale where 1 = normal feces and 5 = severe diarrhea, or a 0 to 3 scale where 0 = normal feces and 3 = severe diarrhea. Due to the subjective nature of visual fecal scoring, it is imperative that the same scorer(s) are utilized for trial entirety and that proper blinding of the fecal scorers to treatments is carried out as to avoid observation bias. Another challenge of visual fecal scoring is determining the threshold that dictates clinical diarrhea, which varies between studies depending on the fecal scoring scale that was used. For example, Sun et al. (2021) scored feces on a 0 to 3 scale, where fecal scores ≥ 1 were classified as diarrhea. Alternatively Li et al. (2019) used a 1 to 4 scale for fecal consistency assessment and considered scores ≥ 3 as diarrhea. This difference in experimental procedures may seem small, but greatly contributes to the variability seen within literature as it relates to fecal consistency. In general, visual fecal scoring is subject to intra- and inter-observer variation (Pedersen and Toft, 2011), and the resulting data is inherently qualitative and ordinal in nature. When assessing a response criterion according to a scale like fecal scoring, the outcomes are not continuous or discrete, which can lead to inaccuracies in statistical analysis, where often these data are analyzed as if they were quantitative and normally distributed. White et al. (2016) reiterated the fact that analyzing qualitative variables, such as fecal score, can be difficult and requires the appropriate use of inferential statistics to avoid reporting misleading results.

Unfortunately, this mistake is commonly made in animal science research and was observed on nearly every account among the studies included in this review. Therefore, analysis of fecal DM can serve as a more objective measurement of fecal consistency and may be more straightforward to analyze statistically given it is quantitative and reasonably fits model assumptions for normality. This methodology involves the drying of fecal samples to calculate the amount of moisture extracted during the drying process, resulting in a final DM % of the sample (Pedersen et al., 2011b). Multiple protocols have been developed for evaluating fecal DM (Klopfenstein et al., 1995; Callan et al., 2007; Partanen et al., 2007) with varying degrees of repeatability and reproducibility. Of the studies reviewed, only one utilized the fecal DM approach, with all other studies implementing some degree of visual fecal scoring (Table 2). Application of fecal DM analysis may be limited due to necessary equipment to conduct analyses and time constraints for collection and drying of samples. Additionally, a pitfall of fecal DM analysis specific to weanling pig studies is the difficulty in collecting appropriate quantities of fecal material required for analysis, given the small amount of feces produced from a newly weaned pig.

Again, the appropriate use of experimental controls is necessary to effectively evaluate fecal consistency as a response criterion. A non-challenged control group is recommended for *in vivo* experiments (National Research Council, 2011), which would allow for direct comparison to groups challenged with ETEC. Table 2 displays ETEC challenge studies evaluating fecal consistency and their respective responses. Unfortunately, nearly all fecal scoring data is presented in the form of figures and statistical information such as standard error of the mean are not provided, making it difficult to quantify the variability in fecal consistency responses seen within the literature. The ordinal nature of this data makes sample size calculation difficult, thus,

fecal DM may be a more suitable response to evaluate. Despite only one ETEC challenge study using the fecal DM approach, there are other published papers in non-challenged pigs where fecal DM analysis was conducted (Chance et al., 2021; Laskoski et al., 2021). Because these data are quantitative, they are typically presented in a form that allows scientists to extract measures of variability and means needed for sample size calculation. Despite being unable to summarize the variability in fecal consistency responses, it is important to note that only three papers included in this review failed to see a statistical difference in fecal consistency upon ETEC challenge. The body of literature strongly favors a worsening in fecal consistency when ETEC challenge is induced, and this response was reported as early as 6 hours post-inoculation (hpi;(Lei and Kim, 2020), and lasted as long as 25 days post-inoculation (dpi;(Sun et al., 2021). Utilizing fecal consistency as an indicator of ETEC challenge efficacy is valuable. However, there is a need for the standardization of the methodologies used for data collection and reporting of results such that statistical information is provided to assess variability for sample size calculation.

Fecal Bacterial Shedding

The normal microbiota-host relationship of the weanling pig prompts the shedding of multiple bacteria every day. In newly weaned piglets, infection with pathogenic bacteria such as ETEC causes an increase in the fecal shedding of that organism, which creates an environment for pathogen transmission to other animals via the fecal-oral route (Pluske, 2013). In the case of ETEC challenge studies, fecal bacterial shedding has been evaluated on several accounts, but with high degrees of variability in the methodologies used. Studies have investigated parameters such as total bacterial shedding (Wojnicki et al., 2019; Smith et al., 2020), total *E. coli* shedding

(Li et al., 2015; Becker et al., 2020), F4 or F18-specific *E. coli* shedding (Hong et al., 2021), and hemolytic coliforms (He et al., 2022). Given *E. coli* shedding can occur even in healthy pigs, the most reliable evaluation of bacterial shedding as an indicator of ETEC infection is suggested to be specific to F4 or F18 strains. Typically, a 3 to 4 day measurement period post-inoculation is required for proper adherence, colonization, and toxin production within the small intestine (Luise et al., 2019b). A notable difference among studies evaluating fecal bacterial shedding, regardless of the specific outcome variable they report, is the method by which bacteria are isolated from the feces. Wojnicki et al. (2019) and Smith et al. (2020) both utilized quantitative real-time polymerase chain reaction (qRT-PCR) technology for fecal bacterial enumeration. Alternatively, studies by Becker et al. (2020), Li et al. (2015), and Li et al. (2019) quantified fecal bacteria via culture methods using selective media. Constraints related to experimental budgets, time, expertise, and required equipment may be the driving force behind which method of evaluation is used. While both procedures are widely accepted in literature, differences in the sensitivity and reproducibility of each procedure are important to consider (Lee et al., 2013). Regardless of the methodology used, the literature suggests that if ETEC infection is carried out effectively, the rate and number of bacteria shed in feces will increase. Perhaps the greatest difficulty in interpreting responses in fecal bacterial shedding stems from the presentation of data. The papers included in this review which evaluated fecal bacterial shedding provided data in the form of bacterial shedding scores, $\log_{10}$ CFU/g, and raw cycle threshold (CT) values, depending on the method of bacterial enumeration used. Directly comparing means and variability across studies is not justified and could lead to inaccurate interpretation of results. Additionally, like fecal consistency, these data are often presented in figure form to demonstrate the bacterial shedding over time, with no measure of variability provided. This causes the

inability to utilize these published studies in power calculation, which is a severe pitfall of the body of literature. Table 3 provides a summary of the challenge models which quantified bacterial shedding and the response observed. Despite being unable to provide quantitative values for the variation in fecal bacterial shedding responses within these papers, Table 3 displays information that may be useful in the planning of future challenge work. Based on the information presented here and among other similar reviews, it appears that fecal bacterial enumeration should be conducted using PCR methods that allow for quantitative detection of F4 or F18 specific ETEC to accurately determine shedding of the pathogen.

Immunological

Immunoglobulins

Immunoglobulins play a pivotal role in the host defense against pathogens such as ETEC. Several immunoglobulins have been looked at as markers of ETEC infection in challenge studies, however, it is important to consider the nature of each immunoglobulin's role in the humoral immune response during infection. Immunoglobulin A (IgA) is primarily found in bodily secretions such as saliva, milk, and as it relates to ETEC infection, intestinal fluid (Tizard, 2009). Secretory IgA (sIgA) is the most prevalent antibody of the intestine, protecting the intestinal tract from microbial colonization by preventing the adherence to the mucosal surface (Mantis and Forbes, 2010). Quantification of IgA has been done on multiple accounts (Table 4) with varying methodologies. Evaluation of sIgA must be done at the mucosal level, thereby requiring the euthanasia of the pig, so many studies evaluate circulating IgA in plasma or serum. Most studies included in this review analyzed IgA concentrations in the serum. The variability for serum IgA was lower than IgA in the intestinal mucosa. Based on included papers, to elicit an

IgA response with ETEC challenge, a final concentration of ETEC inoculum of at least 5×10^9 CFU was required.

In addition to IgA, immunoglobulins G (IgG) and M (IgM) have also been evaluated as indicators of immune function in weanling pigs following ETEC challenge. Immunoglobulin G is the predominant immunoglobulin found in plasma, and the weaning transition can cause a 4-fold reduction in IgG levels within the pig (Butler et al., 2009). Next to IgG, IgM is the second most prevalent immunoglobulin in the serum and is produced in small amounts during a primary immune response (Tizard, 2009). While both immunoglobulins have been studied upon ETEC challenge within the literature, quantifying their levels post-inoculation appear to be rather ineffective given they do not contribute directly to the host's defense against bacterial adhesion at the mucosal level, when compared to IgA (Gaskins, 1998).

When evaluating immunoglobulins following ETEC challenge, consideration should be given to the time point at which sample collection will occur. Generally, once the animal has been exposed to a pathogen, it can take anywhere from 5 to 7 days for antibodies to appear, and typically, the peak of antibody production happens around 10 to 14 days post-infection (Tizard, 2009). Studies have reported responses in IgA production to ETEC as early as 7 dpi and lasting as long as 24 days (Nadeau et al., 2017; Laird et al., 2021). Among papers included in this review, the range in time points where a significant response in IgA was reported either in serum or the intestinal mucosa was rather large. Interestingly, Li et al. (2015) saw a significant increase in circulating IgA as soon as 24 hpi. Studies by Chang et al. (2022) and Trevisi et al. (2011) observed significant responses in IgA much later at 7 dpi. Both Becker et al. (2020) and Sugiharto et al. (2015) evaluated IgA in the intestinal mucosa and observed significant IgA changes at 10 and 8 dpi, respectively. It is apparent that evaluating immunoglobulins,

specifically IgA, can serve as crucial indicators of ETEC infection. The timing of inoculation and the amount of time needed for peak activation of the host's antibody defense system should be considered when evaluating these response criteria. While significant differences in IgA have been reported extremely early following an ETEC infection on rare accounts, it appears that analysis of IgA is most effective at least 7 dpi, and many authors continue evaluating IgA through later stages of infection, as late as 21 dpi. Additionally, the variability surrounding IgA within the literature can partially be attributed to differences in piglet age and laboratory procedures to quantify immunoglobulin presence in both serum and mucosa.

Pro-Inflammatory Cytokines

Pro-inflammatory cytokines are secreted proteins that act as signaling cells during an immune response, communicating with other immune cells of the body to target the pathogen upon infection (Zhang and An, 2007). Previous studies have focused heavily on the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as they have been reported to be primary markers of pathogenic infection in the weanling pig (Zhang et al., 2010). Regulation and secretion of these cytokines is important for the pig to elicit an immune response upon ETEC infection; however, over-stimulation of these proteins, especially TNF-α, can result in reverse pathological effects that lead to inflammation. Therefore, the goal should be to decrease pro-inflammatory cytokine production following ETEC infection, thus, alleviating inflammation (Grimble, 1998). Ljuca et al. (2010) demonstrated that circulating cytokines in the serum are highly correlated to mucosal inflammation, indicating that these parameters could effectively be used to assess intestinal disease. Within the literature, TNF-α and IL-6 are the most predominantly studied pro-inflammatory cytokines as it relates to ETEC

challenge trials, with most authors quantifying their presence in serum. Many studies reported a significant response in both parameters, but there is still a great deal of variability surrounding each response. In part, piglet age at time of sample collection and methodologies to quantify the levels in serum most likely contribute to the variation that is seen within these outcome variables. Generally, the enzyme linked immunosorbent assay (ELISA) is used to quantify parameters such as pro-inflammatory cytokines, and this methodology was used across nearly all studies evaluating TNF-α and IL-6. While the ELISA is considered a reliable and effective measurement tool, considerations for differences in laboratory procedures could potentially explain variability in responses seen between studies. Similar to quantifying levels of immunoglobulins, it is nearly impossible to standardize all factors which can account for variability in the responses seen (i.e., age of piglets, laboratory techniques or methods of evaluation) for immunological parameters. However, there is a need for research to focus on identifying optimal challenge model specifications, such as inoculum dose, timing of inoculation, duration of study, and genetic susceptibility of piglets, such that future experiments can follow a more standardized approach to ETEC infection. Based on the information we've summarized, assessment of TNF- α and IL-6 appear to be the most effective pro-inflammatory cytokines indicative of a response to ETEC. Evaluating these parameters can be done in both serum or mucosa, and most commonly using ELISA methods. Careful consideration of the large degree of variability should be done when conducting sample size calculations from previously published studies. The variability in both TNF- α and IL-6 responses among studies included in this review are presented in Tables 5 and 6, respectively.

Intestinal Morphology and Permeability

It is well-understood that during the weaning transition, villi atrophy can occur, resulting in less mucosal surface area available for absorption of nutrients or for proteins like sIgA to bind to (Pluske, 2013). Evaluation of small intestinal morphology can be a beneficial measurement to assess following ETEC challenge, especially when nutritional intervention is expected to alleviate the detrimental effects that bacterial adhesion and toxin release can have on the intestinal mucosa. However, euthanasia and necropsy are required to obtain such measurements, therefore limiting the ability of some researchers to assess these outcome variables. There are numerous reports in the literature of changes in small intestinal morphology post-inoculation with ETEC. Table 7 summarizes the studies included in this review that evaluated changes in small intestinal morphology. Assessment of morphology includes quantifying the villus height (VH), crypt depth (CD), and the VH:CD ratio by staining intestinal tissue with hematoxylin and eosin, where a larger VH and smaller CD are indicative of a more favorable morphological environment. It has been reported that ETEC infection can have detrimental effects on the small intestinal morphology, subsequently increasing intestinal permeability (Rong et al., 2015). This type of response was common in the reviewed papers, where ETEC infection, regardless of inoculum dose, timing, or other factors impaired villi height (Becker et al., 2020; Choi et al., 2020; Duarte et al., 2020). Interestingly, very few authors reported a significant response in CD post-inoculation. This is supported by Al Masri et al. (2015), who reviewed piglet small intestinal morphology surrounding weaning and suggested there was no clear evidence that CD changes immediately post-weaning or upon pathogenic infection. Including small intestinal morphology as outcome variables appear to be valuable tools to help assess the impacts of ETEC on the weanling pig, especially in studies investigating nutritional interventions that show

promise to alter the intestinal morphology post-weaning. Given morphological changes can occur around weaning even in clinically healthy pigs, the use of non-challenged control pigs would be beneficial when assessing small intestinal morphology following ETEC challenge to determine if changes in morphology are attributed to ETEC.

Aside from the morphological changes, ETEC infection is also suspected to alter the permeability of the small intestine. Within the lumen of the small intestine resides a layer of epithelial cells that serve as the first line of defense against pathogens. Together, these mucosal cells form a barrier that is maintained by the connecting enterocytes, where specialized junctions known as tight junctions (TJs) seal the intercellular spaces between them (Mukiza and Dubreuil, 2013). These TJs are made up of a protein complex consisting of occludin (OCLN), zonula occludens protein-1 (ZO-1) and members of the claudin (CLDN) family of proteins (Puthenedam et al., 2007). Upon ETEC infection, TJs work to maintain epithelial barrier integrity in effort to prevent the passage of endotoxins across the intestinal brush border. This is generally accompanied by alterations in the expression of genes related to TJ proteins. Very few studies evaluated these outcome variables following ETEC infection and reported variable results. Becker et al. (2020) quantified the mRNA abundance of OCLN, ZO-1, and claudin-1 (CLDN-1) in the ileum of pigs at 10 dpi and reported elevated expression of OCLN and ZO-1 in unchallenged pigs compared to those challenged with ETEC, but no response in CLDN1 expression. Conversely, Li et al. (2019) evaluated these parameters 7 dpi and observed a significant increase in CLDN1 and no evidence of differences in OCLN or ZO-1. Work by Choi et al. (2020) found no change in OCLN and ZO-1 post-inoculation and only a tendency for elevated CLDN1, while Kim et al. (2019) saw increased ZO-1 and no difference in OCLN or CLDN1 in the jejunum of pigs. Other techniques to measure epithelial transport more directly

include *ex vivo* experiments using Ussing chambers or *in vivo* assessments via orally administered markers of gut permeability. However, to our knowledge, the application of these procedures in ETEC infected pigs is severely limited. While it is well understood that ETEC infection can have detrimental impacts on small intestinal morphology and permeability, emphasis on alterations in TJ proteins is needed moving forward, considering the relatively low number of studies evaluating these response criteria and the high degree of variability among published literature.

Development of a Sample Size Calculation Tool

After identifying the pertinent response criteria to include in ETEC challenge studies and providing quantitative measures of their responses and variability, our goal was to create a tool that could help researchers effectively compute sample size requirements for future studies using the information compiled in this review. The values for the means and SD from each study and every response criterion described throughout this paper were filed into a Microsoft Excel database. From there, two calculators were built to determine the number of replications needed to detect a statistically significant difference between two treatment groups or based on a specified magnitude of difference. These calculators can be found in Supplemental File 1, where the first tab of the tool provides instructions for its use. However, to better outline how this tool can be used, two example scenarios are featured below.

Scenario 1 (Sample Size Required to Detect a Difference Between Two Groups)

Calculator 1 allows you to determine the number of animals needed per treatment group to detect a significant difference between two groups. This calculator uses the mean and SD values from the ‘Literature Database’ tab, which coincide with the values presented in tables

throughout this review. To begin, you should thoroughly read the information found on the 'Instructions' tab, and then click on the tab labeled 'Calculator 1' to begin your analysis. For this example, we will assume the following:

- The ETEC strain being used for the challenge is F18 (cell D8)
- The significance level (α) will be set at 0.05 (cell D9)
- The power ($1 - \beta$) will be set at 80% (cell D10)
- The control group means will be pulled from column H (Mean Value from Literature)
- We want to determine the number of animals required to detect a significant difference in ADG
- The difference indicative of a biologically relevant effect in ADG has been determined to be 35% following challenge with ETEC F18

The steps to utilize this calculator to generate the required sample size given this scenario are as follows:

1. Select 'F18' as the ETEC type (cell D8)
2. Select '0.05' as the α value (cell D9)
3. Select '80%' as the Power value (cell D10)
4. Copy the 'Mean Value from Literature' for ADG of 303 (cell H17) to your 'Control Mean' (cell D17)
5. Using the magnitude of effect of 35% as declared above, the value for the 'Treatment Group' (cell E17) would be 408

The tool will then calculate the number of animals required to detect a statistically significant difference between these two groups in the 'Pigs Per Treatment Needed' area (cell F17). The calculation for this tool is derived from Dohoo et al. (2003) and is described below.

$$n = 2 \times \left[\frac{\left(Z_{\frac{\alpha}{2}} - Z_{\beta} \right)^2 \times \sigma^2}{(\mu_1 - \mu_2)^2} \right]$$

Where *n* represents the sample size, $Z_{\frac{\alpha}{2}}$ represents the Z critical value for the significance level (α; the probability of a type I error), Z_{β} represents the Z critical value for beta (β; the probability of a type II error), σ^2 represents the standard deviation, μ_1 represents the control group mean, and μ_2 represents the treatment group mean. In this example, the number of animals required per treatment group to detect a statistically significant difference in ADG would be 29 pigs.

Scenario 2 (Sample Size Required to Detect a Significant Difference Given an Effect Size)

Calculator 2 allows you to determine the number of animals required per treatment group given a specified magnitude of effect (i.e., the difference between the control and treatment groups). Since many ETEC challenge studies are conducted in specialized BSL-2 facilities, often there can be constraints on the experimental design due to space availability. In this case, it is critical to understand what the minimum number of replications would be to detect a statistically significant difference in the response criteria you are evaluating. Calculator 2 utilizes a desired magnitude of difference to provide sample size estimates for a series of effect sizes, which helps the user visualize the minimum difference that would be required to see a response. Additionally, this calculator allows the user to determine the maximum number of possible treatment groups that could be utilized in a study given a specified number of pens (assuming that pen is the

experimental unit; regardless of if pigs are housed individually or as a group). Again, this calculator uses the mean and SD values from the 'Literature Database' tab, which coincide with the values presented in tables throughout this review. To begin, you should thoroughly read the information found on the 'Instructions' tab, and then click on the tab labeled 'Calculator 2' to begin your analysis. For this example, we will assume the following:

- The ETEC strain being used for the challenge is F4 (cell D6)
- The significance level (α) will be set at 0.05 (cell D7)
- The power (1 - β) will be set at 80% (cell D8)
- The control mean and standard deviation will be pulled from the 'Literature Database' tab
- We want to determine the number of animals required to detect a significant difference in ADFI
- The difference indicative of a biologically relevant effect in ADFI has been determined to be 15% following challenge with ETEC F4
- In our experimental facility, we have a total of 40 pens to individually house animals (i.e., the animal/pen will be the experimental unit)

The steps to utilize this calculator to generate the required sample size given this scenario are as follows:

1. Select 'F4' as the ETEC type (cell D6)
2. Select '0.05' as the α value (cell D7)
3. Select '80%' as the Power value (cell D8)

4. Select the response criterion of choice (cell D9)
5. The control mean (cell D10) and standard deviation (cell D11) will auto-populate based on the information selected above
6. Enter the desired magnitude of difference of 15% (cell D12)
7. Enter the number of pens as 40 (cell D16)

The tool will provide sample size calculations for 10 different effect sizes. The initial magnitude of difference was set at 15% in cell D12, however, in cells D21 – M21 the range of effect sizes will be calculated. From there, the tool will hold the control mean constant (cells D19 – M19), and calculate the required treatment mean based on the magnitude of difference needed (cells D20 – M20). Next, the number of pigs required per treatment will be calculated in cells D23 – M23 and are highlighted in green. Additionally, the total number of animals needed will be calculated in cells D24 – M24, assuming two treatment groups. Finally, based on the number of pens that were provided in cell D16, the tool will calculate the maximum number of possible treatments that can be used given these experimental constraints. These values will calculate in cells D25 – M25 and any non-feasible values will highlight in red, meaning that the sample size required to detect a statistically significant difference is not feasible given the facility constraints you provided.

This calculator uses the same equation by Dohoo et al. (2003) as mentioned above. For this example, the number of animals required per treatment group to detect a statistically significant difference in ADFI in a facility with 40 pens and an expected magnitude of difference between the control and treatment groups of 15% would be 130 pigs per group. As the magnitude of differences increases from 15% to 150% (cells D21 – M21), the number of animals

required per treatment group becomes smaller. As indicated by the red 'n/a' in cell D25, based on the assumptions provided above, we would not have enough statistical power in this facility to see a statistically significant response in ADFI.

Conclusions

In summary, there is considerable variation in piglet responses to ETEC F4 and F18 infection during challenge experiments. While this review summarized the most frequently evaluated response criteria used during these types of studies and quantified the variability around them; there is still a large magnitude of work needed to standardize a repeatable and reproducible challenge model for ETEC. Many factors contribute to the high degree of variation observed in the literature, which includes, but is not limited to the number of experimental animals needed, the precise dosage and timing of ETEC inoculation, and animal selection criteria. Additionally, there are many other outcome variables that have been studied, but were not included in this review, as their application was more limited. Here we have used the information from this review to quantitatively summarize the responses and variability from ETEC challenge models and generate a tool to allow for more accurate and simplified sample size calculations for future work. While this tool can provide value, it is important to consider it does not include all published ETEC data, nor does it account for external factors such as laboratory practices that could perhaps impact variability of response seen across studies. The complexity of ETEC studies and the inherent difficulty in utilizing their data to guide future models is ever-changing and requires constant reassessment. Additionally, further research surrounding experimental infection with ETEC in the weanling pig is needed to more effectively investigate nutritional or management strategies to mitigate PWD caused by ETEC.

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Table 1.1. Summary of variability in growth performance responses following ETEC challenge in weanling pigs.

	Description of Challenge Model				ADG, g/d		ADFI, g/d		G:F	
Reference	ETEC Type	Inoculum Dose (CFUs)	Inoculation Time (dpw)[2]	Study Length (dpi)[3]	Mean ± SD	% Change[4]	Mean ± SD	% Change[4]	Mean ± SD	% Change[4]
Liu et al., 2010	F4	3×10^{11}	7	7	265 ± 19.0*	-16	392 ± 76.8*	-7	0.67 ± 0.21+	-11
Nyachoti et al., 2011	F4	1×10^{10}	8	7	161 ± 96.0	+79	230 ± 99.0	+50	0.72 ± 0.39	+20
Trevisi et al., 2011	F4	2×10^{10}	7	5-7	138 ± 63.7*	-39	285 ± 36.1*	-16	-	-
Lee et al., 2012	F4	5×10^{9}	14	14	449 ± 39.2*	-31	647 ± 203.3	-16	0.69 ± 0.09	-18
Wu et al., 2012	F4	5×10^{9}	13	6	363 ± 18.1*	+18	595 ± 27.7+	+6	0.62 ± 0.03*	+13
Almeida et al., 2013	F4	9×10^{10}	6	5	143 ± 180.2*	-42	591 ± 545.9	-12	0.27 ± 0.14	-32
Gao et al., 2013	F4	1×10^{9}	1, 3, 5, 7	11	100 ± 48.9*	-29	280 ± 73.5	-13	0.37 ± 0.05*	-16
Khafipour et al., 2014	F4	6×10^{10}	8 – 10	10	245 ± 69.8	-16	333 ± 36.7	-12	0.73 ± 0.22	-4
Yang et al., 2014	F4	1×10^{9}	15	3	323 ± 147.0	-9	374 ± 36.7	-2	0.80 ± 0.47	-11
Li et al., 2015	F4	2×10^{10}	14	27	400 ± 60.9	+8	598 ± 76.7*	+3	0.67 ± 0.05*	+5
Trevisi et al., 2015	F4	2×10^{8}	7	14	201 ± 231.5	-5	-	-	-	-
Chen et al., 2016	F4	5×10^{10}	7	7	198 ± 73.5	+8	351 ± 83.1	+24	0.56 ± 0.15*	+19
Han et al., 2016	F4	3×10^{8}	0	7	142 ± 51.4*	-73	-	-	-	-
Lee et al., 2017	F4	3×10^{10}	7	14	142 ± 59.4*	-	-	-	-	-
Lei et al., 2017	F4	5×10^{10}	8 – 10	14	222 ± 8.9*	+44	298 ± 35.8*	+34	0.74 ± 0.07	+6
Pan et al., 2017	F4	1×10^{11}	9	3	320 ± 45.8*	-21	391 ± 53.6*	-20	0.82 ± 0.05	-1
Koo et al., 2019	F4	5×10^{10}	10	3	376 ± 150.7	-33	590 ± 182.2+	-22	0.67 ± 0.20+	+7
Lopez-Colom et al., 2019	F4	2×10^{9}	7	8	117 ± 256.0	-9	211 ± 129.5	-4	-	-
Luise et al., 2019	F4	2×10^{5}	7	13	125 ± 94.2	+24	313 ± 148.0	+18	0.33 ± 0.35	-33

Choi et al., 2020	F4	5×10^7	7	5	306 ± 149.4[+]	-46	559 ± 105.3	-15	-	-
Lei et al., 2020	F4	5×10^9	22	2	428 ± 47.0[*]	-16	643 ± 40.3	-6	0.66 ± 0.08[*]	-10
Wang et al., 2020	F4	1×10^{11}	15	5	334 ± 40.3	+20	830 ± 57.7[*]	+21	0.40 ± 0.29	-4
Xu et al., 2020	F4	1×10^{10}	7	14	444 ± 83.8[*]	-27	729 ± 104.1[*]	-22	0.61 ± 0.05	-8
Yu et al., 2021	F4	1×10^{12}	19	2	390 ± 36.6	-8	486 ± 19.6[*]	-8	-	-
Kim et al., 2019	F18	3×10^{10}	7	11	214 ± 141.3[*]	-59	437 ± 201.9[*]	-36	0.46 ± 0.27	-33
Li et al., 2019	F18	2×10^{10}	7	7	250 ± 94.8[*]	-41	682 ± 94.9[*]	-31	0.62 ± 0.18	-20
Wojnicki et al., 2019	F18	9×10^{10}	13	10	505 ± 159.0	-10	1,282 ± 785.0	+3	0.39 ± 0.20[*]	-15
Becker et al., 2020	F18	1×10^{10}	7	10	238 ± 31.6[*]	-51	335 ± 31.6[*]	-33	0.63 ± 0.19	-33
Duarte et al., 2020	F18	2×10^9	13	13	355 ± 116[*]	-21	465 ± 82.0	-5	0.76 ± 0.16[*]	-17
Hong et al., 2021	F18	2×10^9	7	13	335 ± 86.6[*]	+78	416 ± 96.7[*]	+70	0.82 ± 0.10	+1
Sun et al., 2021	F18	6×10^9	7	12	435 ± 333.8	-3	664 ± 449.7	+0.39	0.65 ± 0.14	-5
Chang et al., 2022	F18	3×10^{10}	7	14	251 ± 57.2[*]	-50	393 ± 31.7	-3	0.64 ± 0.12[*]	-49
F4 Average	-	9×10^{10}	-	-	260 ± 86.3	-	436 ± 104.8	-	0.62 ± 0.17	-
F18 Average	-	3×10^{10}	-	-	323 ± 127.5	-	584 ± 328.5	-	0.62 ± 0.17	-

[*]Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).
[+]Indicates that a marginally significant difference was observed between the control and treatment groups ($0.05 < P < 0.10$).
[1]Mean ± SD represent the period reported immediately post-inoculation from each study.
[2]Time of inoculation with ETEC, days post-weaning (dpw).
[3]Length of time data was collected following inoculation with ETEC, days post-inoculation (dpi).
[4]The calculated percent change between the control and treatment groups.

Table 1.2. Summary of ETEC challenge studies evaluating fecal consistency as a response criterion.

	Description of Challenge Model						Fecal Consistency Response[4]	
Reference	ETEC Type	Inoculum Dose (CFUs)	Inoculation Time (dpw)[1]	Number of Replicates	Method[2]	Collection Time (dpi)[3]	Pre-Inoculation	Post-Inoculation
Heo et al., 2010	F4	1×10^8	3 – 5	12	FS	0 – 14	-	↑
Liu et al., 2010	F4	3×10^{11}	7	6	FS	0 – 7	-	↑
Nyachoti et al., 2011	F4	1×10^{10}	8	9	FS	0 – 5	-	ND
Trevisi et al., 2011	F4	2×10^{10}	7	6	FS	1 – 7	-	ND
Lee et al., 2012	F4	5×10^9	14	6	FS	0 – 14	-	↑
Almeida et al., 2013	F4	9×10^{10}	6	8	FS	0 – 5	-	ND
Gao et al., 2013	F4	1×10^9	1, 3, 5, 7	6	FS	0 – 11	-	↑
Khafipour et al., 2014	F4	6×10^{10}	8 – 10	5	FS	2, 4, 7	-	↑
Li et al., 2015	F4	2×10^{10}	14	7	FS	7, 14, 21, 28	-	↓
Trevisi et al., 2015	F4	2×10^8	7	10	FS	-12 hpi – 6	ND	↑
Chen et al., 2016	F4	5×10^{10}	7	9	FS	1 – 5	-	↓
Han et al., 2016	F4	3×10^8	0	6	FS	1 – 7	-	↓
Rhouma et al., 2016	F4	1×10^9	7	12	FS	-3 – 35	ND	↑
Lee et al., 2017	F4	3×10^{10}	7	8	FS	0 – 14	-	↑
Lei et al., 2017	F4	5×10^{10}	7	5	FS	-7 – 21	↓	↓
Pan et al., 2017	F4	1×10^{11}	9	6	FS	-9 – 12	ND	↑
Choi et al., 2020	F4	5×10^7	7	6	FS	0 – 54 hpi*	-	↑
Wang et al., 2020	F4	1×10^{11}	15	6	FS	0 – 6	-	↑
Lei et al., 2020	F4	5×10^9	22	5	FS	-21 – 3	ND	↑
Kim et al., 2019	F18	3×10^{10}	7	12	FS	0 – 11	-	↑
Li et al., 2019	F18	2×10^{10}	7	10	FS	-7 – 7	ND	↑
Wojnicki et al., 2019	F18	9×10^{10}	13	12 – 13	FS	-13 – 10	ND	↑

Becker et al., 2020	F18	1×10^{10}	7	8 – 10	FS	0 – 10	-	↑
Duarte et al., 2020	F18	2×10^{9}	7	8	FS	-3 – 20	ND	↑
Smith et al., 2020	F18	9×10^{10}	10	18	DM	7	-	↑
He et al., 2021	F18	6×10^{10}	7	12	FS	0 – 21	-	↑
Sun et al., 2021	F18	6×10^{9}	7	8	FS	-11 – 25	ND	↑
Chang et al., 2022	F18	3×10^{10}	4	9	FS	0 – 14	ND	ND

[*]Hours post-inoculation (hpi).

[1] Time of inoculation with ETEC, days post-weaning (dpw).

[2]Method for evaluation of fecal consistency: FS = fecal scoring; DM = fecal dry matter (DM) analysis.

[3]Length of time data was collected following inoculation with ETEC, days post-inoculation (dpi).

[4]Fecal consistency response following challenge with ETEC: ↑ indicates an increase in diarrhea; ↓ indicates a decrease in diarrhea; ND indicates no statistical difference in diarrhea.

Table 1.3. Summary of ETEC challenge studies evaluating fecal bacterial shedding as a response criterion.

	Description of Challenge Model							Fecal Bacterial Shedding Response[4]	
Reference	ETEC Type	Inoculum Dose (CFUs)	Inoculation Time (dpw)[1]	Number of Replicates	Method[2]	Outcome Variable	Collection Time (dpi)[3]	Pre-Inoculation	Post-Inoculation
Trevisi et al., 2011	F4	2×10^{10}	7	6	Culture	Total *E. coli*	10	-	ND
					PCR	F4 *E. coli*	10	-	ND
Lee et al., 2012	F4	5×10^{9}	14	6	PCR	F4 *E. coli*	2, 7, 14	-	↑
Khafipour et al., 2014	F4	6×10^{10}	8 – 10	5	Culture	Total *E. coli*	0, 11	ND	ND
					PCR	F4 *E. coli*	0, 11	ND	↑
Li et al., 2015	F4	2×10^{10}	14	7	Culture	Total *E. coli*	0, 1, 28	ND	ND
Trevisi et al., 2015	F4	2×10^{8}	7	10	Culture	F4 *E. coli*	0, 10	ND	↑
Chen et al., 2016	F4	5×10^{10}	7	9	Culture	Total coliforms	7, 11	ND	ND
Han et al., 2016	F4	3×10^{8}	0	6	PCR	F4 *E. coli*	1, 3, 7	-	↑
Rhouma et al., 2016	F4	1×10^{9}	7	12	Culture	Total *E. coli*	-1, 1 – 36	ND	ND
					PCR	F4 *E. coli*		ND	↑
Lee et al., 2017	F4	3×10^{8}	7	8	Culture	Total *E. coli*	1, 3, 7, 14	-	↑

Lei et al., 2020	F4	5×10^9	22	5	Culture	Total coliforms	-1, 2	ND	↑
						β- Hemolytic			
Kim et al., 2019	F18	3×10^{10}	7	12	Culture	Coliforms	2, 5, 8, 11	-	↑
Li et al., 2019	F18	2×10^8	7	10	Culture	Hemolytic *E. coli*	-7, 0, 1-3, 5, 7, 8	-	↑
Wojnicki et al., 2019	F18	9×10^{10}	13	12 – 13	PCR	F18 *E. coli*	0, 5, 10	ND	↑
						Total *E. coli*	0, 5, 10	ND	↓
						Total bacteria	0, 5, 10	ND	ND
Becker et al., 2020	F18	1×10^{10}	7	8 – 10	Culture	Total *E. coli*	0, 1-3, 5, 7, 10	ND	↑
Smith et al., 2020	F18	9×10^{10}	10	18	PCR	Total bacteria	0, 7	↓	ND
						Total *E. coli*	0, 7	ND	↓
						F18 *E. coli*	0, 7	ND	↑
Hong et al., 2021	F18	2×10^9	7	10 – 11	PCR	F18 *E. coli*	0, 7, 13	ND	ND

[1]Time of inoculation with ETEC, days post-weaning (dpw).

[2]Method for evaluation of fecal bacterial shedding, polymerase chain reaction (PCR).

[3]Length of time data was collected following inoculation with ETEC, days post-inoculation (dpi).

[4]Fecal bacterial shedding response following challenge with ETEC: ↑ indicates an increase in shedding; ↓ indicates a decrease in shedding; ND indicates no difference in shedding.

Table 1.4. Summary of variability in immunoglobulin responses following ETEC challenge in weanling pigs.

				Response Criteria[1]							
				IgA				IgG		IgM	
	Description of Challenge Model			Mucosal, μg/mg		Serum, mg/dL		Serum, mg/dL		Serum, mg/dL	
Reference	ETEC Type	Inoculum Dose (CFUs)	Number of Replicates	Mean ± SD	% Change[2]	Mean ± SD	% Change[2]	Mean ± SD	% Change[2]	Mean ± SD	% Change[2]
Trevisi et al., 2011	F4	2×10^{10}	6	-		53.8 ± 21.55*	32.1	-	-	-	-
Wu et al., 2012	F4	5×10^{9}	8	0.08 ± 0.01	1.9	17.5 ± 1.58*	10.0	97.3 ± 8.38*	16.1	-	-
Li et al., 2015	F4	2×10^{10}	7	-		43.9 ± 2.06*	9.8	212.0 ± 28.04*	19.7	22.4 ± 5.29*	53.7
Sugiharto et al., 2015	F4	5×10^{7}	3-5	0.72 ± 0.39*	38.8	-	-	-	-	-	-
Trevisi et al., 2015	F4	1×10^{8}	10	-	-	78.5 ± 32.25	11.1	-	-	-	-
Pan et al., 2017	F4	1×10^{11}	6	0.13 ± 0.02*	96.7	-	-	-	-	-	-
Luise et al., 2019	F4	2×10^{5}	14-15	-	-	53.3 ± 34.90	52.6	1,470 ± 868.00	35.0	358.0 ± 209.0	21.2
Xu et al., 2020	F4	1×10^{10}	6	-	-	109 ± 39.00	0.9	738.0 ± 120.02	10.2	78.0 ± 24.49	40.9
Becker et al., 2020	F18	1×10^{10}	8 - 10	1.48 ± 0.96*	77.0	-	-	-	-	-	-
Hong et al., 2021	F18	2×10^{9}	9	-	-	17.2 ± 7.42	3.0	486.4 ± 184.43+	15.9	85.9 ± 29.0	5.6
Chang et al., 2022	F18	3×10^{10}	9	-	-	1.5 ± 0.33*	28.3	150.9 ± 26.43*	33.3	-	-
Median[3]	-	-	-	0.60 ± 0.35	-	46.8 ± 17.38	-	525.8 ± 175.15	-	136.08 ± 66.95	-

*Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).
+Indicates that a marginally significant difference was observed between the control and treatment groups ($0.05 < P < 0.10$).
[1]Response criteria included immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM).
[2]The calculated percent change between the control and treatment groups.
[3]Median values were summarized at the bottom as the best measure of central tendency given the extreme outliers among data reported across studies.

Table 1.5. Summary of variability in tumor necrosis factor-alpha (TNF-α) responses following ETEC challenge in weanling pigs.

				TNF- α			
	Description of Challenge Model			Mucosal, pg/mg		Serum, pg/mL	
Reference	ETEC Type	Inoculum Dose (CFUs)	No. of Replicates	Mean ± SD	% Change[1]	Mean ± SD	% Change[1]
Xiao et al., 2014[2]	-	-	10	-	-	60.7 ± 5.40	9.0
Nyachoti et al., 2011	F4	1×10^{10}	9	-	-	124.1 ± 28.20*	57.3
Lee et al., 2012	F4	5×10^{9}	6	-	-	168.3 ± 6.73*	30.8
Li et al., 2015	F4	2×10^{10}	7	-	-	154.0 ± 15.40*	15.3
Lee et al., 2017	F4	3×10^{10}	8	-	-	93.2 ± 20.36*	64.2
Lei et al., 2020	F4	5×10^{9}	5	-	-	84.8 ± 32.42*	66.7
Wang et al., 2020	F4	1×10^{11}	6	-	-	182.4 ± 23.83*	46.5
Xu et al., 2020	F4	1×10^{10}	6	-	-	86.7 ± 15.41*	19.6
Duarte et al., 2020	F18	2×10^{9}	8	1.00 ± 0.31+	15.2	-	-
Smith et al., 2020	F18	9×10^{10}	18	-	-	235.0 ± 184.70*	25.3
Hong et al., 2021	F18	2×10^{9}	9	-	-	88.9 ± 31.73	1.1
Sun et al., 2021	F18	6×10^{9}	8	0.88 ± 0.65*	29.7	60.5 ± 18.18*	25.1
Chang et al., 2022	F18	3×10^{10}	9	-	-	33.1 ± 6.30*	32.5
Average	-	-	-	0.94 ± 0.48	-	114.3 ± 32.38	-

*Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).
+Indicates that a marginally significant difference was observed between the control and treatment groups ($0.05 < P < 0.10$).
[1]The calculated percent change between the control and treatment group.
[2]Xiao et al. (2014) did not report the specific strain of ETEC or the dose of ETEC inoculum utilized.

Table 1.6. Summary of variability in interleukin-6 (IL-6) responses following ETEC challenge in weanling pigs.

				IL-6			
	Description of Challenge Model			Mucosal, pg/mg		Serum, pg/mL	
Reference	ETEC Type	Inoculum Dose (CFUs)	No. of Replicates	Mean ± SD	% Change	Mean ± SD	% Change
Xiao et al., 2014[1]	-	-	10	-	-	65.3 ± 5.72	10.2
Nyachoti et al., 2011	F4	1×10^{10}	9	-	-	63.5 ± 21.00*	53.4
Lee et al., 2012	F4	5×10^{9}	6	-	-	17.0 ± 3.16*	16.0
Wu et al., 2012	F4	5×10^{9}	8	-	-	3.9 ± 0.25*	36.2
Li et al., 2015	F4	2×10^{10}	7	-	-	25.7 ± 4.05+	27.0
Che et al., 2017	F4	1×10^{9}	9	-	-	58.5 ± 4.32*	38.6
Lei et al., 2020	F4	5×10^{9}	5	-	-	65.5 ± 30.19*	62.9
Wang et al., 2020	F4	1×10^{11}	6	-	-	12.8 ± 0.73*	14.2
Xu et al., 2020	F4	1×10^{10}	6	-	-	116.4 ± 7.27	8.2
Chang et al., 2022	F18	3×10^{10}	9	-	-	46.7 ± 13.08*	41.9
Becker et al., 2020	F18	1×10^{10}	8 – 10	0.6 ± 0.63	47.8	-	-
Duarte et al., 2020	F18	2×10^{9}	8	3.6 ± 1.30*	44.7	-	-
Average	-	-	-	2.1 ± 0.97	-	47.53 ± 8.98	-

*Indicates a statistically significant response was observed ($P < 0.05$).
+Indicates a marginally significant response was observed ($0.05 < P < 0.10$).
[1]Xiao et al. (2014) did not report the specific strain of ETEC or the dose of ETEC inoculum utilized.

Table 1.7. Summary of variability in small intestinal morphology responses after ETEC challenge in weanling pigs.

Location	Ileum			Duodenum			Jejunum		
Measurement	Villus Height, μm	Crypt Depth, μm	VH:CD	Villus Height, μm	Crypt Depth, μm	VH:CD	Villus Height, μm	Crypt Depth, μm	VH:CD
Reference									
Trevisi et al., 2011	217 ± 34*	198 ± 23*	1.12 ± 0.19*	276 ± 34*	240 ± 23*	1.19 ± 0.19*	255 ± 34*	192 ± 23*	1.34 ± 0.19*
Nyachoti et al., 2012	403 ± 30*	278 ± 54	1.45 ± 0.30*	-	-	-	-	-	-
Wu et al., 2012	390 ± 35*	215 ± 16	1.84 ± 0.11*	435 ± 26	223 ± 13	1.93 ± 0.59	434 ± 45	226 ± 17*	1.94 ± 0.02*
Almeida et al., 2013	300 ± 26	223 ± 24*	1.36 ± 0.23	374 ± 60	266 ± 110	1.54 ± 0.74	-	-	-
Yang et al., 2014	565 ± 108	300 ± 54	1.90 ± 0.39	438 ± 78*	359 ± 56*	1.29 ± 0.34*	475 ± 88*	275 ± 27*	1.86 ± 0.54*
Pan et al., 2017	341 ± 33*	223 ± 22	1.54 ± 0.15*	426 ± 50*	244 ± 32	1.76 ± 0.27*	408 ± 41*	229 ± 26	1.79 ± 0.22*
Kim et al., 2019	365 ± 43	215 ± 22	1.70 ± 0.13	441 ± 68	307 ± 66	1.44 ± 0.14	395 ± 116*	241 ± 82	1. 64 ± 0.30
Becker et al., 2020	265 ± 39*	178 ± 31+	1.51 ± 0.29*	-	-	-	-	-	-
Choi et al., 2020	-	-	-	-	-	-	449 ± 95*	262 ± 42	1.98 ± 0.56
Duarte et al., 2020	-	-	-	-	-	-	395 ± 72*	275 ± 30*	1.48 ± 0.25*
Koo et al., 2020	394 ± 53	208 ± 27	1.91 ± 0.25	456 ± 62*	247 ± 34	1.87 ± 0.28*	444 ± 57	208 ± 25*	2.69 ± 0.25*
Lei et al., 2020	379 ± 64*	226 ± 35	1.70 ± 0.34*	475 ± 71*	224 ± 42	1.43 ± 0.45*	344 ± 69	225 ± 28	1.51 ± 0.47
Sun et al., 2021	314 ± 82	258 ± 52*	1.22 ± 0.17	-	-	-	314 ± 56*	231 ± 24*	1.80 ± 0.20*
Chang et al., 2022	351 ± 31*	159 ± 27+	2.28 ± 0.44*	-	-	-	-	-	-
Average	364 ± 52	224 ± 34	1.62 ± 0.26	420 ± 57	267 ± 39	1.53 ± 0.33	389 ± 70	237 ± 33	1.80 ± 0.31

[1]Means and SD represent the period reported immediately post-inoculation for each study

*Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).

Table 1.8. Summary of variability in tight junction gene expression in the ileum and jejunum of weanling pigs following ETEC challenge.

Location				Response Criteria[2]					
				Ileum			Jejunum		
	Description of Challenge Model			OCLN	CLDN1	ZO-1	OCLN	CLDN1	ZO-1
Reference	No. of Replicates	ETEC Type	Inoculum Dose (CFUs)						
Yang et al., 2014	6	F4	1.00 ± 10^{10}	0.91 ± 0.42	1.24 ± 0.56	1.56 ± 0.59	$0.84 \pm 0.29^{*}$	0.83 ± 0.73	$0.64 \pm 0.20^{*}$
Li et al., 2019	10	F18	2.10×10^{10}	1.48 ± 1.68	$1.44 \pm 2.94^{*}$	1.37 ± 0.85	-	-	-
Becker et al., 2020	8 – 10	F18	1.14×10^{10}	$0.57 \pm 1.07^{*}$	0.82 ± 0.59	$0.76 \pm 0.28^{*}$	-	-	-
Choi et al., 2020	6	F4	5.00×10^{7}	-	-	-	0.85 ± 0.37	$0.51 \pm 0.20^{+}$	0.79 ± 0.34
Kim et al., 2019	12	F18	3.00×10^{10}	-	-	-	1.32 ± 0.81	1.27 ± 1.52	$1.42 \pm 1.10^{*}$
Average	-	-	1.45×10^{10}	0.99 ± 1.06	1.17 ± 1.36	1.23 ± 0.57	1.01 ± 0.49	0.87 ± 0.82	0.95 ± 0.55

[*]Indicates a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).

[+]Indicates a marginally significant difference was observed between the control and treatment groups ($0.05 < P < 0.05$).

[1]Data are presented as the mean ± standard deviation in fold change for each response criteria.

[2]Response criteria included occludin (OCLN), claudin-1 (CLDN1), and zonula occludens-1 (ZO-1).

Chapter 2 - Effects of formic acid and glycerol monolaurate on weanling pig growth performance, fecal consistency, fecal microbiota, and serum immunity[2]

Abstract

A total of 350 weanling pigs (DNA 400 × 200; initially, 5.7 ± 0.06 kg BW) were used in a 42-d study with 5 pigs per pen and 14 replicate pens per treatment. At weaning, pigs were allotted to pens in a completely randomized design and pens of pigs were randomly assigned to one of five dietary treatments: 1) negative control (**CON;** standard nursery diet containing only 150 ppm Zn from trace mineral premix and no acidifier); 2) control diet with 3,000 ppm added zinc from ZnO included in phase 1 and 2,000 ppm added zinc from ZnO included in phase 2 **(ZnO)**; 3) control diet with 0.70% formic acid (**FA;** Amasil® NA; BASF, Florham, NJ); 4) control diet with 0.18% glycerol monolaurate (**GML;** Natural Biologics GML, Natural Biologics, Newfield, NY); and 5) control diet with a 1.0% blend of formic acid and glycerol monolaurate (**FORMI;** FORMI 3G, ADDCON GmbH, Bitterfeld-Wolfen, Germany). Pigs were fed treatment diets from d 0 to d 28 and were then fed a common diet from d 28 to d 42.

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From d 0 to d 7, pigs fed ZnO or FORMI had increased ($P = 0.03$) ADG compared to pigs fed CON, with no difference in feed intake ($P > 0.05$). Overall, pigs fed GML had reduced ($P < 0.0001$) ADG compared to those fed the CON, ZnO, or FORMI diets. Fecal DM was evaluated from d 7 to d 28 and there was a treatment × day interaction ($P = 0.04$). Pigs fed GML had a lower fecal DM % on d 7, but a higher fecal DM % on d 14 and 21; however, no differences in fecal DM were observed on d 28. Fresh fecal samples were collected from the same randomly selected pig on d 0 and d 14 (70 pigs total;14 pigs per treatment) for analysis of fecal microbial populations using 16S rDNA sequencing. Dietary treatment did not significantly impact fecal microbiota at the phyla level, but pigs fed ZnO had an increased relative abundance ($P < 0.01$) of the family *Clostridiaceae*. A blood sample was also collected from one pig per pen on d 0 and 14 for analysis of serum IgA, IgG, and TNF-α. There was no evidence that dietary treatment effected IgA, IgG, or TNF-α concentrations. The effect of sampling day was significant ($P < 0.05$), where circulating IgA and TNF-α was increased and IgG was decreased from d 0 to d 14. In summary, there is potential for a blend of formic acid and GML to improve growth performance immediately post-weaning without negatively impacting fecal consistency. Formic acid and GML alone or in combination did not impact fecal microbial populations or serum immune parameters.

Introduction

The period immediately post-weaning is a crucial time in swine production, as pigs undergo many physiological, dietary, and environmental changes (Zheng et al., 2021). Young piglets who have been switched from a milk diet to solid feed endure damage to the intestinal villi and are unable to properly regulate gastric pH resulting in reduced digestive enzyme

secretion and decreased nutrient absorption (Wei et al., 2021). Also during this time, pigs are comingled and potentially exposed to pathogens without a fully-functioning immune system which can lead to post-weaning diarrhea (PWD), one of the most detrimental diseases in the swine industry (Campbell et al., 2013). Collectively, these challenges can severely impact the overall health and growth performance of the animal. One solution to this challenge is the dietary addition of high levels of zinc in the form of zinc oxide (ZnO) during the immediate post-weaning period. When ZnO is included at pharmacological rates near 2,000 to 4,000 ppm, well above the 150 ppm requirement by the pig, a drastic improvement in growth performance and a reduction in PWD have been observed (Hill et al., 2001; Shelton et al., 2011). Despite the effectiveness of pharmacological rates of ZnO, a large portion is excreted in swine manure, which is problematic when the manure is applied to soil as fertilizer because of pollution concerns (Poulsen and Larsen, 1995; Jongbloed and Lenis, 1998; Carlson et al., 2004). Additionally, several studies have linked ZnO supplementation to antimicrobial resistant genes in bacterial pathogens of both animal and human importance, like *E. coli* and *Salmonella* (Yazdankhah et al., 2014; Vahjen et al., 2015; Ciesinski et al., 2018). Due to these concerns, regulatory bodies have begun closely monitoring ZnO application in swine feeding, specifically with the European Union limiting the total Zn concentrations in the diet to 150 ppm beginning in July 2022 (Commission, 2003, 2016). Data suggests that at these levels, the prophylactic effects traditionally seen in the post-weaning period will be diminished; thus, there is a pressing need for ZnO alternatives moving forward (Sales, 2013).

Dietary acidifiers have become a promising solution for preventing PWD and improving growth performance in a world without pharmacological ZnO. While these feed additives were initially used as a preservative, recent work has shown a multitude of positive responses in

growth performance, nutrient digestibility, and gut health of weanling pigs (Walsh et al., 2007; Liu et al., 2018; Tugnoli et al., 2020). A variety of organic and inorganic acids and their salts have been studied as alternatives to ZnO or antibiotics, but their precise mechanism of action has yet to be elucidated. Inclusion of formic acid and its derivatives has resulted in improvements in performance, likely due to the reduction in stomach pH and feed buffering capacity, resulting in increased pepsin activation and digestibility of proteins and amino acids (Luise et al., 2020). Additionally, the piglet microbiome is a complex environment of organisms that plays a large role in the animal's ability to adapt post-weaning. Diet composition represents one of the primary mechanisms to rapidly change the microbial composition along the gastrointestinal (GI) tract. As an example, inclusion of acidifiers like formic acid can limit the colonization of pathogenic bacteria and proliferate commensal microorganisms (Luise et al., 2017). Beyond improving growth performance during the post-weaning period, an emphasis on strengthening the piglet's immune system to prevent clinical disease is of great importance. Medium-chain fatty acids, those of 6 to 12 carbons in length, have been shown to improve immune system development in young animals and act as viral and bacterial pathogen-mitigants in feed (Jackman et al., 2020). Specifically, the monoglyceride, glycerol monolaurate (GML), has demonstrated the ability to act as a potent immunomodulator in the gut and exhibit antimicrobial properties that could limit the colonization of pathogenic bacteria along the GI tract (Lan et al., 2021; Papadopoulos et al., 2022). Despite numerous studies evaluating acidifiers in the post-weaning period, results are highly variable due to inconsistencies in inclusion rates, age and existing health status of pigs, and response criteria evaluated. Generally, a blend of acids tends to be more effective when considering their use in nursery pig diets. Formic acid and GML are of particular interest since formic acid exerts its primary effects in the stomach, while GML tends to

be most beneficial within the small intestine suggesting that the two may act synergistically (Ramırez et al., 2001; Luise et al., 2020). Experiments have been conducted looking at formic acid and GML separately, but there is extremely little data evaluating the two as a blend, despite this combination being available within commercial feed additives. Thus, our objective was to evaluate feeding formic acid and GML alone, or in combination on growth performance, fecal microbiota, fecal consistency, and immune function of weanling pigs. Specifically, this study aimed to determine if these acids could provide additive effects when fed as a blend.

Materials and Methods

Animals and Diets

All experimental procedures adhered to guidelines for the ethical and humane use of animals for research according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2012) and were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4036). The experiment was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, Kansas.

A total of 350 pigs (DNA 400 × 200; Columbus, NE, initially, 5.67 ± 0.06 kg BW) were weaned at an average of 21 d of age and used in a 42-d experiment. Weaning was considered d 0 of the trial and, at this point, pigs were individually weighed and allotted to pens in a completely randomized design. There were 5 pigs per pen and 14 replicate pens per treatment. Each pen had tri-bar floors (1.5 × 1.5 m) and was equipped with a 4-hole dry self-feeder and nipple waterer to supply *ad libitum* access to feed and water. At weaning, pigs were randomized to pens, and pens randomly assigned to dietary treatments. Diets were formulated and manufactured in three dietary phases (phase 1 = d 0 to d 7; phase 2 = d 8 to d 28; phase 3 = d 29 to d 42) such that

experimental diets were fed from d 0 to 28, and a common diet was fed to all pigs from d 29 to 42. Diets were formulated to meet or exceed (NRC, 2012) requirements (Table 1) and contained no antimicrobials. Pigs treated with antimicrobials for any reason were immediately removed from the experiment. Treatments were as follows: 1) negative control (**CON;** standard nursery diet containing 150 ppm Zn from trace mineral premix and no acidifier); 2) control diet with 3,000 ppm added zinc from ZnO included in phase 1 and 2,000 ppm added zinc from ZnO included in phase 2 **(ZnO)**; 3) control diet with 0.7% formic acid (**FA;** Amasil® NA; BASF, Florham, NJ); 4) control diet with 0.18% glycerol monolaurate (**GML;** Natural Biologics GML, Natural Biologics, Newfield, NY); and 5) control diet with a 1.0% blend of formic acid, sodium diformate, and glycerol monolaurate (**FORMI;** FORMI 3G, ADDCON GmbH, Bitterfeld-Wolfen, Germany). All test ingredients were included at the expense of corn in the diet. Individual pig weights and feed disappearance were measured on days 0, 7, 14, 21, and 28, with pen weights collected on d 35 and 42 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F).

Chemical Analysis

Complete diet samples were collected from 10 different feeders per dietary treatment on d 0 and 21 and composite subsamples were analyzed for nutrient composition (Table 2). Assays included DM (method 930.15; AOAC, 2007), crude protein (CP) as N × 6.25 using the combustion method (Nitrogen Determinator; model TruMac N, Leco Corporation, St. Joseph, MI; method 990.03; AOAC, 2007), acid detergent fiber (ADF) (ANKOM Tech. Method 200), Ca (method 985.01; AOAC, 2006), P (method 985.01; AOAC, 2006), Zn (method 985.01; AOAC, 2006), and fatty acid profile (method 996.06; AOAC, 2010).

Fecal Dry Matter and Microbiota Analysis

Fecal samples were collected from the same 3 randomly selected pigs per pen on d 7, 14, 21, and 28 for analysis of fecal DM. Briefly, a sterile, cotton-tipped applicator was gently inserted into the rectum to stimulate defecation. Samples were pooled on a per pen basis to form one composite sample for each collection point and stored at -20° C until analysis. To determine fecal DM, samples were dried for 48 h at 55° C in a forced air oven.

On d 0 and 14, fresh fecal samples were collected from an additional one pig per pen (n = 14 pigs/treatment, 70 pigs total) for fecal microbiota analysis. The same pig was sampled at each time point by inserting a sterile, cotton-tipped applicator into the rectum to stimulate defecation. Samples were collected into sterile, DNA-free centrifuge tubes and stored at -80° C until shipment to a commercial laboratory (MR DNA, Shallowater, TX) for DNA extraction and taxonomic analysis.

Genomic DNA was isolated from each sample using the PowerSoil® DNA Isolation Kit (Qiagen Inc., Valencia, CA). Approximately 250 mg of fecal matter was added to a PowerBeads® (Qiagen Inc., Valencia, CA) tube and homogenized using the PowerLyzer® (Qiagen Inc., Valencia, CA) to induce cell lysis. The purified DNA was then eluted and stored at -20° C until PCR amplification. The 16S universal primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) were utilized to amplify 16S gene of samples on the Illumina NovaSeq (Illumina Inc., San Diego, CA) via the bTEFAP® DNA analysis service originally described by (Dowd et al., 2008a). Each sample underwent a single-step 35 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) under the following conditions: 95° C for 5 minutes, followed by 30 cycles of 95° C for 30 seconds; 53° C for 40 seconds and 72° C for 1 minute; after which a final elongation

step at 72° C for 10 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using calibrated solid phase reversible immobilization methodology (SPRI) beads. Samples were sequenced utilizing the Illumina NovaSeq (Illuminia Inc., San Diego, CA) chemistry following manufacturer's protocols.

Data processing was conducted using a proprietary analysis pipeline (MR DNA, Shallowater, TX). Sequences were depleted of primers, short sequences (< 150bp) were removed, and sequences with ambiguous base calls removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised, and unique sequences identified with sequencing or PCR point errors were removed, followed by chimera removal, thereby providing a denoised sequence or operational taxonomic unit (**OTU**). Final OTUs were taxonomically classified using BLASTn (Altschul et al., 1990) against a curated database derived from NCBI (www.ncbi.nlm.nih.gov). Alpha and beta diversity analyses were conducted as previously described by (Dowd et al., 2008b; Edgar, 2010) using Qiime v.2 (Bolyen et al., 2018). After stringent quality sequence curation, a total of 4,037,671 sequences identified within the Bacteria and Archaea domains were utilized for final microbiota analyses. The average reads per sample was 29,258. For alpha and beta diversity analysis, samples were rarefied to 10,000 sequences.

Serum Immunoglobulin and Pro-Inflammatory Cytokine Analysis

On d 0 and d 14, whole blood samples were collected from one pig per pen (n = 14 pigs/treatment, 70 pigs total). The same pig was sampled at each time point. Samples were collected from the jugular vein into sterile 5 mL vacuum-sealed tubes (Monoject™ Blood Collection Tube, Mansfield, MA) using a 22 g × 1" blood collection needle (Fisher Scientific,

Hampton, NH). Samples were immediately placed on ice until transport to the laboratory for serum separation. Samples were allowed to clot at room temperature for 2 hours before being centrifuged at 2000 × *g* for 10 minutes at 4° C. Serum was then recovered from samples, placed into aliquots for each assay, and stored at -80° C until analysis. Serum was diluted at 1:10,000 and 1:500,000 for analysis of IgA and IgG, respectively. Samples were not diluted for analysis of TNF-α. The intra-assay CV was 6.5%, 3.3%, and 4.1% for IgA, IgG, and TNF-α, respectively. The inter-assay CV was 8.1%, 5.1%, and 5.6% for IgA, IgG, and TNF-α, respectively. Using an enzyme-linked immunoassay (ELISA), concentrations of tumor necrosis factor-α (TNF-α; R&D Systems, Inc., Minneapolis, MN), immunoglobulin A (IgA; Bethyl Laboratories, Montgomery, TX), and immunoglobulin G (IgG; Bethyl Laboratories, Montgomery, TX) were measured according to the kit manufacturer's instructions.

Statistical Analysis

Growth and fecal consistency data were analyzed as a completely randomized design with pen of pigs as the experimental unit. Fecal dry matter data were analyzed as repeated measures over time. All comparisons included Tukey-Kramer multiple comparison adjustments. Additionally, preplanned contrasts were included to compare the means of the negative control and diets containing an additive, as well as the ZnO and acidifier diets. Serum immunoglobulin and pro-inflammatory cytokine data were log transformed before analysis. Data were then analyzed as a completely randomized design with individual pig as the experimental unit. The model included the main effects of dietary treatment, sampling day, and the treatment × day interaction. Data were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Institute, Cary, NC).

Fecal microbiota data were analyzed with individual pig as the experimental unit to represent one sample selected from each pen on each sampling day (d 0 and d 14). The relative abundance was determined as the total proportion of reads for a sample classified into the specific microbial phyla or family. Samples with a low relative abundance (< 0.01%) were excluded from the analysis. Once the relative abundance for each sample was calculated, data were analyzed as a completely randomized design with the model including dietary treatment, sampling day, and their interaction as fixed effects. Data were analyzed as repeated measures given the two sampling time points on d 0 and d 14. Statistical comparisons of observed features and Shannon Diversity indices for each treatment group were conducted using Kruskal-Wallis pairwise comparisons. Beta diversity was analyzed using a weighted UniFrac distance matrix with pairwise analysis of similarities (ANOSIM) used to evaluate differences in microbial communities between groups over time. Data were considered significant if $P < 0.05$ and marginally significant if $0.05 < P < 0.10$.

Results and Discussion

Growth Performance and Fecal Consistency

Growth performance data are presented in Table 4. During the first week post-weaning (d 0 to d 7), pigs fed a diet with either ZnO or FORMI had increased ($P < 0.01$) ADG compared to pigs fed the control diet. There was no evidence of differences in ADFI ($P > 0.05$), but pigs fed a diet supplemented with GML had improved feed efficiency ($P < 0.01$) compared to their counterparts fed both the control and FA diets, while pigs fed ZnO or FORMI were intermediate. Similarly, others have reported improvements in feed conversion post-weaning with the addition of MCFA, like GML (Cera et al., 1989; Hanczakowska et al., 2010; Gebhardt et al., 2020). Due

to their higher water solubility compared to long chain fatty acids (LCFA), MCFA are hydrolyzed rapidly and easily absorbed in the small intestine (Jackman et al., 2020). This makes them a readily available energy source for the young pig during a time when reduced feed intake can cause energy deficiencies, thus potentially explaining the enhanced feed efficiency seen in the first week of the current experiment. However, this response diminished by dietary phase 2 (d 8 to d 28), where we saw a reduction in ADG in pigs fed GML, which appeared to be driven by decreased feed conversion ($P < 0.0001$). While others have reported no effect of GML supplementation on growth performance in the nursery period (Cui et al., 2020; Thomas et al., 2020), the negative growth response that was seen in the current work from d 7 and beyond was unexpected. It is important to note that the addition of some MCFA can pose palatability concerns and have been associated with reductions in feed intake due to their pungent odor (Hanczakowska, 2017). However, the lack of feed intake response in our study does not corroborate this.

From d 8 to d 28, pigs fed ZnO had increased ADG compared to control-fed pigs ($P = 0.01$), while those fed FA or FORMI were intermediate. Despite a marginally significant treatment effect for feed intake ($P = 0.06$), when a contrast statement was used to directly compare ZnO to the three acidifiers, we saw a significant feed intake response ($P < 0.01$), where pigs fed ZnO had increased ADFI. During the entirety of the treatment period (d 0 to d 28) pigs fed ZnO had increased ADG compared to those fed the control, FA, and GML diets, while pigs fed FORMI were intermediate ($P < 0.0001$). This coincides with previous literature, where the growth enhancements from feeding added ZnO are well documented (Hill et al., 2001; Sales, 2013). Feed efficiency was negatively impacted by feeding GML ($P < 0.0001$) compared to a control, ZnO, or FORMI, while feeding FA alone yielded an intermediate response. A common

phase 3 diet was fed to all pigs from d 29 to d 42, where no evidence of differences was observed for ADG, ADFI, or G:F ($P \geq 0.25$). Overall (d 0 to d 42), a reduction in ADG was seen in pigs fed GML compared to a control, ZnO, or FORMI, while those fed FA were intermediate ($P < 0.0001$). No evidence of differences in ADFI or G:F were observed for the overall experiment ($P \geq 0.14$).

Despite many studies evaluating formic acid and GML alone, experiments investigating their efficacy together are scarce. The use of formic acid and its salts to improve growth performance in the post-weaning period was recently reviewed by Luise et al. (2020). While numerous studies have shown favorable growth responses to formic acid, the precise mode of action is still not clear. Literature suggests several mechanisms by which formic acid can improve weanling pig growth performance including the reduction of gastric pH, increased nutrient digestibility, and alterations in the intestinal microbiome leading to increased SCFA production and energy metabolism by the pig (Partanen and Mroz, 1999). During the entirety of this experiment, we saw no evidence that formic acid inclusion impacted piglet growth compared to a negative control. Parameters like gut pH and nutrient digestibility were not measured in our study to explain the lack of response. When formic acid was fed together with GML, there appeared to be no impact on piglet growth compared to a negative control. Within the literature, responses seen when formic acid or MCFA have been included are extremely variable and highly dependent on other factors such as inclusion rate, other diet components, piglet age, and health status. In our experimental diets containing solely formic acid or GML, the concentrations of each acid used were calculated to equal the levels of both formic acid and GML present within the 1% blend (FORMI), in effort to detect any additive effects if present. In the first week post-weaning, there was no evidence of differences in growth performance between pigs fed formic

acid and GML alone or a blend of the two. However, by week 2, pigs fed FORMI had improved ADG compared to those fed GML alone, and this response was consistent throughout the remainder of the trial, which may indicate that the effect of GML can be enhanced when paired with formic acid.

A treatment × day interaction was observed for fecal DM ($P = 0.04$). On d 7, pigs fed a diet with GML had a lower fecal DM % ($P = 0.04$) compared to pigs fed all other treatments. Interestingly, by d 14, this response had shifted, and pigs fed GML had a higher fecal DM % compared to their counterparts, indicating firmer fecal consistency. This response was observed through d 21, but by d 28 there were no differences in fecal DM. Feeding pharmacological levels of ZnO has been shown to reduce post-weaning diarrhea because of its ability to limit the colonization of pathogenic bacteria along the GI tract (Hu et al., 2013a; Hu et al., 2013b). Certain MCFA, like lauric acid (C12:0) are known to have antimicrobial properties, and the efficacy is considerably greater when in monoglyceride form (Kabara et al., 1972; Bergsson et al., 2002). Meanwhile, Pettigrew (2006) reported that due to the reduction in gastric pH from formic acid supplementation, alterations in the bacterial population within the stomach and subsequent portions of the GI tract could be achieved. However, literature is highly variable as it relates to the efficacy of acidifiers to control PWD, primarily due to differences in inclusion rates and the blends of acids used. Within the first week post-weaning, pigs fed GML had a reduced fecal DM percentage. It is difficult to clearly explain this response; however, we speculate that a change in intestinal bacterial populations not observed in fecal analysis, or an incorrect electrolyte balance could be contributing factors. However, while we observed statistically significant differences in fecal DM percentage across treatments, the numeric difference was still quite small, making it challenging to draw conclusions about the incidence of clinical diarrhea.

Further investigation into the mode of action of dietary acidifiers is needed to fully elucidate their impacts on piglet fecal consistency.

Fecal Microbiota

Most sequences were classified into the phyla Firmicutes and Bacteroidetes on d 0 and 14 (Figure 2), which is consistent with previous literature studying microbial populations at different portions of the pig gastrointestinal tract (Yang et al., 2017; Li et al., 2018; Gebhardt et al., 2020). We observed no evidence of differences ($P > 0.05$) in microbial phyla as a result of dietary treatment; however, a significant sampling day effect ($P \leq 0.01$) was seen for several of the analyzed phyla. The mean relative abundance of Actinobacteria, Spirochaetes, and Firmicutes increased from d 0 to 14, while the relative abundance of Proteobacteria and Synergistetes decreased. The relative abundance of Bacteroidetes did not differ from d 0 to d 14 ($P = 0.71$). Our data does not suggest that feeding formic acid and GML altered the microbial composition at the phyla level, rather a change over time was observed. This alteration in the microbes present immediately after weaning until two weeks post-weaning was expected, as it is well understood that the microbiome shifts drastically with time and along the GI tract (Isaacson and Kim, 2012). In contrast to the current work, Yang et al. (2021) reported an increase in the relative abundance of Bacteroidetes over time. These authors also found that microbes in the phyla Proteobacteria were highly abundant after birth, but decreased post-weaning, which coincides with our data.

Microbial populations at the family level are shown in Figure 3. A marginally significant treatment × day interaction ($P = 0.07$) was observed for the family *Ruminococcaceae*, where pigs fed the CON and ZnO diets had a decreased relative abundance from d 0 to d 14, while those fed FA, GML, or FORMI had increased *Ruminococcaceae*. A dietary treatment effect was seen for

the relative abundance of *Clostridiaceae* ($P = 0.01$), where samples from pigs fed ZnO had increased *Clostridiaceae* compared to those fed CON or FA, with pigs fed a diet with GML or FORMI being intermediate. When considering the piglet microbiota, the weaning transition is associated with dysbiosis that can be identified by reduced abundance of microbes within the Clostridia class, like *Clostridiaceae* (Gresse et al., 2017). Additionally, in dogs, *Clostridiaceae* are associated with increased protein and fat digestibility (Bermingham et al., 2017); however, nutrient digestibility was not measured in the current work. Sampling day impacted the relative abundance of several families. An increase ($P \leq 0.01$) in the families *Paludibacteraceae, Spirochaetaceae, Erysipelotrichaceae, Prevotellaceae, Lachnospiraceae,* and *Lactobacteriaceae* was observed from d 0 to d 14, while the family *Enterobacteriaceae* decreased ($P = 0.03$) and *Bacteroidaceae* tended to decrease ($P = 0.08$). This is consistent with Frese et al. (2015), who studied changes in the microbiome from suckling to post-weaning.

The alpha diversity of microbes present within a sample are commonly assimilated according to the richness (quantity) and evenness (dispersion) (Prehn-Kristensen et al., 2018). Based on the number of observed features, the alpha diversity of the samples from pigs fed CON, FA, GML, or FORMI was less ($P < 0.05$) on d 0 compared to d 14, while there was no evidence of a difference in the alpha diversity from pigs within the ZnO treatment. Generally, a higher diversity is indicative of a more healthy microbiome (Lozupone et al., 2012). Our data indicate that supplementing pharmacological ZnO had the least impact on the number of microbes present in fecal samples. Conversely, Vahjen et al. (2011) reported an increase in these parameters when weanling pigs were supplemented with 3,000 ppm ZnO, but these samples were taken from the ileum rather than fecal samples as seen in the current work. However, it is important to not only consider the quantity of microbes, but also the dispersion of them

according to taxonomic classification. The analysis of the Shannon Diversity indices, which accounts for species evenness in addition to species richness, only detected a significant difference within samples from pigs fed either GML or FORMI over time ($P < 0.05$), which suggests that GML supplemented alone or with formic acid can alter the structure of the microbial community in piglet feces. Beyond evaluating differences in microbial populations within a given sample, comparison between samples is considered Beta diversity (Liu et al., 2021). No evidence of differences in beta diversity was seen across dietary treatments ($P \geq 0.25$). However, there was a difference ($P < 0.01$; Figure 5) in the beta diversity of all samples between d 0 and d 14, which further validates the taxonomic differences over time within our samples.

It is important to consider that it is nearly impossible to determine a 'standard' microbiota composition post-weaning, given the literature is extremely inconsistent due to differences in genetics, diet composition, weaning age, environment, and experimental methodologies. The microbiota differs substantially throughout the gastrointestinal tract (Zhao et al., 2015), therefore it is difficult to elucidate how dietary acidifiers like formic acid or GML impact the microbiome as a whole. Fecal analysis may only represent a small portion of the microbial population present within an animal, so there could be drastic differences in these samples compared to those from other areas such as the intestine or stomach. We would expect both formic acid and glycerol monolaurate to be most effective in the upper GI tract, therefore, only analyzing fecal microbes is certainly a limitation of the current work. Future studies should focus on how these additives can alter the intestinal microbiota specifically. The lack of treatment differences in our data suggest that the changes in growth performance may not have been directly caused by alterations in the piglet fecal microbial populations.

Serum IgA, IgG, and TNF-α Concentrations

There was no evidence of a dietary treatment × sampling day interaction or a main effect of dietary treatment for any serum parameters ($P > 0.05$), therefore these data are not shown. However, sampling day impacted IgA, IgG, and TNF-α concentrations ($P < 0.05$), therefore these data are presented as the calculated change in the targeted parameter from d 0 to d 14. We observed an increase ($P < 0.05$) in serum IgA and TNF-α levels from d 0 to d 14 for all pigs, while the concentration of IgG decreased ($P < 0.05$), regardless of dietary treatment.

The weaning transition predisposes piglets to immunological challenges and intersects with the time at which passive immunity from the sow's milk declines (de Groot et al., 2021). Therefore, strengthening the pigs immune system post-weaning is crucial to maintaining health and growth performance. Others have shown GML to exhibit strong immunomodulatory properties by decreasing the production of certain cytokines and reducing gut inflammation (Zhang et al., 2016; Zhang et al., 2018). While formic acid is not known to directly impact immune function, the symbiotic relationship between the gut microbiota and mucosal immunity is important to consider. Formic acid may indirectly enhance the weaned piglets immune response by altering the GI microbial populations to a more favorable environment (Luise et al., 2020). While we did not observe a significant effect of dietary treatment on serum immune parameters, other studies have reported different findings. Ren et al. (2020) challenged 25-day old piglets with enterotoxigenic *E. coli* and found that a blend of formic acid and monolaurin resulted in lower plasma TNF-α by down-regulating the expression of toll-like receptor 4 (TLR4), ultimately attenuating gut inflammation triggered by the ETEC. Inclusion levels of formic acid and GML were 0.60% and 0.20%, respectively, while the current study included formic acid at 0.70% and GML at 0.18%. With similar levels of both formic acid and GML, we

did not observe a significant change in serum immunity; however, Ren et al. (2020) collected samples post ETEC challenge and pigs in the current study were clinically healthy. More recently, Li et al. (2022) fed weanling pigs either a basal control diet or the basal diet supplemented with 0.10% GML and observed increased IL-10 and reduced TNF-α, both indicative of enhanced immune function. Pigs used in the current study were from a facility with no known pathogen challenges, which would potentially explain the lack of response seen in serum parameters due to dietary treatment. However, the increased circulating TNF-α observed in all pigs from d 0 to d 14 may suggest an inflammatory response was taking place. Future work should evaluate immunoglobulin and pro-inflammatory cytokine levels at a more localized site, such as the small intestine, and perhaps quantify gene expression to gain a more functional understanding of how these acidifiers impact immune status.

Conclusions

In summary, feeding a blend of formic acid and GML showed promise to improve weanling pig growth performance immediately post-weaning. However, the inclusion of GML alone reduced ADG and feed conversion for the entirety of the nursery period, resulting in lighter BW by the end of the experiment. We observed no evidence that feeding formic acid or GML alone or in combination impacted fecal microbial populations or immune status. Further investigation into the use of this acidifier blend to benefit growth performance, the microbiota of the GI tract, and mucosal immunity is warranted.

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Table 2.1. Composition of phase 1, phase 2, and phase 3 basal diets (as-fed basis).

	Dietary Phase[1]		
Item	Phase 1	Phase 2	Phase 3
Ground corn	40.08	45.98	65.50
Soybean meal, 47.5% CP	17.30	22.75	28.45
Corn DDGS, 7.5% oil	5.00	10.00	-
Spray dried whey	25.00	10.00	-
Fish meal	3.00	-	-
Enzymatically treated soybean meal[2]	-	4.5	-
Spray dried bovine plasma	4.00	-	-
Fat	3.00	3.00	2.00
Calcium carbonate, 38.5% Ca	0.65	0.85	0.75
Monocalcium phosphate, 21.5% P	0.55	1.00	1.10
Salt	0.30	0.55	0.60
L-Lys HCL	0.35	0.50	0.55
DL-Met	0.15	0.17	0.21
L-Thr	0.13	0.18	0.23
L-Trp	0.03	0.04	0.05
L-Val	0.07	0.08	0.16
Zinc oxide	Varied	Varied	-
Formic acid[3]	Varied	Varied	-
Glycerol monolaurate (GML), 90% purity[4]	Varied	Varied	-
Formic acid and GML, 1.0% blend[5]	Varied	Varied	-
Vitamin premix w/ phytase[6]	0.25	0.25	0.25
Trace mineral premix[7]	0.15	0.15	0.15
Total	100.00	100.00	100.00
Calculated analysis[8]			
SID amino acids, %			
Lys	1.40	1.35	1.30
Ile:Lys	57	57	53
Leu:Lys	110	118	111
Met:Lys	36	35	36
Met and Cys:Lys	57	57	57
Thr:Lys	63	64	63
Trp:Lys	19.3	20	19.3
Val:Lys	70	70	70
His:Lys	33	34	35

Total Lys, %	1.54	1.49	1.43
ME, kcal/kg	3,440	3,353	3,383
NE, kcal/kg	2,594	2,449	2,534
SID Lys:NE, g/Mcal	5.39	5.51	5.13
CP, %	21.1	21.2	19.9
Ca, %	0.74	0.75	0.65
P, %	0.69	0.67	0.61
STTD P, %	0.48	0.42	0.49

[1]Treatment diets were fed to 350 pigs [DNA 400 × 200 (Columbus, NE); initially 5.67 ± 0.06 kg BW] for 28 d in a 2-phase feeding program with 5 pigs per pen and 14 pens per treatment. A common phase 3 diet was fed to all pigs from d 29 to d 42.
[2]HP300 (Hamlet Protein, Findlay, OH).
[3]Formic acid included at 0.70% in phase 1 and 2 (Amasil-NA; BASF Corp., Florham, NJ).
[4]Glycerol monolaurate (guaranteed ≥ 95% purity) included at 0.18% in phase 2 and 2 (GML; Natural Biologics, Newfield, NY).
[5]A 1.0% blend of formic acid and glycerol monolaurate was included in phase 1 and 2 (FORMI-3G; ADDCON GmbH, Bitterfeld-Wolfen, Germany).
[6]Premix provided per kg of premix: 4,409,249 IU vitamin A; 551,156 IU vitamin D3; 17,637 IU vitamin E; 1,764 mg vitamin K; 15.4 mg vitamin B12; 19,842 mg niacin; 11,023 mg pantothenic acid; and 3,307 mg riboflavin.
[7]Premix provided per kg of premix: estimated release of 0.12% STTD P; 110 g Fe from iron sulfate; 110 g Zn from zinc sulfate; 26.4 g Mn from manganese oxide; 11 g Cu from copper sulfate; 198 mg I from calcium iodate; 198 mg Se from sodium selenite.
[8]NRC (2012).

Table 2.2. Analyzed composition of phase 1 and phase 2 experimental diets (as-fed basis)[1]

	Dietary Treatment[2]				
Analyzed composition, %[3]	CON	ZnO	FA	GML	FORMI
Phase 1					
DM, %	88.04	87.89	88.11	88.31	87.95
CP, %	20.30	20.70	21.40	21.00	21.00
Fat, %	5.14	5.41	5.48	5.51	5.50
ADF, %	2.50	2.40	2.90	2.50	2.50
Calcium, %	0.75	0.80	0.83	0.86	0.81
Phosphorous, %	0.67	0.70	0.71	0.73	0.73
Zinc, ppm	168	2,840	309	113	139
Lauric acid (C:12), g/100 g	< 0.01	< 0.01	< 0.01	0.10	0.13
Phase 2					
DM, %	86.92	87.11	86.65	87.04	86.79
CP, %	18.50	19.80	19.60	18.50	20.20
Fat, %	4.74	5.48	4.88	4.98	4.63
ADF, %	3.70	3.9	4.00	3.80	3.80
Calcium, %	0.85	0.78	0.66	0.58	0.64
Phosphorous, %	0.63	0.7	0.63	0.53	0.65
Zinc, ppm	284	1,900	188	146	192
Lauric acid (C:12), g/100 g	< 0.01	< 0.01	< 0.01	0.13	0.17

[1] Treatment diets were fed to 350 pigs [DNA 400 × 200 (Columbus, NE); initially 5.67 ± 0.06 kg BW] for 28 d in a 2-phase feeding program with 5 pigs per pen and 14 pens per treatment.

[2]Dietary treatments included: basal diet (CON); basal diet with 3,000 ppm added Zn from ZnO in phase 1 and 2,000 ppm added Zn from ZnO in phase 2; basal diet with 0.70% formic acid (Amasil-NA, BASF Corp., Florham, NJ); basal diet with 0.18% glycerol monolaurate (GML guaranteed ≥ 95% purity; Natural biologics, Newfield, NY); basal diet with 1.0% blend of formic acid and GML (FORMI-3G, ADDCON GmbH, Bitterfeld-Wolfen, Germany).

[3]Complete diet samples were collected from the same 10 randomly selected feeders on d 0 and d 21. Samples were pooled by day and subsampled, then submitted to Midwest Laboratories (Omaha, NE) for proximate and fatty acid analysis.

Table 2.3. Analyzed composition of phase 3 common diet (as-fed basis)[1]

Analyzed composition, %[2]	Diet
DM, %	87.23
CP, %	19.50
Fat, %	4.53
ADF, %	3.20
Calcium, %	0.61
Phosphorous, %	0.57
Zinc, ppm	136.0
Lauric acid (C:12), g/100 g	< 0.01

[1] A common diet was fed to 350 pigs [DNA 400 × 200 (Columbus, NE); initially 5.67 ± 0.06 kg BW] from d 29 to d 42 with 5 pigs per pen and 14 pens per treatment.
[2]Complete diet samples were collected from the same 10 randomly selected feeders on d 28 and d 42. Samples were pooled and subsampled, then submitted to Midwest Laboratories (Omaha, NE) for proximate and fatty acid analysis.

Table 2.4. Effect of formic acid and glycerol monolaurate alone or in combination nursery pig growth performance[1]

							P - value		
Item;	CON	ZnO[2]	FA[3]	GML[4]	FORMI[5]	SEM	Treatment	Control vs. Additives[6]	Zinc Oxide vs. Acids[7]
BW, kg									
d 0	5.62	5.69	5.77	5.66	5.61	0.06	0.340	0.335	0.916
d 7	5.96	6.21	6.09	6.18	6.21	0.06	0.033	0.005	0.501
d 14	7.30^{ab}	7.74^{a}	7.29^{b}	7.21^{b}	7.64^{ab}	0.11	0.003	0.171	0.007
d 21	10.17^{bc}	11.40^{a}	10.15^{bc}	9.54^{c}	10.72^{ab}	0.18	< 0.0001	0.154	< 0.0001
d 28	13.95^{bc}	15.34^{a}	13.97^{bc}	13.18^{c}	14.58^{ab}	0.21	< 0.0001	0.166	< 0.0001
d 35	18.69^{b}	20.16^{a}	18.59^{bc}	17.83^{c}	19.15^{b}	0.22	< 0.0001	0.328	< 0.0001
d 42	23.77^{b}	25.14^{a}	23.24^{bc}	22.50^{c}	24.24^{ab}	0.32	< 0.0001	0.975	< 0.0001
Phase 1 (d 0 to 7)									
ADG, kg/d	0.05^{b}	0.09^{a}	0.06^{ab}	0.07^{ab}	0.09^{a}	0.02	0.003	0.003	0.146
ADFI, kg/d	0.11	0.13	0.13	0.11	0.14	0.01	0.162	0.146	0.619
G:F	0.40^{b}	0.62^{ab}	0.45^{b}	0.69^{a}	0.62^{ab}	0.06	0.002	0.003	0.606
Phase 2 (d 8 to 28)									
ADG, kg/d	0.38^{b}	0.42^{a}	0.38^{ab}	0.33^{c}	0.40^{ab}	0.01	< 0.0001	0.642	< 0.0001
ADFI, kg/d	0.58	0.63	0.58	0.57	0.59	0.02	0.058	0.344	0.007
G:F	0.65^{a}	0.67^{a}	0.64^{a}	0.59^{b}	0.68^{a}	0.01	< 0.0001	0.446	0.026
Overall Treatment (d 0 to 28)									
ADG, kg/d	0.29^{bc}	0.33^{a}	0.30^{bc}	0.27^{c}	0.32^{ab}	0.01	< 0.0001	0.247	< 0.0001
ADFI, kg/d	0.46	0.50	0.47	0.46	0.48	0.01	0.120	0.291	0.027
G:F	0.64^{a}	0.67^{a}	0.63^{ab}	0.59^{b}	0.67^{a}	0.01	< 0.0001	0.947	0.008
Common Phase 3 (d 29 to 42)									
ADG, kg/d	0.70	0.70	0.68	0.67	0.69	0.02	0.247	0.212	0.137
ADFI, kg/d	0.98	0.99	0.98	0.94	0.98	0.03	0.555	0.938	0.400
G:F	0.73	0.71	0.69	0.72	0.71	0.02	0.732	0.325	0.921
Overall Experiment (d 0 to 42)									
ADG, kg/d	0.43^{ab}	0.45^{a}	0.42^{bc}	0.40^{c}	0.44^{ab}	0.01	< 0.0001	0.929	0.001
ADFI, kg/d	0.63	0.66	0.64	0.62	0.64	0.04	0.846	0.879	0.860
G:F	0.68	0.69	0.66	0.65	0.69	0.01	0.139	0.896	0.017

[abc]Means within a row that do not share a common superscript differ ($P < 0.05$).

[1]A total of 360 weanling pigs (DNA 200 × 400, initially 5.67 ± 0.06 kg BW) were used in a 42-d growth study with 5 pigs/pen and 14 replicates/treatment.
[2]Added Zn in the form of zinc oxide was provided at 3,000 ppm in phase 1 and at 2,000 ppm in phase 2.
[3]Formic acid (Amasil-NA; BASF Corp. Florham, NJ) was included in the diet at 0.70% in both phase 1 and phase 2.
[4]Glycerol monolaurate (guaranteed ≥ 95% purity) (Natural Biologics, Newfield, NY) was included in the diet at 0.18% in both phase 1 and phase 2.
[5]FORMI-3G (Addcon Gmbh, Bitterfeld-Wolfen, Germany) was included in the diet at 1.0% in both phase 1 and phase 2.
[6]Contrast statement used to evaluate means from pigs fed the Control compared to those fed ZnO, Formic Acid, Glycerol Monolaurate, and FORMI-3G.
[7]Contrast statement used to evaluate means from pigs fed ZnO compared to those fed Formic Acid, Glycerol Monolaurate, and FORMI-3G.

Figure 1. Effect of dietary treatments on weanling pig fecal dry matter percentage.

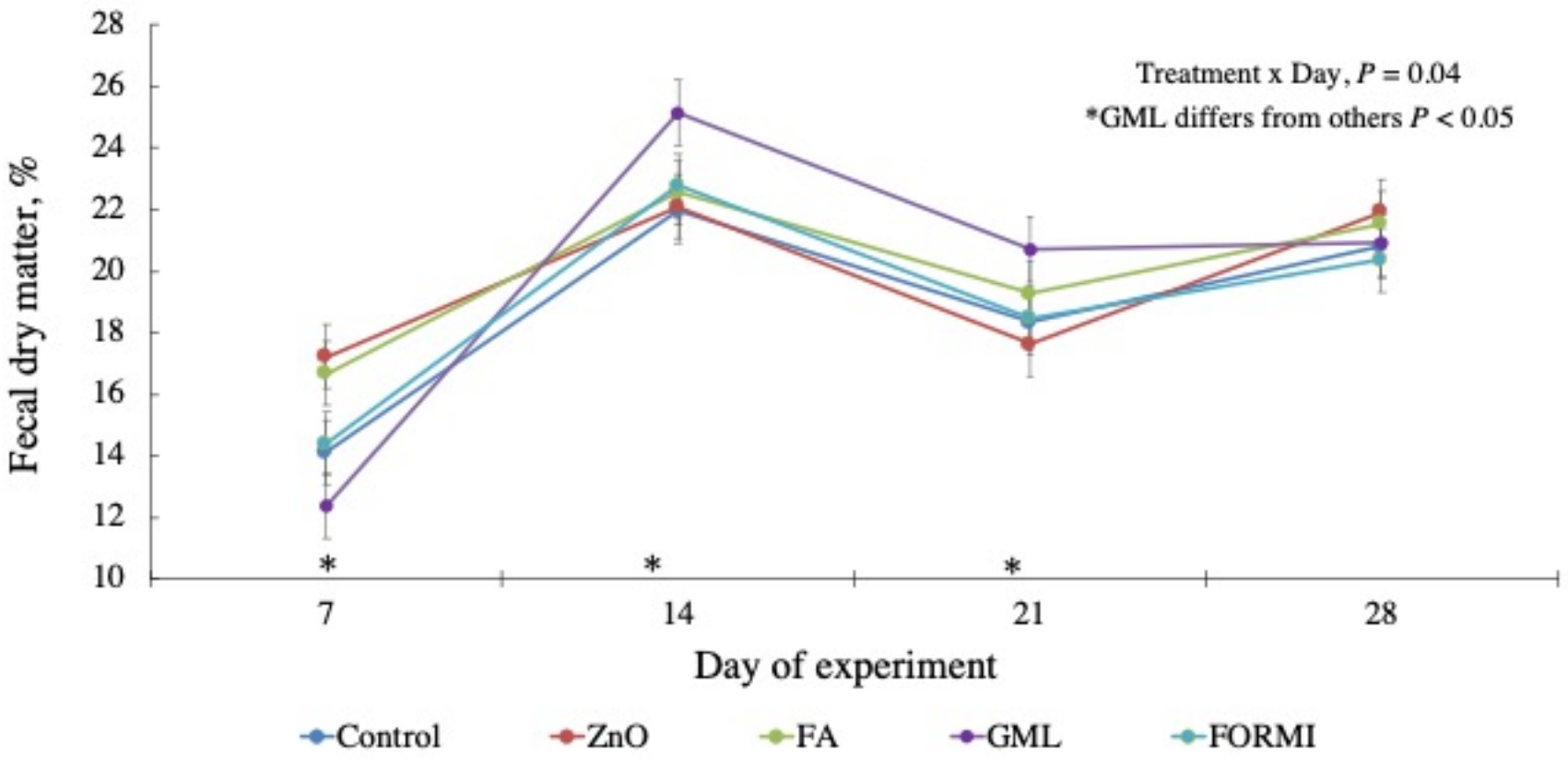


Figure 2. Relative abundance of microbial phyla by day presented as a proportion of all reads for a specific sample classified into the designated phyla.

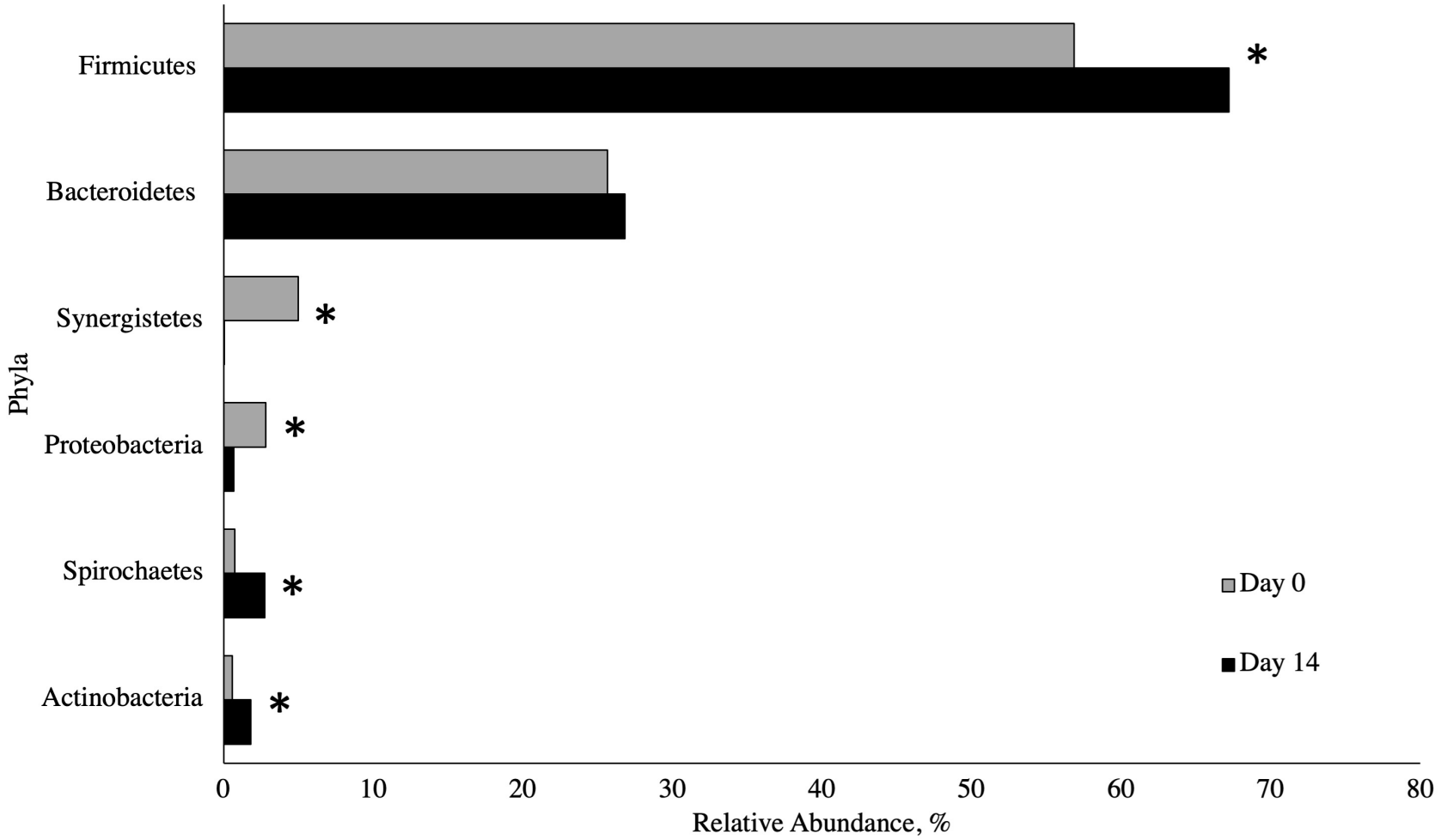


Figure 3. Relative abundance of microbial families by day presented as a proportion of all reads for a specific sample classified into the designated family.

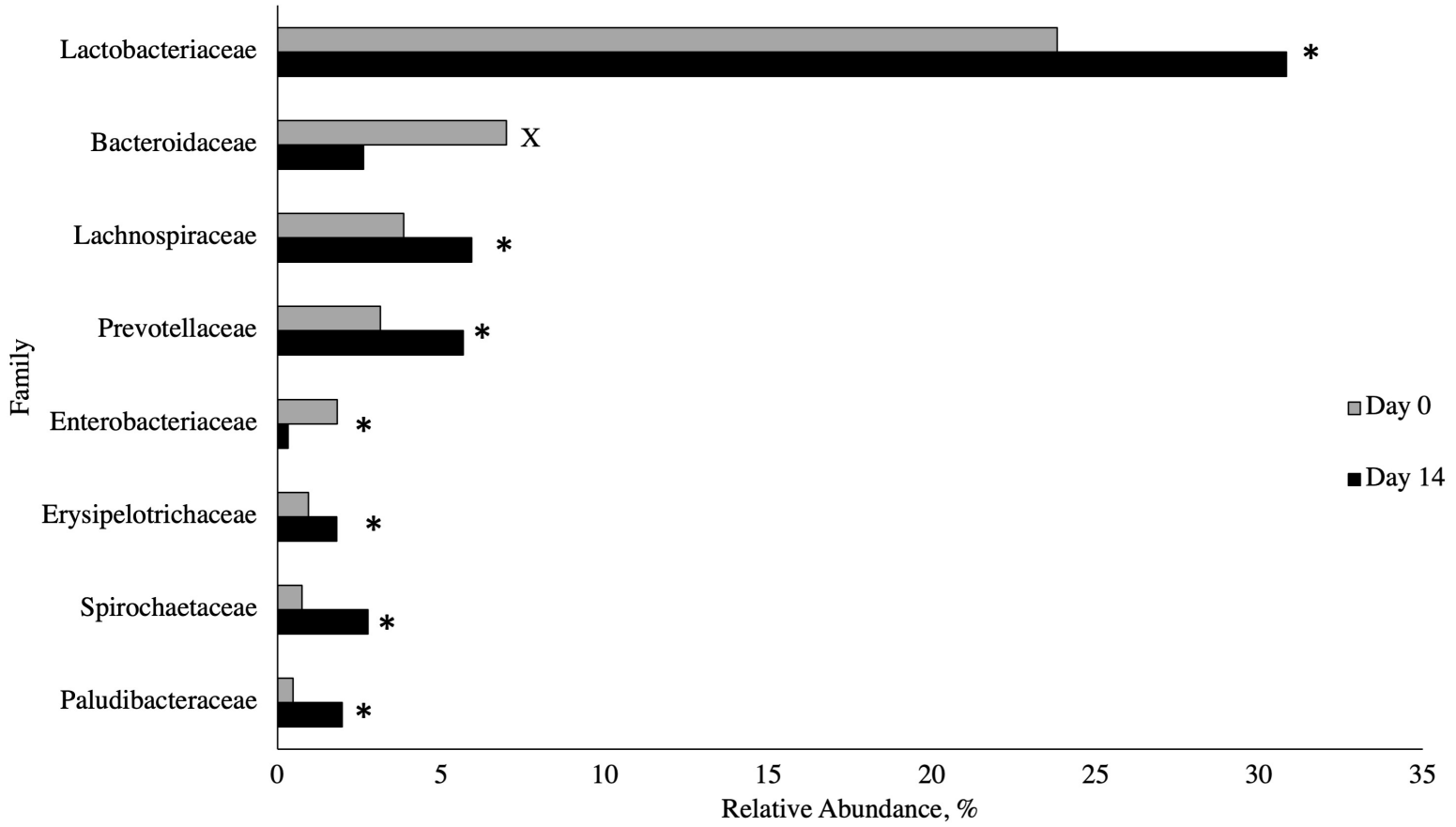
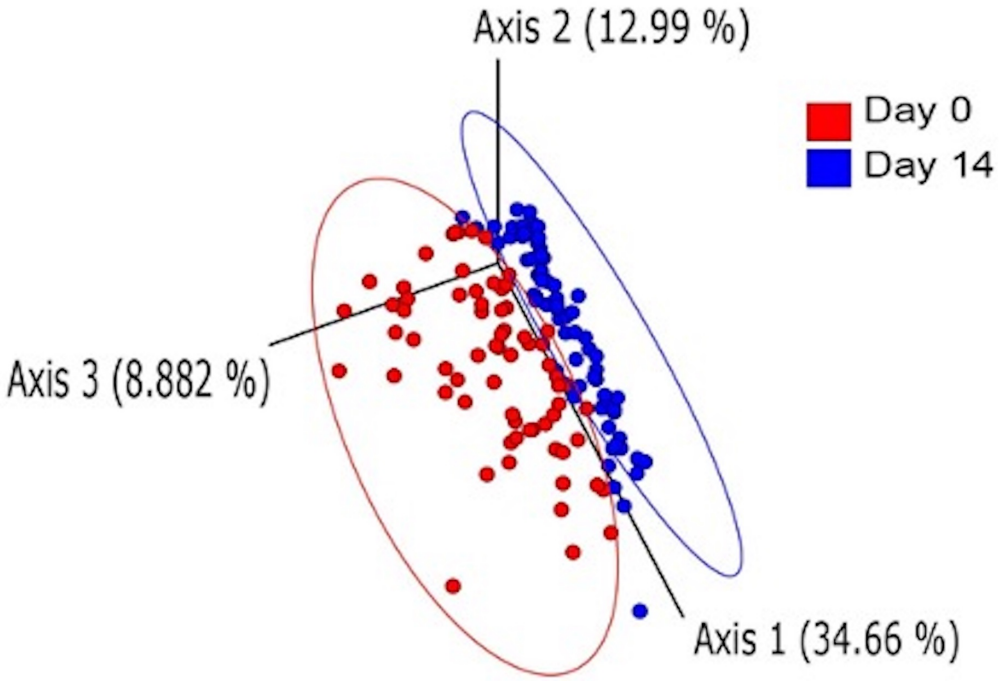


Figure 4. Principal coordinate plot of the microbial community structure between samples over time.



Chapter 3 - Evaluation of a microencapsulated form of zinc oxide on weanling pig growth performance, fecal zinc excretion, and small intestinal morphology

Abstract

A total of 300 pigs (DNA 200 × 400; initially 6.0 ± 0.08 kg BW) were used in a 42-d study to determine the effects of a microencapsulated form of zinc oxide on weanling pig growth performance, fecal zinc excretion, and small intestinal morphology. At weaning, pigs were randomly allocated to pens and pens randomly assigned to dietary treatments with 5 pigs per pen and 12 pens per treatment. Dietary treatments were: 1) negative control (CON**;** standard nursery diet containing 110 ppm Zn from trace mineral premix); 2) control diet with 400 ppm added Zn from ZnO (Low-ZnO); 3) control diet with 3,000 ppm added Zn from ZnO included in phase 1 and 2,000 ppm added Zn from ZnO included in phase 2 (High-ZnO); 4) control diet with 400 ppm added Zn from microencapsulated ZnO (Low-MZnO**;** Vetagro S.p.A., Reggio Emilia, Italy); 5) control diet with 3,000 ppm added Zn from microencapsulated ZnO in phase 1 and 2,000 ppm added Zn from microencapsulated ZnO in phase 2 (High-MZnO**;** Vetagro S.p.A., Reggio Emilia, Italy). Pigs were weighed and feed disappearance determined to evaluate ADG, ADFI and G:F. On d 10 and d 28, fecal samples from 2 pigs per pen were collected for fecal Zn concentrations and on d 28, 30 pigs (n = 6/treatment) were euthanized, and small intestinal tissues were collected to evaluate the villus height and crypt depth.. For the entire treatment period (d 0 to d 28) there was no evidence of differences in ADG, ADFI, or G:F ($P > 0.05$). During the common phase 3 (d 28 to 42) pigs fed the negative control, High-MZnO, or Low-MZnO had improved ($P < 0.0001$) ADG compared to pigs fed High- or Low-ZnO, which was

driven by an increase in ADFI ($P < 0.0001$). For the entire experiment (d 0 to 42), pigs fed Low-ZnO or High-ZnO had reduced ($P < 0.0001$) ADG compared those fed the negative control. A significant treatment × day interaction ($P = 0.04$) was observed for fecal Zn concentrations, where the level of Zn excreted in the feces was dependent on the sampling day in pigs fed a low level of ZnO or low level of microencapsulated ZnO, while excretion of Zn did not differ between d 10 and 28 in pigs fed a negative control, high level of ZnO, or high level of microencapsulated ZnO. There was no evidence ($P > 0.05$) that small intestinal morphology differed significantly between treatments.

Introduction

The post-weaning period is one of the most crucial times in swine production as the stress induced on the young pig can negatively impact performance and health, sometimes resulting in mortality. During this time when the piglet's digestive capabilities are still limited, the animal is drastically shifted from a liquid milk diet to solid feed, comingled with other pigs, and potentially exposed to pathogens (Campbell et al., 2013; Gebhardt et al., 2020; Wensley et al., 2021). Collectively, these nutritional and environmental changes can lead to a decrease in performance and clinical illness such as post-weaning diarrhea (PWD). While various nutritional and management strategies have been implemented to help lessen the negative ramifications of the post-weaning period, the addition of supplemental Zn in the form of ZnO to the diet has been proven one of the most effective (Sales, 2013). While the weanling piglet's Zn requirement is around 100 mg/kg (NRC, 2012), when included at much higher levels, near 2,000 to 4,000 ppm, improvements in growth performance and fecal consistency have been determined (Hill et al., 2001; Shelton et al., 2011). These inclusion levels, termed pharmacological, are typically used

for the first two weeks post-weaning. While the positive benefits of this feeding practice are well documented, there is scrutiny on the swine industry to reduce its reliance on pharmacological ZnO for various reasons. Since ZnO has a relatively low bioavailability, the excess Zn not absorbed by the pig is excreted in the manure that is eventually applied to the soil as fertilizer. Over time, there is potential for Zn to accumulate in the soil and pose risk to the environment. In addition to potential environmental concerns, studies have demonstrated a possible linkage of pharmacological ZnO to the development of antimicrobial resistant genes. Collectively, these issues have prompted regulatory action in some countries. For instance, the European Union placed a limit of 150 ppm total dietary Zn in 2022 (Commission, 2016). While these measures have not been enforced globally yet, the swine industry is still constantly evaluating other avenues to improve post-weaning growth performance and PWD in the instance that pharmacological ZnO supplementation becomes obsolete.

One potential method to reduce ZnO inclusion that could potentially still provide the positive benefits associated with ZnO supplementation is the use of microencapsulated ZnO. As this technology further develops, the hypothesis that lower inclusion of microencapsulated ZnO can yield similar benefits to pharmacological levels of ZnO has been examined, but on few accounts. While there is some data to suggest potential for microencapsulated ZnO to serve as a viable alternative to pharmacological ZnO, there is still variability in the responses observed among literature, primarily due to differences in inclusion levels, methodologies for microencapsulation of the ZnO, and the age and existing health status of experimental animals. Thus, our objective was to further evaluate feeding a microencapsulated form of ZnO in place of pharmacological ZnO. Specifically, we aimed to determine the effects of microencapsulated ZnO on piglet growth performance, fecal zinc excretion, and small intestinal morphology.

Materials and Methods

Animals and Diets

All experimental procedures adhered to guidelines for the ethical and humane use of animals for research according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2012) and were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4678). The experiment was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, Kansas.

A total of 300 pigs (DNA 400 × 200; Columbus, NE, initially, 6.0 ± 0.08 kg BW) were weaned at an average of 21 d of age and used in a 42-d experiment. Weaning was considered d 0 of the trial and at this point pigs were individually weighed and allotted to pens in a completely randomized design. There were 5 pigs per pen and 12 replicate pens per treatment. Each pen had tri-bar floors (1.5 × 1.5 m) and was equipped with a 4-hole dry self-feeder and nipple waterer to supply *ad libitum* access to feed and water. At weaning, pigs were randomized to pens and pens were randomly assigned to one of five dietary treatments. Diets were formulated and manufactured in three dietary phases (phase 1 = d 0 to d 10; phase 2 = d 11 to d 28; phase 3 = d 29 to d 42) such that experimental diets were fed from d 0 to 28, and a common diet was fed to all pigs from d 28 to 42. Diets were fed in pelleted form in phase 1 and in mash form in phases 2 and 3. Diets were formulated to meet or exceed (NRC, 2012) requirements with the exception of SID Lys, which was formulated to 1.40% in the phase 1 diet (Table 1) and contained no antimicrobials. Treatments were as follows: 1) negative control (CON; standard nursery diet containing 110 ppm Zn from trace mineral premix); 2) control diet with 400 ppm added zinc from ZnO (Low-ZnO); 3) control diet with 3,000 ppm added zinc from ZnO included in phase 1 and 2,000 ppm added zinc from ZnO included in phase 2 (High-ZnO); 4) control diet with 400

ppm added zinc from microencapsulated ZnO (Low-MZnO; Vetagro S.p.A., Reggio Emilia, Italy); 5) control diet with 3,000 ppm added zinc from microencapsulated ZnO in phase 1 and 2,000 ppm added zinc from microencapsulated ZnO in phase 2 (High-MZnO; Vetagro S.p.A., Reggio Emilia, Italy). All test ingredients were included at the expense of corn in the diet. Individual pig weights and feed disappearance were measured on days 0, 10, 14, 21, and 28, with pen weights collected on d 35, and 42 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F).

Chemical Analysis

Complete diet samples were collected from 10 different feeders per dietary treatment on d 0 and 21 and composite subsamples were analyzed for nutrient composition (Table 1). Assays included DM (method 930.15; AOAC, 2007), crude protein (CP) as N × 6.25 using the combustion method (Nitrogen Determinator; model TruMac N, Leco Corporation, St. Joseph, MI; method 990.03; AOAC, 2007), fat (AOAC 2003.05), acid detergent fiber (ADF) (ANKOM Tech. Method 200), Ca (AOAC 985.01, 2006), P (AOAC 985.01, 2006), and Zn (AOAC 985.01, 2006).

Fecal Zinc Content

Fresh fecal samples were collected from the same 2 randomly selected pigs per pen on d 10 and 28 (end of dietary phase 1 and 2, respectively) via rectal massage for analysis of Zn concentrations. Samples were pooled on a per pen basis to form one composite sample for each collection point and frozen at -20° C until sample preparation. Samples were dried in a 55° C forced-air oven for 48-h, ground to pass through a 1 mm screen, and shipped to a commercial laboratory (Midwest Labs, Omaha, NE) for zinc analysis (method 985.01; AOAC, 1996).

Small Intestinal Morphology

At the conclusion of dietary phase 2 (d 28 of the study), a subset of pigs (n = 30 total, 6 per treatment) closest to the treatment average BW were euthanized via captive bolt for collection of small intestinal tissue samples for morphological analysis. Briefly, once opened viscerally a 2 cm segment of ileum was harvested approximately 10 cm from the ileocecal valve and fixed in 10% buffered formalin. Samples were stored at room temperature until further processing. A 4 μm cross-section of ileum was then trimmed, embedded, routinely processed, and stained with hematoxylin and eosin per animal, and ten consecutive full-sized villi located within the mesenteric border were identified for the analysis. When incomplete, folded and/or damaged villi were observed within the area of analysis, they were excluded and a matching number of full-sized villi adjacent to the area of evaluation were included. For each villus, the villus height (VH) and crypt depth (CD) were measured using an Olympus BX53 bright light microscope coupled with a mounted Olympus DP47 camera and analyzed using Olympus cellSens 1.18 software (Olympus Life Sciences, Tokyo, Japan). The villus height to crypt depth ratio (VH:CD) was calculated. Assessment and measurement of villi was performed by a single veterinary pathologist.

Statistical Analysis

All data were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Institute, Cary, NC). Growth and fecal zinc data were analyzed as a completely randomized design with pen of pigs as the experimental unit, while individual pig was the experimental unit for intestinal morphology data. Growth and intestinal morphology data included treatment as a fixed effect in the model, while fecal Zn data included the main effects of dietary treatment, sampling day, and their interaction. All comparisons included Tukey-Kramer multiple

comparison adjustments. Data were considered significant if $P < 0.05$ and marginally significant if $0.05 < P < 0.10$.

Results and Discussion

Growth Performance

Growth performance data are presented in Table 4. During the first dietary phase (d 0 to 10), there was no evidence of differences ($P \geq 0.13$) in ADG, ADFI, or G:F across treatments. A similar response was observed during dietary phase 2 (d 11 to 28), where no statistically significant differences ($P \geq 0.27$) in growth performance were detected. Collectively, the lack of response seen during dietary phases 1 and 2 resulted in no evidence of differences ($P \geq 0.12$) in any growth performance parameters for the entirety of the treatment period (d 0 to d 28). From d 29 to 35, all pigs were fed a common phase 3 diet that contained no supplemental ZnO. During this timeframe, we observed that pigs who had previously been fed conventional ZnO had poorer ADG ($P < 0.0001$) compared to pigs fed the negative control or either dose of microencapsulated ZnO. This response appears to have been driven by feed intake, where pigs fed the negative control diet or microencapsulated ZnO had significantly greater ADFI ($P < 0.0001$) relative to their contemporaries fed conventional ZnO. Dietary treatment did not significantly impact feed conversion ($P = 0.19$) during this period. For the entirety of the experiment (d 0 to 42), pigs fed conventional ZnO at either a low or pharmacological dose, had significantly reduced ADG ($P < 0.0001$) compared to pigs fed a negative control diet or a diet containing a low or high dose of microencapsulated ZnO. This response appears to be driven by statistical differences in ADFI, where pigs fed a high dose of microencapsulated ZnO had increased ADFI ($P = 0.02$) compared to those fed either level of conventional ZnO, while pigs fed the negative control or a low dose

of microencapsulated ZnO were intermediate. Feed efficiency did not change ($P = 0.38$) during the entire experiment. Piglet BW was not significantly different across dietary treatments at experiment d 0, 10, or 28; however, by d 35, pigs fed a high level of microencapsulated ZnO were significantly heavier ($P < 0.01$) than those fed a high dose of conventional ZnO, with all other treatments being intermediate. By the end of the 42-day experiment, pigs fed either dose of conventional ZnO were significantly lighter ($P < 0.05$) than pigs fed the negative control, or both levels of microencapsulated ZnO.

Typically, pharmacological supplementation of ZnO to the weanling pig diet takes place during the first few weeks post-weaning (Canibe et al., 2022). This can be explained by the multifunctional mechanism of action of ZnO that coincides with the delicate weaning transition. While not perfectly understood, ZnO is thought to positively impact the pigs gastrointestinal tract and immune system during the post-weaning period by increasing nutrient digestibility and enzyme secretion, enhancing small intestinal barrier integrity and absorptive capacity, regulating pro- and anti-inflammatory cytokines, and providing a slight antimicrobial effect (Bonetti et al., 2021). These proposed benefits have been cited responsible for the benefits in growth performance with pharmacological ZnO supplementation immediately post-weaning across the body of literature (Hill et al., 2001; Sales, 2013). In the current study, no enhancements in growth performance were seen during the first 28-d post-weaning when pigs were fed pharmacological levels of conventional ZnO. While this type of response is quite uncommon among published literature, there are reports of either minimal or negative responses to pharmacological ZnO supplementation. For instance, in a study by Hill et al. (2001), early-weaned pigs (15 days of age) were fed 2,000 ppm ZnO and no growth response was observed during the first week post-weaning. While weaning age has been shown to play a role in the

piglet's ability to overcome the weaning transition, pigs in the current study were weaned older than pigs in the study conducted by Hill et al. (2001). It is important to note that the analyzed values of Zn in both the High ZnO and High-MZnO treatments was lower than formulated in phase 1 (3,000 ppm formulated vs. approximately 2,000 ppm analyzed), which could perhaps explain the lack of response seen immediately post-weaning. There were also no discernable differences in growth performance as a result of feeding either level of a microencapsulated ZnO during this time. This refutes previous work by Grilli et al. (2015), where weanling pigs were fed 3,000 ppm ZnO and either 300 or 800 ppm microencapsulated ZnO. These authors reported similar growth performance in pigs fed microencapsulated ZnO to those fed pharmacological ZnO. However, similar to our findings, Shen et al (2014) observed no statistically significant differneces in growth performance between pigs fed a microencapsulated form of ZnO or ZnO.

Following administration of dietary treatments from d 0 to 28, all pigs were placed on a common diet with no additional ZnO from d 29 to 42. During this time, pigs that had previously been fed a diet with conventional ZnO at either low or pharmacological levels had reduced growth performance compared to pigs previously fed the negative control or a diet with microencapsulated ZnO at low or high levels. This response was observed during the entirety of the common period, and ultimately resulted in a similar response for the entire trial. There are extremely limited published data to corroborate the negative response in gain and feed intake of pigs previously fed conventional ZnO during the common phase. Studies by Batson et al. (2021) and Feldpausch et al. (2018) found that pigs fed conventional ZnO during an experimental period had poorer feed conversion after pigs were switched to a common diet, but no significant ramifications were observed on ADG or ADFI. In an experiment by Poulsen (1989), pigs were fed 4,000 ppm ZnO and had reductions in both ADFI and ADG, which were attributed to

potential zinc toxicity. According to Burrough et al. (2019), pigs experiencing zinc toxicosis can have reduced ADG and G:F; however, chemical analysis of diets in the current experiment validate that Zn levels were substantially lower than that of Poulsen (1989), nor did pigs experience other clinical signs indicative of Zn overload, such as lameness, depression, or elevated serum zinc (data not shown). We are unable to clearly explain the response observed during the common period and subsequently, the overall trial as it relates to piglet growth performance. Further investigation into how a microencapsulated form of ZnO can impact weaned pig performance should be conducted.

Fecal Zinc Content

Fecal Zn levels are shown in Table 5 and are presented as the ppm of Zn detected in the fecal sample on a DM basis. A significant sampling day × treatment interaction ($P = 0.04$) was observed for fecal Zn content, where the level of Zn excreted in the feces was dependent on the sampling day in pigs fed a low level of ZnO or low level of microencapsulated ZnO, while excretion of Zn did not differ between d 10 and 28 in pigs fed a negative control, high level of ZnO, or high level of microencapsulated ZnO.

There are concerns that pharmacological ZnO supplementation can result in accumulation of Zn in the soil when swine manure is applied as fertilizer. It has been shown that the bioavailability of ZnO is much lower than other organic sources of Zn, thus, the portion of Zn not absorbed by the pig is then excreted in the feces (Poulsen and Larsen, 1995; Buff et al., 2005). While data to support long-term environmental toxicity from swine manure is rather scarce, regulatory action has been taken in some parts of the world to limit ZnO supplementation at levels in excess of nutritional requirements such that excretion of Zn in swine manure is reduced. One of the hypothesized advantages of microencapsulated ZnO is that lower inclusion

levels can be fed compared to ZnO. In this case, inclusion of microencapsulated ZnO can be lower than the 2,000 to 4,000 ppm that are typically used when adding conventional ZnO to the weanling pig diet (Hill et al., 2001). Our study design inherently allowed for the direct comparison of ZnO and microencapsulated ZnO at two inclusion levels, either a low dose (400 ppm added Zn) or pharmacological dose (2,000 and 3,000 ppm added Zn). We are unable to clearly explain the significant interaction observed in the current study. It is important to note limitations of our methodology, as we did not account for Zn intake or differences in fecal output between treatments and across sampling days. Samples were analyzed on a DM basis and not adjusted. While we know that fecal excretion of Zn increases linearly with Zn intake (Hansen et al., 2023), future work evaluating Zn excretion should consider factors such as Zn intake and differences in fecal output.

Small Intestinal Morphology

The villus height, crypt depth, and villus height: crypt depth ratio were evaluated in both the proximal and distal ileum. No evidence of differences in in proximal ileal VH ($P = 0.34$), CD ($P = 0.52$), and VH:CD ($P = 0.49$) were observed between dietary treatments. Likewise, there was no evidence of differences in distal ileal VH ($P = 0.63$), CD ($P = 0.53$), or VH:CD ($P = 0.80$) between dietary treatments.

The gastrointestinal architecture of the newly weaned pig is often underdeveloped, and notably, villi atrophy can occur during the post-weaning period (Pluske, 2013). Studies have reported morphological improvements in the small intestine of weaned pigs fed pharmacological levels of ZnO, whereas the villus height was increased (Li et al., 2001; Zhu et al., 2017). However, it is well-known that ZnO supplementation at pharmacological levels does not consistently increase absorption of Zn (Poulsen and Larsen, 1995; Krebs, 2000). Therefore, it is

theorized that microencapsulated ZnO can allow for a slower and more controlled release of Zn along the small intestine, thereby increasing its ability to be absorbed and impact intestinal health. Previous studies have tested this hypothesis and reported significant improvements in small intestinal morphology in pigs fed various doses of microencapsulated ZnO compared to a negative control, with no differences relative to conventional ZnO fed at pharmacological doses (Grilli et al., 2015; Kim et al., 2015; Lei and Kim, 2018). Conversely, we observed no histological alterations in the ileum as a result of feeding both conventional ZnO or microencapsulated ZnO. These results were unexpected, but perhaps corroborate the lack of growth performance differences observed throughout the experimental period. In effort to capitalize on the stress of weaning and avoid time to allow potential enterocyte proliferation and villi recovery, pigs in the current study were euthanized and ileal tissue samples collected at 14 days post weaning (dpw). It is difficult to conclude that sampling day explains our lack of differences, as others have evaluated small intestinal morphology at similar time points and observed significant changes due to microencapsulated ZnO supplementation (Grilli et al., 2015). However, many authors have reported findings similar to ours, whereas both conventional and microencapsulated forms of ZnO had no apparent impact on the morphological environment of the small intestine (Jang et al., 2014; Kwon et al., 2014; Park et al., 2015). The variability in results across the literature can be explained by differences in dosage, piglet health status, and experimental conditions. Pigs in the current work were housed in a research facility with no known health challenges, which could potentially explain why no differences in small intestinal morphology were observed.

Conclusions

In summary, feeding ZnO or a microencapsulated form of ZnO at both low and high inclusion levels did not alter the growth performance of pigs immediately post-weaning. However, pigs fed ZnO at both a low or high level had reduced performance during the late nursery compared to those fed either level of microencapsulated ZnO or a control diet. Additionally, small intestinal morphology was not impacted by supplementing either form of ZnO at both a low and high level. Fecal excretion of Zn across dietary treatments was dependent on the sampling day, but pigs fed pharmacological levels of ZnO had numerically greater excretion of Zn compared to all other treatments.

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Table 3.1. Composition of phase 1, phase 2, and phase 3 basal diets (as-fed basis).

	Dietary Phase[1]		
Item	Phase 1	Phase 2	Phase 3
Ground corn	44.61	49.71	65.65
Soybean meal, 47.5% CP	17.83	24.60	28.30
Whey permeate, 80% lactose	10.00	-	-
Spray dried whey	10.00	10.00	-
Corn DDGS, 7.5% oil	5.00	7.50	-
Enzymatically treated soybean meal[2]	4.00	3.50	-
Fish meal	2.50	-	-
Spray dried bovine plasma	2.00	-	-
Choice white grease	1.00	1.00	2.00
Calcium carbonate, 38.5% Ca	0.60	0.85	0.75
Monocalcium phosphate, 21.5% P	0.75	0.85	1.10
Salt	0.30	0.50	0.60
L-Lys HCL	0.45	0.55	0.55
DL-Met	0.20	0.22	0.21
L-Thr	0.19	0.20	0.23
L-Trp	0.03	0.04	0.05
L-Val	0.10	0.08	0.16
Vitamin premix w/ phytase[3]	0.25	0.25	0.25
Trace mineral premix[4]	0.15	0.15	0.15
Choline chloride, 60%	0.04	-	-
Zinc oxide	Varied	Varied	-
Zincoret S[5]	Varied	Varied	-
Total	100.00	100.00	100.00
Calculated analysis[6]			
SID amino acids, %			
Lys	1.40	1.35	1.30
Ile:Lys	56	56	53
Leu:Lys	114	119	111
Met:Lys	36	37	36
Met and Cys:Lys	57	58	57
Thr:Lys	65	63	63
Trp:Lys	18	19	19.3
Val:Lys	72	67	70
His:Lys	33	34	35
Total Lys, %	1.56	1.51	1.43

ME, kcal/kg	3,321	3,359	3,383
NE, kcal/kg	2,512	2,134	2,534
SID Lys:NE, g/Mcal	4.10	4.02	5.13
CP, %	21.7	21.2	19.9
Ca, %	0.71	0.70	0.65
P, %	0.65	0.63	0.61
STTD P, %	0.46	0.40	0.49

[1]Treatment diets were fed to 300 pigs [DNA 400 × 200 (Columbus, NE); initially 6.03 ± 0.08 kg BW] for 28 d in a 2-phase feeding program with 5 pigs per pen and 12 pens per treatment. A common phase 3 diet was fed to all pigs from d 29 to d 42.
[2]HP300 (Hamlet Protein, Findlay, OH).
[3]Premix provided per kg of premix: Ronozyme HiPhos (DSM Nutritional Products, Basel, Switzerland) provided 2,027 FTU/kg of feed and an estimated release of 0.12% STTD P4,409,249 IU vitamin A; 551,156 IU vitamin D3; 17,637 IU vitamin E; 1,764 mg vitamin K; 15.4 mg vitamin B12; 19,842 mg niacin; 11,023 mg pantothenic acid; and 3,307 mg riboflavin.
[4]Premix provided per kg of premix: 110 g Fe from iron sulfate; 110 g Zn from zinc sulfate; 26.4 g Mn from manganese oxide; 11 g Cu from copper sulfate; 198 mg I from calcium iodate; 198 mg Se from sodium selenite.
[5]Zincoret S (Vetagro S.p.A., Reggio Emilia, Italy).
[6]NRC (2012).

Table 3.2. Analyzed composition of phase 1 and phase 2 experimental diets (as-fed basis)[1].

	Dietary Treatment[2]				
Analyzed composition, %[3]	CON	Low-ZnO	High-ZnO	Low-MZnO	High-MZnO
Phase 1					
DM, %	88.74	88.11	87.99	87.84	87.65
CP, %	19.00	18.30	18.9	18.90	19.5
Fat, %	3.53	3.53	3.43	3.43	3.83
ADF, %	2.60	2.50	2.40	2.50	2.60
Calcium, %	0.75	0.69	0.70	0.77	0.89
Phosphorous, %	0.64	0.62	0.63	0.69	0.71
Zinc, ppm	178	572	2210	458	2110
Phase 2					
DM, %	87.86	87.43	87.74	87.76	88.03
CP, %	21.5	20.8	21.5	21.5	19.4
Fat, %	3.33	3.26	3.61	3.47	3.85
ADF, %	4.60	4.90	4.40	4.40	4.30
Calcium, %	0.76	0.66	0.79	0.91	1.15
Phosphorous, %	0.55	0.51	0.60	0.62	0.59
Zinc, ppm	127	391	2060	782	1370

[1] Treatment diets were fed to 300 pigs [DNA 400 × 200 (Columbus, NE); initially 6.0 ± 0.08 kg BW] for 28 d in a 2-phase feeding program with 5 pigs per pen and 12 pens per treatment.
[2]Dietary treatments included: negative control basal diet with no added ZnO (CON); basal diet with 400 ppm added Zn from ZnO in phases 1 and 2 (Low-ZnO); basal diet with 3,000 ppm added Zn from ZnO in phase 1 and 2,000 ppm added Zn from ZnO in phase 2 (High-ZnO); basal diet with 400 ppm added Zn from microencapsulated ZnO in phases 1 and 2 (Low-MZnO); and basal diet with 3,000 ppm added Zn from microencapsulated ZnO in phase 1 and 2,000 ppm added Zn from microencapsulated ZnO in phase 2 (High-MZnO).
[3]Complete diet samples were collected from the same 10 randomly selected feeders on d 0 and d 21. Samples were pooled by day and subsampled, then submitted to Midwest Laboratories (Omaha, NE) for proximate and zinc analysis.

Table 3.3. Analyzed composition of phase 3 common diet (as-fed basis)[1]

Analyzed composition, %[2]	Diet
DM, %	86.07
CP, %	20.10
Fat, %	3.78
ADF, %	3.50
Calcium, %	0.73
Phosphorous, %	0.61
Zinc, ppm	103

[1] A common diet was fed to 300 pigs [DNA 400 × 200 (Columbus, NE); initially 6.0 ± 0.08 kg BW] from d 29 to d 42 with 5 pigs per pen and 12 pens per treatment.
[2]Complete diet samples were collected from the same 10 randomly selected feeders on d 28 and d 42. Samples were pooled and subsampled, then submitted to Midwest Laboratories (Omaha, NE) for proximate and zinc analysis.

Table 3.4. Growth performance of pigs fed a diet containing a low or high level of added zinc in the form of either free zinc oxide (ZnO) or a microencapsulated zinc oxide[1].

	Dietary Treatment[2]						
Item;	CON	Low ZnO	High ZnO	Low M-ZnO	High M-ZnO	SEM	*P*-value
BW, kg							
d 0	6.00	6.06	6.00	6.07	6.07	0.083	0.94
d 10	7.28	7.35	7.29	7.35	7.49	0.117	0.71
d 28	14.22	13.90	13.92	14.60	14.62	0.296	0.24
d 35	18.31^{abc}	17.29^{bc}	16.84^{c}	18.57^{ab}	18.89^{a}	0.383	< 0.01
d 42	23.59^{a}	21.40^{b}	20.77^{b}	23.65^{a}	24.38^{a}	0.477	< 0.0001
Phase 1 (d 0 to 10)							
ADG, kg/d	0.13	0.13	0.13	0.13	0.14	0.008	0.63
ADFI, kg/d	0.18	0.21	0.21	0.22	0.21	0.010	0.13
G:F	0.68	0.61	0.62	0.60	0.70	0.033	0.14
Phase 2 (d 11 to 28)							
ADG, kg/d	0.36	0.32	0.36	0.38	0.37	0.010	0.81
ADFI, kg/d	0.55	0.58	0.52	0.53	0.51	0.025	0.27
G:F	0.66	0.64	0.71	0.72	0.74	0.039	0.28
Overall treatment (d 0 to 28)							
ADG, kg/d	0.30	0.28	0.28	0.30	0.30	0.009	0.26
ADFI, kg/d	0.45	0.46	0.44	0.45	0.45	0.013	0.70
G:F	0.66	0.62	0.65	0.67	0.68	0.017	0.12
Overall common (d 28 to 35)							
ADG, kg/d	0.67^{a}	0.54^{b}	0.51^{b}	0.65^{a}	0.69^{a}	0.017	< 0.0001
ADFI, kg/d	0.91^{ab}	0.74^{c}	0.77^{bc}	0.95^{a}	1.00^{a}	0.036	< 0.0001
G:F	0.74	0.73	0.67	0.69	0.69	0.024	0.19
Overall Experiment (d 0 to 42)							
ADG, kg/d	0.41^{a}	0.36^{b}	0.36^{b}	0.41^{a}	0.42^{a}	0.010	< 0.0001
ADFI, kg/d	0.60^{ab}	0.55^{bc}	0.54^{c}	0.60^{ab}	0.61^{a}	0.018	0.02
G:F	0.70	0.67	0.65	0.68	0.68	0.015	0.38

^{abc}Means within a row that do not share a common superscript differ significantly ($P < 0.05$).

[1]A total of 300 weanling pigs [DNA 400 × 200 (Columbus, NE); initially 6.0 ± 0.08 kg BW] were used in a 42-d growth study with 5 pigs/pen and 12 replicates/treatment.

[2]Dietary treatments included: negative control basal diet with no added ZnO (CON); basal diet with 400 ppm added Zn from ZnO in phases 1 and 2 (Low-ZnO); basal diet with 3,000 ppm added Zn from ZnO in phase 1 and 2,000 ppm added Zn from ZnO in phase 2 (High-ZnO); basal diet with 400 ppm

added Zn from microencapsulated ZnO in phases 1 and 2 (Low-MZnO); and basal diet with 3,000 ppm added Zn from microencapsulated ZnO in phase 1 and 2,000 ppm added Zn from microencapsulated ZnO in phase 2 (High-MZnO).

Table 3.5. Effect of feeding microencapsulated zinc oxide (ZnO) on weanling pig fecal Zn excretion (ppm Zn, DM basis)[1].

	Dietary Treatment[2]						
Sampling Day	CON	Low-ZnO	High-ZnO	Low-MZnO	High-MZnO	SEM	Treatment × Day, *P* =
d 10	2,260^{cde}	4,560^{bc}	9.,960^{a}	5,830^{b}	6,960^{b}	629.5	0.04
d 28	624^{e}	1,791^{de}	9,413^{a}	2,659^{cd}	6,045^{b}		

[1]A total of 300 pigs [DNA 200 × 400 (Columbus, NE); initially 6.03 ± 0.08 kg BW] were used in a 42-day experiment with 5 pigs per pen and 12 pens per treatment. On d 10 and d 28, a fresh fecal sample was collected from the same two randomly selected pigs per pen for zinc analysis.

[2]Dietary treatments were: negative control basal diet with no added ZnO (CON); basal diet with 400 ppm added Zn from ZnO in phases 1 and 2 (Low-ZnO); basal diet with 3,000 ppm added Zn from ZnO in phase 1 and 2,000 ppm added Zn from ZnO in phase 2 (High-ZnO); basal diet with 400 ppm added Zn from microencapsulated ZnO in phases 1 and 2 (Low-MZnO); and basal diet with 3,000 ppm added Zn from microencapsulated ZnO in phase 1 and 2,000 ppm added Zn from microencapsulated ZnO in phase 2 (High-MZnO).

Chapter 4 - Impacts of a post-transport/pre-processing rest period on the growth performance, anthelmintic efficacy, and serum metabolite changes in cattle entering a feed yard[3]

Abstract

A total of 80 crossbred, high-risk heifers (initially 250 ± 4.2 kg BW), were transported from an Oklahoma City, Oklahoma sale barn to the Kansas State University Beef Cattle Research Center. Cattle were unloaded and randomly placed into one of four receiving pens and provided *ad libitum* hay and water. Each pen was randomly assigned to one of four rest-times before processing: 1) immediately upon arrival **(0)**; 2) after a 6-h rest period **(6)**; 3) after a 24-h rest period **(24)**; and 4) after a 48-h rest period **(48)**. After all cattle were processed, heifers were allotted into individual pens with *ad libitum* access to a receiving ration and water. Heifers were weighed individually on d 0, 7, 14, 21, 28 and 35 to calculate average daily gain **(ADG)**. Feed added and refusals were measured daily to determine dry matter intake **(DMI)**. A fecal egg count reduction test and analysis of blood serum metabolites were also conducted. All data were analyzed using the GLIMMIX procedure of SAS (v. 9.4, Cary, NC) with individual animal as the experimental unit.

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[3]This work has been published in *Translational Animal Science:* Dahmer, P. L., C. A. Zumbaugh, M. E. Reeb, N. B. Stafford, Z. T. Buessing, K. G. Odde, J. S. Drouillard, A. J. Tarpoff, and C. K. Jones. 2022. Impacts of a post-transport/pre-processing rest period on the growth performance, anthelmintic efficacy, and serum metabolite changes in cattle entering a feed yard. Transl. Anim. Sci. 6(3)doi: 10.1093/tas/txac085.

Processing time did not impact ($P > 0.05$) heifer BW or ADG. From d 0 to 35, DMI decreased linearly ($P = 0.027$) as rest time increased. The number of days for heifers to reach a DMI of 2.5% BW was linearly increased ($P = 0.023$) as rest time increased. There was no evidence of differences ($P \geq 0.703$) among rest times for feed efficiency. While morbidity did not differ between treatments ($P > 0.10$), mortality increased linearly ($P = 0.026$) as the time of rest increased. A significant processing time × day interaction ($P < 0.0001$) was observed for the prevalence of fecal parasites, where the percentage of positive samples was significantly lower 14-d after anthelmintic treatment, regardless of the processing time. Serum IBR titer for heifers processed at either 0 or 6-h upon arrival was significantly higher ($P < 0.01$) on d 35 compared to d 0. Heifers processed after a 48-h rest period had significantly higher glucose values ($P < 0.01$) on d 0 compared to heifers processed at 0, 6, or 24-h. In summary, rest time prior to processing did not impact receiving calf growth performance. A 6-h rest period upon arrival appeared to be most beneficial to DMI. Anthelmintic treatment at processing reduced the parasitic load in heifers processed at all times. Vaccine titer did not increase after initial processing in heifers processed 24- or 48-h after arrival, indicating the seroconversion of IBR antibodies during the longer rest period.

Introduction

Beef cattle are exposed to stress at multiple points throughout their life. While producers try to limit these instances, some, like transportation, are unavoidable. Transportation of cattle in the United States occurs in many facets such as movement through livestock auctions, to feedlots, and eventually to processing facilities. This means cattle can be transported once, up to five or more times in their lifetime (Schwartzkopf-Genswein and Grandin, 2014). The stress induced from transport can predispose calves to dehydration, reduced feed intake, inhibition of

immune function, and increased susceptibility to bovine respiratory disease **(BRD)** (Van Engen et al., 2018). This disease, caused by both viral and bacterial agents, is responsible for substantial economic loss to the beef industry, totaling an estimated $1 billion, annually (NAHMS, 2013). Many methods have been adopted to decrease the severity of transport stress in newly received cattle. Preconditioning cattle by ensuring adequate weaning time prior to transport, vaccinating, castrating, dehorning, and treating with anthelmintics has been proven extremely effective (Duff and Galyean, 2007). In many cases, cattle are sourced from various locations and previous nutrient and health status is unknown. Therefore, management of cattle upon receiving also plays an integral role in their health and performance after arrival. Appropriately vaccinating, deworming, and treatment with antibiotics is part of a successful receiving protocol. Likewise, providing newly received cattle with a nutrient-dense diet can combat their reduced feed intake (Loerch and Fluharty, 1999). Additionally, rest time during long transport of cattle has been studied, but data is variable regarding its benefits to animal stress-levels and performance upon receiving (Melendez et al., 2021; Cooke et al., 2013; Marti et al., 2017). Delaying processing upon arrival to a feedlot is also an area of interest to counteract the stress associated with transport. Once received to a feedlot, cattle are typically placed into a receiving pen and allowed to rest, which is then followed by processing and placement into feedlot pens (Thomson et al., 2015). However, few studies have evaluated different rest times under controlled conditions. This lack of recent data prompted the current study, where we hypothesized that allowing calves rest time upon arrival would improve calf health and feedlot performance. Thus, our objectives were to evaluate the impact a post-transport rest period had on calf growth performance, mortality, and morbidity. This study also aimed to determine if a rest period affected calf response to anthelmintics and blood serum metabolites.

Materials and Methods

Animals and Experimental Design

All experimental procedures adhered to the guidelines for the ethical and humane use of animals for research according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4279).

A total of 80 crossbred heifers (initially 250 ± 4.2 kg BW) were transported approximately 482 km from an Oklahoma City, Oklahoma sale barn to the Kansas State University Beef Cattle Research Center (Manhattan, Kansas) via semi-truck, with a total transit time of approximately six hours. Heifers were considered high-risk and originated from a geographic area high in parasites. Upon arrival, heifers were unloaded and as they came off the trailer were placed into one of four pens with free-choice water and alfalfa hay in a completely randomized design. Each pen of heifers (n = 20) was then randomly assigned to one of four treatments of varying rest-times before processing: 1) immediately upon arrival **(0)**; 2) after a 6-h rest period **(6)**; 3) after a 24-h rest period **(24)**; and 4) after a 48-h period **(48)**. Processing was considered d 0 for the trial. At processing, all heifers were tagged, weighed, and subcutaneously injected with 1.0 ml/50 kg BW moxidectin (Cydectin®, Bayer Animal Health, Shawnee Mission, KS) and orally dosed with 1.0 ml/50 kg BW oxfendazole (Synanthic®, Boehringer Ingelheim Animal Health, St. Joseph, MO). Heifers were also subcutaneously injected with 1.1 ml/45 kg BW tulathromycin (Draxxin®, Zoetis Animal Health, Parsippany, NJ), 2 ml of a recombinant *Mannheimia haemolytica* leukotoxoid vaccine (Nuplura® PH, Elanco Animal Health, Greenfield, IN), and 2 ml of a modified-live virus vaccine (Titanium® 5, Elanco Animal Health, Greenfield, IN) containing infectious bovine rhinotracheitis **(IBR)**, bovine viral diarrhea (types 1

and 2), bovine respiratory syncytial virus, and parainfluenza 3. Finally, heifers were implanted with 140 mg trenbolone acetate and 14 mg of estradiol (Revalor-H®, Merck Animal Health, Whitehouse Station, NJ). After processing, cattle were returned to their receiving pen until all cattle had been processed at 48-h after arrival to the facility. Heifers were then placed into individual pens across two separate barns, with each pen containing an automatic waterer and feed bunk to provide *ad libitum* access to feed and water. Heifers were fed a standard receiving ration twice daily with feed refusals recorded. The diet was supplied as a total mixed ration **(TMR)**, that met or exceeded all NASEM (2016) requirements. Diets consisted of 40% dry rolled corn, 30% ground alfalfa hay, 26% corn silage, and 4% receiving supplement (DM basis; Table 1). All animals were monitored daily for any health abnormalities. Any treatments were determined by staff in accordance with the facility's standard operating procedures and all moralities had necropsies conducted at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS). All animals treated or removed from the trial were recorded, with morbidity analysis including first pull, second pull, third pull, and chronic.

Data Collection

Heifers were weighed individually on d 0, 7, 14, 21, 28 and 35 to calculate average daily gain **(ADG)**. Feed was individually weighed and delivered to each heifer twice daily, with refusals collected and weighed daily to determine dry matter intake (**DMI)**. On d 0 (processing) and d 35, blood samples were collected via the coccygeal vein from each heifer using sterile 15-mL vacutainer tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood samples were immediately placed on ice and transported to the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS) where serum was separated by centrifugation at 1,000 × *g* for 30 min and then analyzed for infectious bovine rhinotracheitis **(IBR)** titer via serum neutralization

antibody test. Additionally, samples were analyzed for biochemical parameters via spectrophotometry using the Cobas c501 (Roche Diagnostics, Indianapolis, IN). In order to evaluate the efficacy of anthelmintic treatment, fresh fecal samples were collected via rectal grab on d 0 (processing) and d 14, placed on ice, and immediately transported to the Kansas State University Veterinary Diagnostic Laboratory for analysis of fecal parasites. First, semiquantitative analysis was conducted as described by Garcia et al. (2017), to determine the density of organisms in samples with a positive result. Using a microscope, each sample was given a density score according to the following: 1) rare/occasional (2 to 5 organisms per entire 22 × 22-mm coverslip area); 2) scanty/light/few (2 or fewer eggs or larvae/5 to 10 fields); 3) moderate (3 to 9 eggs or larvae/field); 4) numerous/heavy/many (10+ eggs or larvae/field). Then, a fecal egg count reduction test (FECRT) was conducted according to Gasbarre et al. (2009) using a modified Wisconsin Sugar Floatation Technique in order to determine the number of eggs per gram of feces. A subsample of 10 heifers/treatment was collected at processing and snap frozen in liquid nitrogen for subsequent analysis.

Statistical Analysis

All data were analyzed as a completely randomized designed using the GLIMMIX procedure of SAS (v. 9.4, Cary, NC) with individual animal as the experimental unit. The statistical model included the random effects of 'barn' and 'location within barn'. All comparisons included Tukey-Kramer multiple comparison adjustments. For growth performance, morbidity, and mortality data, pre-planned polynomial contrasts were conducted to evaluate linear and quadratic trends. For fecal parasite data and blood metabolite data, the model included the main effects of treatment and sampling day, as well as their interaction. Results were considered significant if $P < 0.05$ and a tendency if $0.05 < P < 0.10$.

Results and Discussion

Growth Performance, Mortality, and Morbidity

Processing time did not impact ($P \geq 0.624$) heifer BW or ADG for the duration of the experiment. From d 0 to d 14, there was a linear inverse relationship between DMI and time of rest ($P = 0.012$), where DMI decreased as the time of rest before processing increased. Likewise, for the overall experiment (d 0 to d 35), DMI decreased linearly ($P = 0.027$) as the rest time increased. Heifer DMI as a % of BW from d 0 to 14 decreased linearly ($P = 0.020$) as time of rest increased; however, this impact was only marginally significant ($P \geq 0.061$) for the remainder of the trial. The number of days for heifers to reach a DMI of 2.5% BW was linearly increased ($P = 0.023$) as time of rest increased, with heifers processed at 0, 6, 24, or 48 hours requiring 18, 15, 18, and 20 d to reach this parameter, respectively. The main effect of rest time significantly impacted ($P = 0.038$) the percentage of heifers that reached a targeted DMI of 2.5% BW by d 14 of the experiment, where 25.0, 60.0, 52.6, and 23.5% of cattle reached this parameter after 0, 6, 24, and 48 hours of rest prior to processing, respectively. Feed efficiency did not differ ($P \geq 0.70$) between rest times. While morbidity did not differ between treatments ($P > 0.10$), mortality increased linearly ($P = 0.026$) as the time of rest increased. This increase was due to the loss of two experimental animals in the 48-h treatment on d 2 of the study.

Our data suggest that delaying processing time upon arrival does not impact growth performance of newly received feedlot cattle. However, processing cattle at 6-h upon arrival appeared to be the most beneficial to improve DMI. Heifers processed at 6-h had increased DMI for the duration of the experiment and took the fewest days to reach a targeted DMI of 2.5% BW. It is known that newly received cattle at a feed yard have reduced DMI (Loerch et al., 1999; Colombo et al., 2021). Calves that are healthy and unstressed can consume up to 3% of their

BW, while high-risk, highly stressed cattle tend to consume 1.5% or less during the initial two weeks after receiving (Reinhardt and Thomson, 2015). Heifers in the current study were considered high-risk, but all cattle had a DMI well-beyond 1.5% BW two weeks into the experiment. By d 14, we saw a linear decrease in DMI as calves were processed beyond 6-h. Additionally, cattle were not allotted to their experimental pens until all calves had been processed at 48-h post-arrival. Each receiving pen provided free-choice hay and water, but no concentrate was provided until all cattle had been processed at 48-h. Much work has been done assessing how this time frame impacts digestion and rumen function of cattle and their ability to adapt to the receiving diet (Duff and Gaylean, 2007; Gilbery et al., 2007; Smock et al., 2020). Limited ruminal fermentative capacity **(RFC)** is a potential factor responsible for limited feed intake in cattle deprived of feed due to transport. Several researchers have reported reduced ability of rumen microbes to ferment substrate after 48-h without feed (Baldwin, 1967; Cole and Hutcheson, 1981). Since all calves were feed-deprived for an equal amount of time, the increased DMI in calves processed at 6-h indicates their ability to adapt to the receiving diet sooner compared to their contemporaries in this study, however, we don't have a clear explanation for why this occurred. Unfortunately, other parameters like RFC or digestibility were not measured to assess a potential mechanism of action behind the increase in DMI in heifers processed at 6-h. An important limitation of the current work was how cattle were fed. Unlike a traditional feedlot setting, heifers were penned and fed individually, which is not completely indicative of normal industry practice.

Anthelmintic Efficacy

Fecal parasitic data are presented in Table 3. The percentage of positive samples for overall fecal parasite prevalence was significantly lower ($P < 0.0001$) 14-d after anthelmintic

treatment when compared to the count on d 0. The processing time × day interaction was significant ($P \leq 0.04$) for the semiquantitative density of Strongyle, Eimeria, Trichuris, and Strongyloides organisms, where their density was significantly lower 14-d after anthelmintic treatment. Finally, only Strongyle and Eimeria eggs were identified using the FECRT, and the processing time × day interaction was again significant ($P \leq 0.01$), where number of eggs per g of feces was lower on d 14 compared to d 0.

We hypothesized that anthelmintic treatment at processing would reduce the presence of fecal parasites by d 14, regardless of when cattle were processed upon arrival. The World Association for Advancement of Veterinary Parasitology (WAAVP) has established guidelines for conducting FECRT and suggest that anthelmintic efficacy be determined at a 90% reduction threshold (Coles et al., 1992). The presented data indicate that treatment with both moxidectin and oxfendazole were effective at reducing fecal parasites. Cattle across all treatments were at or above this threshold at initial sampling on d 0 (94.7, 90.0, 100.0, and 93.3% prevalence for the 0, 6, 24, and 48-h processing times, respectively), but were significantly reduced and fell below the 90% threshold by sampling on d 14 (21.1, 20.0, 11.1, and 40.0% prevalence for the 0, 6, 24, and 48-h processing times, respectively). These results were expected and coincide with other work studying anthelmintic efficacy (Utley et al., 1974; Ives et al., 2007; Fazzio et al., 2016). Gastrointestinal parasitism is a leading cause of reduced performance in newly received feedlot cattle, and prior environment plays a large role in this, as cattle that have been backgrounded on pasture have increased exposure to larvae which can prompt further infections (Griffin et al., 2018). The heifers in the current study originated from a geographic location high in parasites, therefore, the large parasite burden on d 0 was expected. Parasitic infections can lead to reduced intake, digestibility, and other physiological mechanisms which can thereby negatively impact

animal health, performance, and economic efficiency (Perry et al., 1999). Since it is well established that anthelmintic treatment is often effective, producers should use tools like FECRT to determine their treatment protocols. Additionally, future work evaluating anthelmintic treatments at varying processing times and anthelmintic resistance should be done.

Blood Serum Metabolites

Serum metabolite data is presented in Table 3. While a significant processing time × day interaction was observed for nearly all parameters ($P < 0.05$), only few differences were biologically significant. Serum IBR titer for heifers processed at either 0 or 6-h upon arrival was significantly higher ($P < 0.01$) on d 35 compared to d 0. This response was expected, as these cattle were vaccinated immediately or shortly after arrival. Interestingly, no difference in IBR titer was observed ($P > 0.05$) between d 0 and d 35 for heifers processed at either 24 or 48-h upon arrival, indicating that these cattle may have been exposed to virus during transport or the rest period, and had time to seroconvert antibodies to the virus before vaccination. Heifers processed after a 48-h rest period had significantly higher glucose values ($P < 0.01$) on d 0 compared to heifers processed at 0, 6, or 24-h, however, this parameter was standardized across processing treatments by d 35. Other researchers have found that transit stress can result in increased blood glucose (Galyean et al., 1981; Damtew et al., 2018), thus, the concentration found in heifers processed 48-h upon arrival might suggest this rest period prompted more stress on the animals. However, the current work did not look at more stress-specific hormones, such as cortisol, which could provide explanation to the observed glucose differences and stress levels. Additionally, heifers across all processing times had increased ($P < 0.0001$) sorbitol dehydrogenase **(SDH)** from d 0 to d 35. While increases in SDH are most commonly associated with hepatocellular injury, the observed levels were not outside of normal biological ranges.

Conclusions

In summary, rest time prior to processing did not impact receiving calf growth performance. These data suggest that 6 hours, or approximately 1 hour of rest per hour of transport time, was the most beneficial to maximizing DMI during the first 14 d after arrival to the feedlot. Anthelmintic treatment at processing reduced the parasitic load in all heifers, regardless of their rest time upon arrival. Vaccine titer did not increase after initial processing in heifers processed 24- or 48-h after arrival, indicating the seroconversion of IBR antibodies during the longer rest period. Continued research with increased replication and more industry-standard experimental conditions should be conducted in order to further validate how rest time prior to processing can affect the health and growth performance of cattle entering a feed yard.

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Table 4.1. Ingredient composition and nutrient analysis of total mixed ration (TMR) fed to heifers from d 0 to d 35.

Ingredient, % DM	TMR
Corn silage	26.0
Alfalfa hay, ground	30.0
Dry rolled corn	40.0
Receiving supplement[1]	4.0
Nutrient analysis, % DM	
Ether extract, %	3.13
Crude protein, %	12.50
Calcium, %	0.65
Phosphorous, %	0.29
Neutral detergent fiber, %	27.91
Acid detergent fiber, %	19.78

[1]Receiving supplement was formulated with: ground corn (42.8%); soybean meal, dehulled (34.0%); urea, 46% N (9.3%); limestone (6.7%); salt (5.7%); Rumensin-90 (0.38%); and trace mineral premix (0.88%). Trace mineral premix provided: copper (250 mg/kg); manganese (499 mg/kg); selenium (2.46 mg/kg); zinc (749 mg/kg); vitamin A 0.17% (30,000 IU).

Table 4.2. Impact of time of processing on feedlot heifer growth performance, mortality, and morbidity[1].

	Processing Time After Arrival, hr[2]					*P* =		
Item;	0	6	24	48	SEM	Treatment	Linear	Quadratic
Weight, kg								
d 0	250	252	246	252	5.9	0.858	0.980	0.473
d 14	269	270	266	271	6.2	0.949	0.896	0.654
d 35	301	306	300	303	6.8	0.902	0.992	0.835
ADG, kg/d								
d 0 to 14	1.3	1.3	1.5	1.3	0.15	0.879	0.750	0.493
d 14 to 35	1.5	1.7	1.6	1.5	0.15	0.624	0.693	0.509
d 0 to 35	1.5	1.5	1.5	1.5	0.08	0.678	0.945	0.311
DMI, kg/d								
d 0 to 14	5.2[ab]	5.4[a]	5.1[ab]	4.9[b]	0.14	0.031	0.012	0.635
d 14 to 35	9.0	9.4	8.7	8.5	0.31	0.150	0.072	0.937
d 0 to 35	7.4	7.8	7.2	7.0	0.21	0.057	0.027	0.956
DMI, % of BW								
d 0 to 14	2.11	2.16	2.09	1.93	0.068	0.091	0.020	0.344
d 14 to 35	3.37	3.50	3.29	3.15	0.129	0.239	0.075	0.782
d 0 to 35	2.98	3.10	2.97	2.80	0.098	0.183	0.061	0.426
G:F								
d 0 to 14	0.25	0.24	0.29	0.26	0.030	0.645	0.507	0.368
d 14 to 35	0.17	0.18	0.18	0.18	0.015	0.891	0.626	0.936
d 0 to 35	0.20	0.20	0.21	0.21	0.010	0.703	0.375	0.471
Days to 2.5% BW DMI	18[ab]	15[b]	18[ab]	20[a]	1.3	0.030	0.023	0.393
Prevalence, %								
Mortality	0.0	0.0	0.0	10.5	3.57	0.096	0.026	0.236
Morbidity	0.0	0.0	5.3	0.0	2.60	0.382	0.806	0.113
Cattle to 2.5% BW by d 14	25.0	60.0	52.6	23.5	11.56	0.038	0.354	0.025

[ab]Means within a row that do not share a common superscript differ $P < 0.05$.

[1]A total of 80 mixed-breed, high-risk heifers were used in a 35-d experiment with 1 heifer per pen and 20 replicates per treatment.

[2]Cattle were processed at either 0, 6, 24, or 48 hours after their arrival to the research facility.

Table 4.3. Impact of processing time after arrival on feedlot heifer fecal parasites at d 0 and 14 d after anthelmintic administration[1].

	Processing Time After Arrival, hr[2]				SEM	
Item;	0	6	24	48		Day, *P* =
Prevalence, %					8.69	< 0.0001
d 0	94.7[a]	90.0[a]	100.0[a]	93.3[a]		
d 14	21.1[b]	20.0[b]	11.1[b]	40.0[b]		
Parasitic load, semiquantitative density[3]						
Strongyle					0.25	< 0.0001
d 0	2.7[a]	3.0[a]	2.8[a]	2.6[a]		
d 14	0.2[b]	0.4[b]	0.1[b]	0.2[b]		
Eimeria					0.19	< 0.0001
d 0	2.5[a]	2.3[a]	2.9[a]	2.0[ab]		
d 14	0.4[b]	0.4[b]	0.3[bc]	0.2[c]		
Trichuris					0.09	< 0.0001
d 0	2.4[a]	2.5[a]	2.6[a]	2.4[a]		
d 14	0.3[b]	0.4[b]	0.3[b]	0.2[b]		
Strongyloides					0.019	0.043
d 0	0.06[a]	0.07[a]	0.06[a]	0.05[a]		
d 14	0.00[b]	0.00[b]	0.00[b]	0.00[b]		
Moniezia					0.031	0.297
d 0	0.04	0.03	0.01	0.08		
d 14	0.01	0.02	0.00	0.00		
Giardia					0.051	0.084
d 0	0.11	0.09	0.10	0.13		
d 14	0.00	0.01	0.00	0.05		
Parasitic load, eggs/g of feces[4]						
Strongyle					128.0	0.0009
d 0	263[a]	261[a]	411[a]	325[a]		
d 14	1[b]	6[b]	0[b]	1[b]		
Eimeria					97.2	< 0.0001
d 0	135[a]	129[a]	204[a]	152[a]		
d 14	4[b]	1[b]	15[b]	6[b]		

[ab]Means within response criteria that do not share a common superscript differ $P < 0.05$.

[1]A total of 80 mixed-breed, high-risk heifers were used in a 35-d experiment with 1 heifer per pen and 20 replicates per treatment.

[2]Cattle were processed at either 0, 6, 24, or 48 hours after their arrival to the research facility.

[3]Semiquantitative analysis was conducted as described by Garcia et al. (2017). Scores indicated: 1) rare/occasional: 2 to 5 organisms per entire 22 × 22-mm coverslip area; 2) scanty/light/few: 2 or fewer eggs or larvae/5 to 10 fields; 3) moderate: 3 to 9 eggs or larvae/field; 4) numerous/heavy/many: 10+ eggs or larvae/field.

[4]On d 0 and d 14 a fecal egg count reduction test was conducted to determine the number of eggs per g of feces as an indication of anthelmintic efficacy.

Table 4.4. Impact of processing time after arrival on IBR titer and serum biochemical parameters[1].

	Processing Time After Arrival, hr[2]				SEM	Treatment × Day, *P* =
	0	6	24	48		
Blood parameter						
IBR Titer, 1:X[3]					15.2	0.0006
d 0	8[b]	1[b]	54[ab]	54[ab]		
d 35	64[a]	70[a]	47[ab]	31[ab]		
Glucose, mg/dL					7.3	0.0002
d 0	82[bc]	76[bc]	68[c]	108[a]		
d 35	83[bc]	85[abc]	83[abc]	96[ab]		
Urea Nitrogen, mg/dL					0.9	< 0.0001
d 0	12[b]	18[a]	16[a]	17[a]		
d 35	9[b]	10[b]	10[b]	9[b]		
Creatinine, mg/dL					0.10	0.0008
d 0	1.2[ab]	1.2[ab]	1.2[ab]	1.3[a]		
d 35	0.9[b]	0.9[b]	1.0[b]	1.1[ab]		
Total Protein, g/dL					0.15	< 0.0001
d 0	7.4[a]	7.4[a]	7.3[ab]	7.3[ab]		
d 35	6.7[c]	6.7[c]	6.8[bc]	6.8[bc]		
Albumin, g/dL					0.07	0.563
d 0	3.3	3.2	3.3	3.4		
d 35	3.3	3.3	3.2	3.2		
Globulin, g/dL					0.15	< 0.0001
d 0	4.1[a]	4.1[a]	4.0[ab]	3.9[abc]		
d 35	3.4[cd]	3.4[d]	3.6[bcd]	3.6[bcd]		
Total Ca, mg/dL					10.12	0.0002
d 0	9.2[bc]	9.1[c]	9.2[bc]	10.1[a]		
d 35	9.7[abc]	9.6[abc]	9.6[abc]	9.9[ab]		
P, mg/dL					0.39	< 0.0001
d 0	8.5[b]	10.2[a]	8.8[ab]	7.9[b]		
d 35	7.7[bc]	8.0[bc]	7.9[bc]	7.0[c]		
Na, mmol/L					0.7	0.0005

d 0	145[a]	143[b]	143[b]	143[b]		
d 35	142[b]	142[b]	142[b]	143[ab]		
K, mmol/L					6.39	< 0.0001
d 0	5.7[b]	5.5[b]	6.4[a]	5.9[ab]		
d 35	5.5[b]	5.2[b]	5.5[b]	5.7[b]		
Cl, mmol/L					0.8	< 0.0001
d 0	104[a]	100[b]	96[c]	94[c]		
d 35	96[c]	97[c]	96[c]	97[c]		
Bicarbonate, mmol/L					1.1	0.0008
d 0	19[b]	22[ab]	22[ab]	18[b]		
d 35	22[ab]	23[a]	23[a]	22[ab]		
Anion Gap, mmol/L					1.2	< 0.0001
d 0	29[bc]	27[c]	32[b]	37[a]		
d 35	30[bc]	29[bc]	30[bc]	30[bc]		
Na:K Ratio					0.7	< 0.0001
d 0	26[a]	26[a]	23[b]	25[ab]		
d 35	26[a]	27[a]	26[a]	26[ab]		
Aspartate transaminase P5P, U/L					14.2	0.255
d 0	127	118	134	123		
d 35	140	105	111	100		
Alkaline phosphatase, U/L					17.5	< 0.0001
d 0	112[c]	120[c]	142[bc]	119[c]		
d 35	208[a]	204[ab]	199[ab]	201[ab]		
Gamma glutamyl transferase, U/L					1.7	0.016
d 0	9[ab]	9[ab]	5[b]	7[ab]		
d 35	10[ab]	9[ab]	10[ab]	13[a]		
Sorbitol dehydrogenase, U/L					2.18	< 0.0001
d 0	6.5[b]	10.2[b]	3.6[b]	4.5[b]		
d 35	20.9[a]	18.7[a]	18.2[a]	19.5[a]		
Creatine kinase, U/L					825	0.375
d 0	1,360	1,094	1,041	1,625		
d 35	2,889	694	1,631	605		
Total bilirubin, mg/dL					0.02	< 0.0001

d 0	0.2[ab]	0.2[bc]	0.3[a]	0.2[bc]		
d 35	0.1[c]	0.1[c]	0.1[c]	0.1[c]		
Direct bilirubin, mg/dL					0.01	0.058
d 0	0.1	0.1	0.1	0.1		
d 35	0.1	0.1	0.1	0.1		

[abc]Means within the same row that do not share a common superscript differ, $P < 0.05$

[1]A total of 80 mixed-breed, high-risk heifers were used in a 35-d experiment with 1 heifer per pen and 20 replicates per treatment.

[2]Cattle were processed at either 0, 6, 24, or 48 hours after their arrival to the research facility.

[3] Serum samples were analyzed for infectious bovine rhinotracheitis (IBR) titer via serum neutralization antibody test with the means displayed as the ratio of serum: dilutant where no antibodies remained detectable within the sample.

Chapter 5 - Professional skill development and understanding of research methods: Student perceptions of their undergraduate research (UGR) experience in animal science

Abstract

The undergraduate research (UGR) experience has been shown to enhance student learning gains, however, there is little evidence to demonstrate how UGR impacts student perceptions of professional and research skill development. A total of 167 undergraduate students in the Department of Animal Sciences and Industry at Kansas State University completed an anonymous, retrospective post-then-pre-test to assess their perceptions of how the UGR experience impacted the development of professional skills and research competence. The survey instrument was formulated from the Undergraduate Research Student Self-Assessment with slight modifications and consisted of 4 sections containing a total of 35 questions. Students participating in either an independent (student paired with faculty member in 1:1 ratio) or course based (approximately 20 students paired with 1 faculty member) UGR experience completed the assessment at the end of the academic semester following conclusion of the UGR project. A comparison group of students not completing any form of UGR were also surveyed. Data were analyzed using the GLIMMIX procedure of SAS with student as the experimental unit. Students completing a course-based UGR experience reported significant increases ($P < 0.02$) in professional skill development across a variety of areas compared to students not completing UGR, while those who participated in an independent UGR experience were intermediate. No evidence of differences ($P > 0.12$) was observed for student gains in the areas of time management, working well under pressure, and working independently. Students participating in

course based UGR reported increased ($P < 0.0001$) gains across all skill areas related to research methods compared to those not completing UGR. Those completing an independent UGR experience also demonstrated greater gains ($P < 0.0001$) compared to students not participating in UGR for all statements about research methods except when asked about their ability to analyze data and ability to use basic statistics to understand data. Based on student responses, completion of an UGR experience has positive implications for professional skill development and comprehension of how science is practiced. These data suggest there are differences in students' perception of skill development from their UGR experience. Future research should increase the number of respondents and participating institutions as well as utilize more objective measurements of student learning gains.

Introduction

Undergraduate research (UGR) experiences have been shown to provide positive outcomes for students, faculty, and research universities altogether (Bauer and Bennett, 2003; Lopatto, 2004; Hunter et al., 2007). These experiences can serve as a valuable tool to develop skills and enhance learning outcomes, while exposing students to graduate school opportunities. More specifically, literature suggests that UGR can improve students critical thinking (Kardash, 2000), expand their understanding of how science is conducted (Russell et al., 2007), and prepare them for higher education or a career in science, technology, engineering and mathematics (Eagan Jr et al., 2013).

During the 2015-2016 academic year, a course based UGR model was implemented in the Department of Animal Sciences and Industry at Kansas State University to meet the rapidly growing student demand for research opportunities. This model was provided in addition to the

pre-existing, individualized UGR experience where a student was paired 1:1 with a faculty mentor. Jones and Lerner (2019) tracked student critical thinking gains as a quality measure of the newly established model and reported no differences relative to the conventional independent UGR experience, indicating that course based UGR could serve to expose a greater number of students to research while maintaining the tangible benefits associated with these experiences. Since its inception, the course based UGR model has grown to accommodate approximately 60 students each semester, conducting research across a variety of Animal Science disciplines including swine nutrition, poultry nutrition, beef cattle nutrition, and meat science. While enhancing student critical thinking remains a targeted outcome of the UGR experience, other areas of student skill development and comprehension are important to quantify. Literature surrounding students' professional growth as a result of UGR is limited. Additionally, a primary learning outcome of UGR is to instill a fundamental knowledge of scientific practice in participants. While quantitatively measuring students' understanding of research methods can be determined via assignments, projects, or other forms of assessment, these are often difficult to standardize across disciplines. Moreover, for internal evaluation of UGR experiences, surveying students can serve as a simple yet reliable tool to identify gaps in student learning and help cultivate plans for improvement. Thus, the objective of this study was to evaluate student perceptions of two types of UGR experiences as it relates to development of professional skills and their understanding of research methods vs. their peers who did not conduct any form of UGR.

Materials and Methods

Survey Design and Instrumentation

All experimental procedures (IRB #10727) were determined by the Kansas State University Research compliance office as exempt under the criteria set forth in the Federal Policy for the Protection of Human Subjects, 45 CFR 46.101(b)(1)(i).

An anonymous survey was designed to evaluate student gains resulting from their undergraduate research experience based on the Undergraduate Research Student Self-Assessment (URSSA), with slight modifications. Originally created by Hunter et al. (2009), the URSSA tool is a publicly available online survey modeled after the Student Assessment of Their Learning Gains (SALG) instrument and focuses on four primary constructs related to student gains in 'Skills', 'Thinking and Working Like a Scientist', 'Personal Gains', and 'Attitudes and Behaviors as a Researcher'. The URSSA was used for the current research due to its accessibility, adaptability, and validity. The free survey is housed on the SALG website (www.salgsite.org), where the original template of the instrument contains 134 questions but can be modified as needed. Using the URSSA template, we created a shortened version of the instrument by extracting questions that would allow for the assessment of student gains related to both professional skills and their understanding of research methodologies. Additionally, the URSSA contains questions that ask students how their research experience has impacted their thoughts related to pursuing further education or their career goals.

The final instrument included five sections of questions. Section 1 asked students to rate how much they had gained in areas related to professional skills over the course of the semester using a 4-point Likert scale ranging from no gain to a lot of gain. Section 2 asked the same question regarding student perceptions of their gains, but in areas related their knowledge of

research methods using the same scale. Section 3 asked students to rate how much they agreed with a series of statements related to their confidence and preparedness for future study or employment using a 5-point Likert scale ranging from strongly disagree to strongly agree. Section 4 asked students how likely they would be to pursue different educational or employment opportunities related to science, technology, engineering, or mathematics at the time of assessment using a 5-point Likert scale ranging from extremely unlikely to extremely likely. Finally, section 5 asked students a series of demographic questions.

Survey Distribution and Data Analysis

A total of 167 undergraduate students in the Department of Animal Sciences and Industry at Kansas State University (Manhattan, KS) completed the survey. Because the instrument was designed as a post-then-pre-assessment, the survey was administered to students the last week of the semester, after the conclusion of the UGR experience. Student participants had completed either an individual UGR experience or course based UGR. A third group of students who had not completed any form of UGR were asked to take the survey to serve as a comparison group. Student responses were coded to a numeric value so that data were treated as continuous and normally distributed for the purpose of statistical analysis. Data were analyzed using the GLIMMIX procedure of SAS (v. 9.4, Cary, NC) with individual student as the experimental unit and Tukey-Kramer adjustments included for all pairwise comparisons. Results were considered significant if $P < 0.05$ and marginally significant if $0.05 < P < 0.10$.

Results and Discussion

Justification for Survey Modifications and Use

The URSSA is designed as a retrospective post-then-pre-test to assess student gains following their research experience. Literature suggests that this model can help reduce response

shift bias, increase the survey's validity, and in a practical sense, help reduce response fatigue by only requiring participants to take the survey once (Howard, 1980). Additionally, the URSSA was evaluated by Weston and Laursen (2015) to examine its validity, where these authors suggested that the instrument serves as a reliable way to assess students' development of skills from the UGR experience. We made modifications to the original URSSA instrument to help further reduce response fatigue and better fit our research objectives. Specifically, we reduced the scale of sections 1 and 2 from the original 6-point to a 4-point scale since it has been shown that this smaller scale is easier to understand and requires less effort, appealing more to younger and less motivated respondents (Nemoto and Beglar, 2014). The objective of this research was not to evaluate the efficacy of the URSSA as a tool for UGR program evaluation; however, the instrument was used to assess student perceptions of their gains as a result of their UGR experience.

Student Gains in Professional Skills

The UGR experience serves as a high impact educational opportunity to enhance student learning, and the value of these transformational experiences has been cited on multiple accounts (Hunter et al., 2007; Russell et al., 2007; Linn et al., 2015). No different than faculty or graduate student-led research, the primary end-product of UGR is still the generation of information that can be used to make data-driven decisions within the scientific community. However, UGR should also develop knowledge and a variety of skills within student participants as an additional outcome. Researchers have demonstrated the impacts that UGR can have on student critical thinking (Kardash, 2000; Jones and Lerner, 2019), but few have focused on other skills that can be uncovered and developed during the UGR experience. Upon completion of an undergraduate degree, most students will either enter the workforce or pursue some form of graduate or other

professional degree. In fact, as of the 2021-2022 academic year in Department of Animal Sciences and Industry at Kansas State University, approximately 63% of students were employed and 35% were furthering their education beyond a bachelor's degree. Historically, UGR has been cited as a significant pathway to graduate school for those who participate (Hathaway et al., 2002). While this remains true, UGR experiences have grown to serve a broader range of student demographics and goals post-graduation, including those who forego furthering education beyond a bachelor's degree and choose direct employment. Work by St. Louis et al. (2021) focused on enhancing soft skills referred to as "21st century skills" in college graduates to make them more marketable to future employers. These skills, including decision making, communication and collaboration, information literacy, life/career management, and self-confidence were all cited by employers as applied skills lacking in today's graduates (Baird and Parayitam, 2019; Rios et al., 2020).

Student perceptions of their gains in professional skills over the course of the semester are presented in Table 1. Students completing a course based UGR experience reported significant gains in most professional skills ($P < 0.02$) compared to those not completing UGR, while students completing independent UGR were intermediate. Learning gains were not observed in only three traits, time management, working well under pressure, and working independently ($P > 0.05$). Little work has been done to evaluate the development of these "21st century skills" as a result of UGR; however, Hunter et al. (2007) interviewed undergraduate students completing an apprenticeship-style UGR experience and found that student's perception of their professional skills were higher following UGR. Our results corroborate this, but there are notable differences between these studies including the discipline of the UGR, data collection procedures (survey vs. interview), and the number of participants. Regardless, while a goal of

animal science curriculum is developing content expertise, the data presented here suggest that UGR experiences can extend well beyond this and simultaneously enhance professional skills within students.

While the primary objective of our study was to assess the outcome of all research projects, regardless of type, it was important to our objective to directly compare students conducting independent vs. course-based research. We found no differences ($P > 0.05$) between students completing independent UGR or course based UGR in most professional skills. This was consistent with our hypothesis because the final outcomes and assessments of these two forms of UGR are the same – students must compile their findings in the form of a scientific abstract, research poster, and research presentation at the conclusion of the project. Thus, it is not surprising that the only professional skill in which students completing independent UGR and no UGR differed significantly was preparing an oral presentation. Student responses indicated that the areas of time management, working well under pressure, and working independently were not skills that developed differently as a result of UGR experience. Among both forms of UGR, students spend the first two-thirds of the semester conducting the research and collecting data, with the final third dedicated to data analysis, dissemination, and preparation of a research abstract, poster, and oral presentation. A large portion of the workload inherently falls at the end of the semester-long project; therefore, it is interesting that students responses reported no differences ($P > 0.05$) in gains related to time management or working well under pressure. Additionally, we saw no evidence ($P > 0.05$) that UGR significantly impacted students perception of their ability to work independently, which was unexpected given students conducting independent UGR bear the project responsibility solely, while course based UGR students share this with a graduate student mentor and their peers. In a related skill area, Jones

and Lerner (2019) found no difference in students' perceptions of their ability to work as a team when comparing participants of individual and course based UGR experiences.

Student Gains in Competence of Research Methods

The UGR experience is designed to increase students' understanding of how to conduct a research project (Russell et al., 2007). Within our department, student learning outcomes from UGR are centered around developing an understanding of how to formulate research questions, generate hypotheses, conduct a sound experiment, and comprehend the resultant data. Objective measurement of these outcomes is conducted at the completion of the project via assessment of the student's abstract, research poster, and research presentation, but it can be difficult to directly compare student's performance across project disciplines. However, for internal program assessment and direction for future growth, it is important to quantify how students feel about their UGR experience.

Student perceptions of their gains related to research skill development are shown in Table 2. Students participating in course based UGR reported significantly increased ($P < 0.0001$) gains across all skill areas compared to those not completing UGR. Those completing an independent UGR experience also demonstrated significantly enhanced gains ($P < 0.05$) compared to students not participating in UGR for all statements except when asked about their ability to analyze data and ability to use basic statistics to understand data. We hypothesized that students completing a UGR experience, regardless of the format, would have enhanced understanding of research methods as indicated by their self-assessment. Our data indicate that students are developing an understanding of the proposed learning outcomes stated above.

Student Perceptions of Future Education or Career Goals

At the end of the survey, students were asked a series of questions related to their educational and career goals following their UGR experience. When asked how much they agree with the statement "I am confident about my interest in my current field of study", there were no discernable differences ($P = 0.36$) between students completing either form of UGR and those completing no UGR. Similarly, when asked the same question regarding the statements, "I feel prepared to pursue an advanced degree, if desired", "I feel prepared to enter the workforce, if desired", and "I feel as though I need more clarification on what I would like to do post-graduation", there was no evidence of differences ($P \geq 0.49$) between comparison groups. When students were asked how likely they were to pursue a graduate program in a field related to science, technology, math, or engineering or pursue a different professional degree or certification as a teacher, there were no statistical differences ($P \geq 0.18$) as a result of UGR experience. These findings differ from previous studies that suggest UGR experiences provide students with clarification on their future as it relates to pursuing graduate education or a career in STEM (Russell et al., 2007; Eagan Jr et al., 2013). Interestingly, when students were asked how likely they were to work in a science lab, those who had completed an independent UGR experience were significantly more likely to do so ($P = 0.02$) compared to their contemporaries completing no UGR or course based UGR. This result was anticipated, given that students participating in an independent UGR typically spend more time working one-on-one with their mentor in a lab setting throughout the duration of their project. Meanwhile, because of the larger student to mentor ratio in course based UGR experiences, these students are less likely to gain direct skills in a laboratory setting and tend to spend a greater amount of time during the project on-farm collecting more applied data.

Conclusions and Implications

In summary, the data presented here suggest that UGR experiences can impact student perceptions of their learning gains related to professional skills and research competence. Specifically, among the students surveyed within our department, course based UGR appears to have a greater influence on the development of soft skills deemed important by future employers. However, when it comes to enhancing students' abilities to comprehend how science is conducted, both independent and course based UGR can have positive impacts. The current work has limitations which include an unequal number of students represented within each comparison group, a relatively short period of data collection, and a lack of objective measurement. Future research should focus on increasing both the number of students surveyed and the semesters that data are collected in effort to assess how these perceptions may change over time. Additionally, incorporating more precise quantitative measures to assess student learning gains would be beneficial. Since this research is based off students' own perceptions of their gains, it is difficult to extrapolate our findings to the broader population outside of our institution. However, here we have provided evidence that utilizing a post-then-pre-test can identify differences in student satisfaction with their UGR experience.

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Table 5.1. Student perceptions of learning gains related to professional skill development.

Statement	No UGR (n = 111)	Independent UGR (n = 9)	Course UGR (n = 47)	SEM	P-value
Preparing an oral presentation	1.56[b]	2.23[a]	2.31[a]	0.23	< 0.0001
Communicating information to someone outside my field	1.77[b]	2.08[ab]	2.36[a]	0.21	< 0.0001
Conducting observations	1.74[b]	2.15[ab]	2.53[a]	0.21	< 0.0001
Understanding journal articles	1.77[b]	2.25[ab]	2.54[a]	0.22	< 0.0001
Interpreting and critically evaluating marketing literature	1.46[b]	1.83[ab]	2.35[a]	0.25	< 0.0001
Managing my time	2.03	2.31	2.09	0.22	0.468
Self-confidence	1.77[b]	2.08[ab]	2.11[a]	0.24	0.019
Ability to think critically about complex problems	2.03[b]	2.23[ab]	2.40[a]	0.20	0.001
Evaluate others' work and provide constructive feedback	1.45[b]	1.92[ab]	2.31[a]	0.24	< 0.0001
Listen effectively	2.02[b]	2.31[ab]	2.34[a]	0.22	0.012
Work well under pressure	1.94	2.35	2.11	0.23	0.101
Productive as a team member	1.67[b]	2.23[ab]	2.28[a]	0.26	< 0.0001
Work independently	2.28	2.54	2.47	0.20	0.107
Accept critique	1.81[b]	2.08[ab]	2.49[a]	0.21	< 0.0001
Respect and acknowledge others' contributions	2.02[b]	2.15[ab]	2.57[a]	0.21	<0.0001
Attention to detail	2.21[b]	2.15[b]	2.48[a]	0.19	0.013

[ab]Means within a row lacking a common superscript differ, $P < 0.05$.

[1]Student survey responses to the question, “Compared to the beginning of the semester, please rate how much you have gained in the following areas” using a 4-point Likert scale consisting of 1: no gain; 2: a little gain; 3: moderate gain; and 4: a lot of gain.

Table 5.2. Student perceptions of learning gains related to research skill development.

Statement	No UGR (n = 111)	Independent UGR (n = 9)	Course UGR (n = 47)	SEM	P-value
Understanding the ethics of conducting research	1.66[b]	2.33[a]	2.72[a]	0.22	< 0.0001
Ability to analyze data	1.76[b]	2.25[ab]	2.47[a]	0.23	< 0.0001
Formulating a research question	1.50[b]	2.33[a]	2.56[a]	0.24	< 0.0001
Identifying limitations of research methods and designs	1.44[b]	2.25[a]	2.48[a]	0.24	< 0.0001
Understanding the theory and concepts guiding a research project	1.51[b]	2.33[a]	2.49[a]	0.24	< 0.0001
Ability to summarize research findings	1.79[b]	2.5[a]	2.58[a]	0.23	< 0.0001
Drawing conclusions	1.85[b]	2.42[a]	2.43[a]	0.21	< 0.0001
Writing a scientific abstract	1.06[b]	2.5[a]	2.73[a]	0.24	< 0.0001
Creating a research poster	1.01[b]	2.42[a]	2.74[a]	0.25	< 0.0001
Ability to use basic statistics to understand data	1.63[b]	2.08[ab]	2.49[a]	0.23	< 0.0001
Confidence in my ability to do well in future science courses	1.87[b]	2.58[a]	2.44[a]	0.21	< 0.0001

[ab]Means within a row lacking a common superscript differ, $P < 0.05$.

[1]Student survey responses to the question, "Compared to the beginning of the semester, please rate how much you have gained in the following areas" using a 4-point Likert scale consisting of 1: no gain; 2: a little gain; 3: moderate gain; and 4: a lot of gain.

Table 5.3. Student perceptions of their confidence and preparedness for future study or employment.

Statement	No UGR (n = 111)	Independent UGR (n = 9)	Course UGR (n = 47)	SEM	P-value
I am confident about my interest in my current field of study	4.19	4.00	4.00	0.27	0.359
I feel prepared to pursue an advance degree, if desired	3.89	4.08	3.78	0.27	0.487
I feel prepared to enter the workforce, if desired	3.80	4.08	3.86	0.27	0.603
I feel that I need more clarification on what I would like to do post-graduation	3.37	3.31	3.30	0.32	0.908

[1]Student survey responses to the question, "Please rate how much you agree with the following statements" using a 5-point Likert scale consisting of 1: strongly disagree; 2: disagree; 3: neither agree or disagree; 4: agree; and 5: strongly agree.

Table 5.4**.** Students perceptions of various advanced degrees or employment opportunities.

Statement	No UGR (n = 111)	Independent UGR (n = 9)	Course UGR (n = 47)	SEM	P-value
Pursue a graduate program in a field related to science, math, or engineering	3.13	3.31	3.38	0.38	0.396
Pursue a different professional degree	3.50	2.92	3.69	0.40	0.175
Pursue certification as a teacher	1.98	2.46	2.17	0.33	0.271
Work in a science lab	2.77[b]	3.77[a]	2.91[b]	0.34	0.022

[ab]Means within a row lacking a common superscript differ, $P < 0.05$.
[1]Student survey responses to the question, "At the current time, how likely are you to consider each of the following" using a 5-point Likert scale consisting of 1: extremely unlikely; 2: somewhat unlikely; 3: neither likely or unlikely; 4: somewhat likely; and 5: extremely likely.